Study on the diversity of denitrification bacteria treating with wastewater by using PPGC filler on SBMBBR at low temperature

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Abstract. Aiming at the problem of the low removal efficiency of biological nitrogen-removing of low temperature waste-water, using Polyurethane Porous Gel Carrier (PPGC)-SBMBBR treated low temperature sewage, in compared with conventional SBR,and viaing Miseq high-throughput sequencing technology in analysis of the differences of microbial diversity and abundance of structure on the two reactors of activated sludge, revealed dominant nitrogen-removing bacterium improving the treatment efficiency of low temperature sewage. The results shows that the removal efficiency of the effluent nitrogen and the sludge sedimentation rate of (PPGC)-SBMBBR reactor are significantly improved under the water temperature (6.5±1℃). Adding the filler can contribute to improvement of bacterial diversity and relative abundance of nitrification and denitrification bacterium in the activated sludge system. The main relative abundance of ammonia oxidizing bacteria (AOB),nitrite oxidizing bacteria (NOB),anaerobic denitrifying bacteria, and aerobic denitrifying bacteria in (PPGC)-SBMBBR(R2) are significantly better than SBR (R1),and the R2 reactor can independently enrich the nitrifying bacteria and the aerobic denitrifying bacteria, such as Nitrospira, Hydrogens, Pseudomonas, and Zoogloea. The total relative abundance of dominant and nitrifying denitrifying bacterium increases from 28.65% of R1 to 60.23% of R2, providing a microbiological reference for improving the efficiency of biological nitrogen removal in low temperature waste-water.

1 Introduction

The adsorption settle-ability of activated sludge process and the growth effect of denitrification bacterium are significantly affected by some factors, such as the low temperature (≤10℃). So the treatment effect of biological denitrification of domestic sewage under low temperature is poor, it means that achieving the emission standard is difficult [1]. Bio-film is often used in low temperature sewage treatments which aim at enriching denitrification bacterium by extending the sludge residence times. Polyurethane Porous Gel Carrier (PPGC) is one kind of polyurethane compoundings with other polymer materials, which has the features of high hydrophilicities, better bacterial attachments. And it is a kind of new type gel filler suiting to 1-50℃ temperature, and the nitrification efficiency can reach 600-1250 gNH_3-N/m^3·d. At present, the most of researches about bio-film process is limited to improving the effect of biological denitrification of low temperature waste-water by changing factors after adding external fillers [2-4], however, there are few studies on the biological diversity and structural abundance of biological nitrogen-removing in bio-film reactors.

High-throughput sequencing has the characteristics of high speed, precision, and sensitivity [5-6], which has been widely used in the analysis of microbial community structure in sludge [7-8]. The author used Miseq high-throughput sequencing technology for sequencing the dominant nitrifying and denitrifying bacteria in SBR and PPGC-SBMBBR which treated low-temperature sewage (6.5±1℃). It aimed to analysis the diversity of bacteria and the structural abundance changes of the activated sludge in the two reactors. In order to provide a microbiological reference on improving the nitrogen removal efficiency from low temperature waste-water (6.5±1℃), thereby effective solving the problem of low biological nitrogen removal efficiency of low temperature sewage.

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2 Materials and methods

2.1 Test equipment and operating conditions

An SBR reactor (R1) was compared with a PPGC-SBMBBR reactor (R2) filled with Polyurethane Porous Gel Carrier. Polyurethane Porous Gel Carrier was selected from an environmental protection company in China, and its dosing ratio was 30%. Seeing table 1 for specific parameters.

The effective volumes of the two reactors were 13.8L and sampling ports (drainage ports) were evenly set. The aerator pump was connected to the bottom of the aerator disc to fully aerate, and the air flow-meter control the reactors dissolves oxygen. The activated sludge was concentrated from Shenyang municipal sewage plant. After successful starting-up, the activated sludge was operated in anoxic/aerobic alternate mode (Anoxic DO= (1.5±0.5) mg/L; Aerobic DO=(6±1) mg/L), the sludge concentration was controlled at 3500-4200mg/L, and the pH value is between 7-7.5.The reactors was operated at a low temperature (6.5±1℃) for two cycles per day. The main reaction required 660min, the anaerobic process was 180min, the aerobic process was 420min, and the precipitation for 60min.

The test using influent was artificial simulated sewage: COD was 350mg/L, NH_4^+-N was 35mg/L, and TP was 5mg/L. Main ingredients have many species, including glucose, ammonium chloride, potassium dihydrogen phosphate, anhydrous calcium chloride, magnesium sulfate, sodium bicarbonate, two kinds of trace elements.

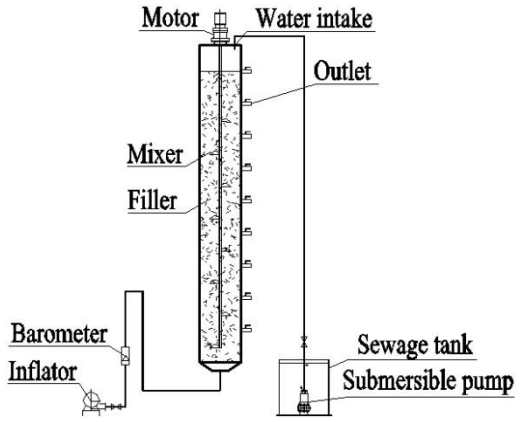

Fig. 1. Testing device and process flow

Table 1. Surface performance parameters of packing

project	size(mm)	Packing density(kg/m3)	Specific surface area(m2/m3)
PPGC	16.8×16.8	40	4000

2.2 Analysis method

2.2.1 Water quality analysis [9]

There are five indicators for water quality testing, and the corresponding testing methods are:NH_4^+-N (nacht reagent spectrophotometry), NO_2^--N (N-(1-naphthyl)-ethylenediamine spectrophotometry), TN(ultraviolet spectrophotometry with alkaline potassium persulfate digestion), DO value(portable analyzer), and water temperature (mercury thermometer).

2.2.2 High-throughput analysis

50mL of activated sludge in SBR and PPGC-SBMBBR reactors under stable aeration for more than 5 minutes was taken as samples and stored at -8℃. Extracting total DNA of activated sludge bacteria samples, two samples would do PCR amplification in 16s rRNA V3 V4 area, and the bacteria primers were NAAGAACACGTTCGGTCACCTCAGCACACTTGT GAATGTCATGGGATCCAT [10]. The obtained sequences were filtered and processed to obtain effective sequences with Illumina MiSeq system sequencing. The effective sequences with a similarity of 97% were divided into different OTU. The representative sequences were selected and compared with the Silva database for annotation, cluster analysis and strain diversity analysis.

3 Results and discussion

3.1 Treatment effect of reactor operation

After the two sets of reactors were operated for 26 days, the operating effects were shown in Table 2. The removal rates of NH_4^+-N, NO_2^--N and TN in the PPGC-SBMBBR reactor (R2) are higher than those in the conventional SBR reactor (R1). This indicates that R2 can optimize the water effluent effect compared with R1 under the same conditions of low water temperature, and the addition of the filler may change the microbial community structure. In order to analyze the reasons, the microbial diversity of the activated sludge samples in the two reactor systems is considered.

Table 2. Treatment effect of plant operation

3.2 Bacterial diversity analysis

project	Effluent concentration/(mg/L)			Removal rate/%			Sludge settling rate(m/h)
	NH4+-N	NO2--N	TN	NH4+-N	NO2--N	TN	
R1	10.77	0.065	26.8	71.8	73.2	50.3	0.156
R2	2.71	0.020	11.9	92.9	91.7	75.3	0.232

As shown in table 3, the effective sequences of R1 and R2 bacterial sequencing are 78642 and 71401, and the OTU are 2671 and 3695. The coverage index is positively correlated with the authenticity of samples. The coverage index of this sequencing reached above

0.97, indicating high reliability of the data. The Chao index value is positively correlated with the abundance of bacterial community, and the Shannon index value is positively correlated with the diversity of bacterial community. These indexes can reflect the diversity of bacterium from different aspects. As shown in table 3, R2 has more diversities and abundance than R1. This may be due to the release of humic acids and silicates from different microorganisms growing on the surface of the filler, which alters the structure and abundance of the bacterial flora in the activated sludge. [11] Thus R2 increases specific functional species and relative abundance than R1.

Table 3. Bacterial Diversity Analysis

sample	Number of sequences	OTU index	Coverage index	Chao index	Shanon index
R1	78642	2671	0.98	11167.21	4.48
R2	71401	3695	0.97	11236.45	5.58

3.3 Analysis of differences in structure abundance of bacterial flora

3.3.1 Phylum, class

Figure 2(a) lists the dominant phylums of R1 and R2 activated sludge samples and sample sequencing can obtain that bacterium of R1 and R2 all have 19 phylums. The categories whose abundance ratio is more than 1% are 9 bacterium in total, including Proteobacteria, Bacteroidetes, Verrucomicrobia, Candidatus Saccharibacteria, Acidobacteria, Planctomycetes, Chloroflexi, Gemmatimonadetes and Actinobacteria. R2 has one more phylum than R1:Nitrospirae (1.07%) and its total relative abundance reached 91.37% of R1 and 94.04% of R2.It means they are the dominant bacterium in their sludge sample [12].It can be seen that the addition of filler can contribute to the enrichment of nitrifying bacteria in the activated sludge system at low temperature. The relative abundance of dominant bacterium of R2 is different from that in R1.Proteobacteria, Bacteroidetes, Candidatus Saccharibacteria and Acidobacteria change from 26.79%, 10.95%, 33.74% and 7.41% of R1 to 43.1%, 17.44%, 12.31% and 8.27% of R2.Planctomycetes, Chloroflexi, Verrucomicrobia, Gemmatimonadetes, and Actinobacteria change from 4.72%, 1.76%, 2.34%, 2.11%, 1.55% of R1 to 3.02%, 2.43%, 2.35%, 2.77%, 1.28% of R2,which are not significant and dominant in the system.

The total relative abundance of Proteobacteria, Bacteroidetes, Candidatus Saccharibacteria and Acidobacteria in R1 and R2 are 78.88% and 81.12%. The three phylums are analyzed from the class classification level, as shown in figure 2(b). Gammaproteobacteria, Betaproteobacteria and Deltaproteobacteria are the largest floras in the relative abundance, and the relative abundance are 8%, 7.58%, 3.55% of R1 and 16.3%, 14.24% and 5.88% of R2. Dominant flora of Proteobacteria are mostly facultative heterotrophic bacterium with organic matter as carbon source and they are major participants in pollutant degradation in sewage treatment systems [13-14], as well as important flora for nitrogen removal [15]. Sphingobacteriia and Cytophagia are dominant classes in Bacteroidetes. The relative abundance of Sphingobacteriia in R1 and R2 are 6.15% and 13.33%. The relative abundance of Cytophagia in R1 and R2 are 2.58%and1.33%.CandidatusSaccharibacteria_unclassified is the dominant class of Candidatus Saccharibacteria, and its relative abundance in R1 and R2 are consistent with Candidatus Saccharibacteria, which are 33.74% and 12.31%,respectively.In Acidobacteria,Gp10 and Gp4 are dominant class and their total relative abundance are 5.83% of R1 and 6.03% of R2. There are 14 classes of R1 and 16 of R2 with relative abundance more than 1%. The unique bacterium in R2 are Nitrospira (1.07%), verrucomicrobiae (1.07%), Ignavibacteria (0.8%) and bacteroides (0.77%). Thus, at the level of phylum and class, adding filler has obvious influence on the structure and abundance of bacteria community in activated sludge system.

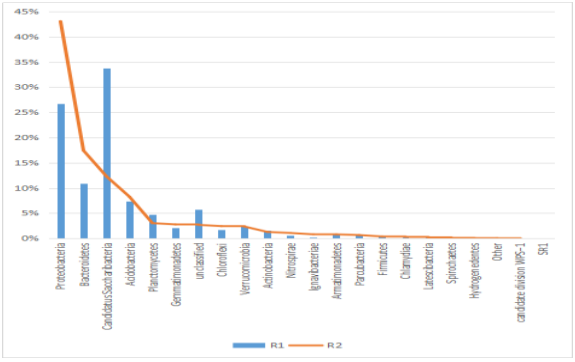

Fig. 2(a). Dominant strains (phylum)

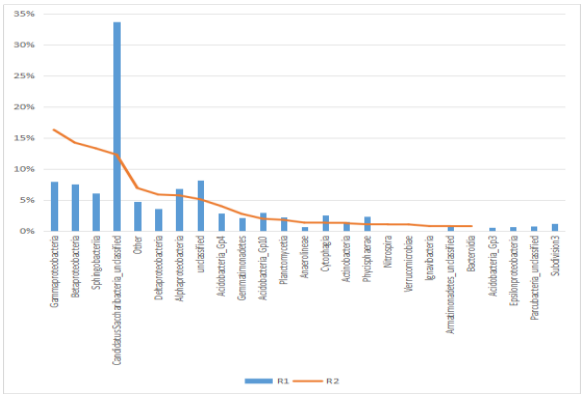

Fig. 2(b). Dominant strains (class)

3.3.2 family, genus

Further in-depth analysis was made at the level of family and genus, as shown in Fig. 3. There are 16 and 19 strains with relative abundance more than 1%, and their relative abundance are different in R1 and R2. The corresponding effects of the dominant strains are shown

in table 4. The dominant families of two samples are Candidatus saccharibacteria_uncharibacteria with the relative abundance of 33.74% in R1 and 12.31% in R2. Daigger etal [16] found that Candidatus Saccharibacteria _unclassified is the major bacteria that may induce sludge swelling at low temperature (≤10℃). Saprospiraceae is a kind of Phosphorus removal bacteria that can secrete Extra-cellular Polymeric Substances and metabolize glucose, galactose, acetate etc [17]. Its Extra-cellular Polymeric Substances can reduce the sedimentation performance of sludge [18], and the abundance increases from 1.97% of R1 to 5.44% of R2. In line with table 2, the results of R1 and R2 activated sludge sedimentation rates are consistent with the Candidatus Saccharibacteria abundance distribution and Saprospiraceae abundance distribution. Dokdonella, Dechloromonas, Planctomycetaceae, as the dominant AOB (ammonia-oxidized bacteria) group, change from 1.47%, 1.01%, 1.42% of R1 to 3.99%, 3.8%, 1.39% of R2, and the total relative abundance of the dominant AOB in R1 and R2 is respectively 3.9% and 9.18%, and it is the reason that R2 NH_4^+-N removal rate reached 92.9%, which is higher than the R1 NH_4^+-N removal rate of 21.1%. Rhodocyclaceae and Anaerolineaceae, as dominant NOB , increases their relative abundances from 2.77% and 1.07% of R1 to 7.62% and 1.36% of R2, and Hydrophaga, Pseudoxanthomonas, Nitrospira and Zoogloea are found only in R2 and its relative abundance are 1.11%, 1.08%, 1.07%,and 0.96%.The total relative abundances of advantage NOB are respectively 3.84% and 13.2% in R1 and R2 ,and it is the reason that the R2 NO_2^--N removal rate reached 91.7%, which is higher than R1 NO_2^--N removal rate of 22.5%. To sum up, in R1 and R2, the total relative abundance of advantage nitrifying bacterium are respectively 7.37%, 22.38%, and it shows that adding filler is beneficial to the enrichment of nitrifying bacteria in the system at low temperature. The relative abundance of Sphingobacteriia is respectively 6.15% and 13.33% in R1 and R2, and that of Xanthomonadaceae is respectively 3.77% and 11.30% in R1 and R2, which are dominant anaerobic denitrifying species in R1 and R2.The total relative abundance of dominant anaerobic denitrifying bacteria in R1 and R2 are 14.29% and 28.82%, indicating that adding filler can obviously enrich anaerobic denitrifying bacteria in activated sludge system. Rhodocyclaceae, Dechloromonas, Dokdonella, as the dominant aerobic denitrifying bacteria in R1 and R2, increase from 2.77%, 1.01%, and 1.47% of R1 to 7.62%, 3.80%, and 3.99% of R2. Hydrogenophaga, Pseudoxanthomonas, Zoogloea etc are only existed in R2, and the relative abundance are 1.11%, 1.08% and 0.96%. The total relative abundance of dominant aerobic denitrifying bacteria in R1 and R2 are 10.66% and 25.28%. This bacterium are grown under aerobic conditions, even under the high DO also have denitrifying activity. NH_4^+-N can be converted to nitrogen gas by the bacterium, also won't produce NO_2^--N/NO_3^--N accumulation. They can remove COD and have a short growth cycle, high growth rate and strong adaptability to environment and other ecological function [19]. Its relative abundance in R2 is more than twice R1, indicating that adding the filler can obviously improve the system of heterotrophic nitrification-aerobic denitrifying bacteria enrichment effect. This may be due to the surface of the filler is enriched by the species represented by the genus saprospiraceae to produce a large number of EPS under the low temperature operating conditions [20-21]. The 21%-50% contents of EPS are humic acid and other poly-acids, which are the main nutrient sources of aerobic denitrifying bacteria. The total relative abundance of dominant nitrifying denitrifying bacteria are respectively 28.65% of R1 and 60.23% of R2, which is the reason that the TN removal rate of R2 is higher than that of R1 by 25%. Combining with the waste-water treatment effect and the species abundance distribution shows that under the condition of low temperature (6.5±1℃), the dominant flora of R2 is richer than R1, and the relative abundance distribution is relatively uniform (11 of R1 and 16 of R2 species with specific ratio ≥1%).It indicates that the addition of fillers can help to increase the diversity and the relative abundance of nitrifying and denitrifying bacteria in the activated sludge system for providing a good bacterial environment of biological nitrogen removal in low temperature waste-water.

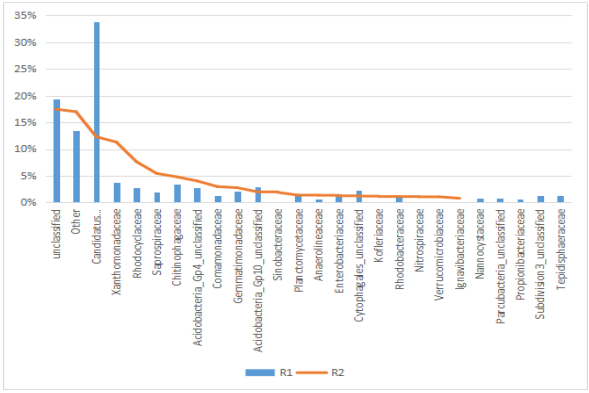

Fig. 3(a). Dominant strains (families)

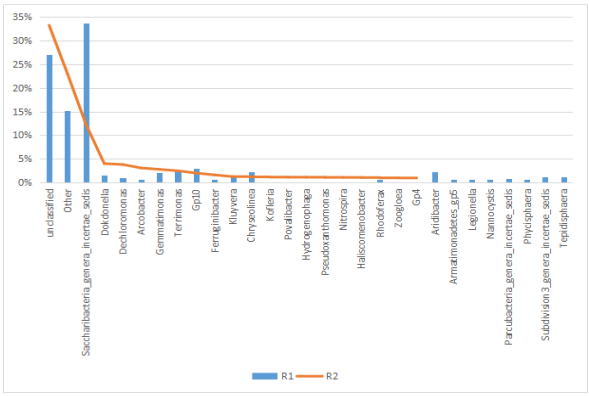

Fig. 3(b). Dominant strains (families, genera)

Table 4. Superiority Strains and Their Functions

Bacterial species	Role
Cytophagaceae	nitroge and phosphorus [22]
Sphingobacteriaceae	Denitrification [23]
Saprospiraceae	nitrogen and phosphorus
Hydrogenophaga	heterotrophic nitrification -

	aerobic denitrification [24]
Dechloromonas	Aerobic denitrification, ammonia oxidation
Zoogloea	aerobic denitrification [25]
Rhodocyclaceae	Heterotrophic nitrification aerobic denitrification
Terrimonas	aerobic denitrification [26]
Gemmobacter	denitrification
Nitrospira	nitrification
Arcobacter	autotrophic denitrification
Comamonadaceae	aerobic denitrification [27]
Xanthomonadaceae	Denitrification [28]
Chitinophagaceae	denitrification
Rhodobacteraceae	denitrification
Ferruginibacter	denitrification
Enterobacteriaceae	aerobic denitrification [29]
Planctomycetaceae	ammonia oxidation
Anaerolineaceae	metabolize organic carbon and nitrification [30]
Aridibacter	Denitrification [31]
Dokdonella	aerobic denitrification, nitrosation
Pseudoxanthomonas	heterotrophic nitrification - aerobic denitrification

4 Conclusion

At low temperature (6.5±1℃), the PPGC-SBMBBR reactor (R2) filled with Polyurethane Porous Gel Carrier has better treatment effect, especially the nitrogen removal effect and sludge settling ratio are significantly improved. The effluent quality, removal rate, and sludge settling rate of NH_4^+-N, NO_2^--N and TN are respectively 2.71mg/L and 92.9%; 0.02mg/L and 91.7%; 11.9mg/L and 75.3%; 0.232m/h.

The addition of fillers can increase the diversity and relative abundance of nitrifying and denitrifying bacteria in the activated sludge system to provide a good bacterial environment for biological nitrogen removal in low temperature sewage.

At the level of the phylum and the class, the dominant bacterium of SBR(R1) and PPGC-SBMBBR(R2) mainly have four kinds: Proteobacteria, Bacteroides, Candidatus Saccharibacteria and Acidophilus. They increase from 26.79%, 10.95%, 33.74% and 7.41%of R1 to 43.1%, 17.44%, 12.31%, and 8.27% of R2. The species of bacteria in R1 and R2 whose the relative abundance are more than 1% are 9 and 10, and the unique bacterial phylum in R2 is nitrifying bacteria (1.07%). Gammaproteobacteria, Betaproteobacteria, and Deltaproteobacteria are the dominant bacterium in Proteobacteria, which increases from 8%, 7.58%, and 3.55% of R1 to 16.3%, 14.24%, and 5.88% of R2.The species of bacterium whose the relative abundance are more than 1% in R1 and R2 are 14 and 16, and the unique bacterial bacterium in R2 are Nitrospira (1.07%), Verrucomicrobiae (1.07%), Ignavibacteria (0.81%), and Bacteroides (0.77%).

At the family and genus level, R1, R2 dominant AOB (ammonia oxidizing bacteria) are Dokdonella etc, and the total relative abundance are respectively 3.9% and 9.18%. R1, R2 dominant NOB (nitrite oxidizing bacteria) are Rhodocyclacea etc, and total relative abundance is respectively 3.47% and 13.2%. Nitrospira only in R2 is 1.07%.The total relative abundance of the dominant nitrifying strains of R1 and R2 are respectively 7.37% and 22.38%.The dominant anaerobic denitrifying bacteria of R1 and R2 are Sphingomonas and Xanthomonas, the total relative abundances are respectively 15.87% and 28.82%. The dominant heterotrophic nitrification-aerobic denitrifying bacteria in R1 and R2 are respectively Rhodobacteraceae, Dechloromonas, Dokdonella, which increase from 2.77%, 1.01%, and 1.47% of R1 to 7.62%, 3.80%, and 3.99% of R2. Hydrogenobacter, Pseudomonas, Zoogloea are R2 dominant aerobic denitrifying bacteria, and the relative abundance are respectively 1.11%, 1.08%, and 0.96%. The total abundance of dominant aerobic denitrifying bacteria in R1 and R2 are 10.66% and 25.28%. The dominant bacteria species with relative abundance more than 1% in R1 and R2 are respectively 11 and 16. The total relative abundance of R1 and R2 dominant nitrification and denitrifying bacteria are 28.65% and 60.23%.

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