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Analysis of Microbial Molecular Ecology Techniques in Constructed Rapid Infiltration System

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ABSTRACT: The microbial molecular ecology techniques, which were developed on the basis of molecular, were applied in studying the bacteria in Constructed Rapid Infiltration (CRI) system. These techniques are very efficient in better describing the bacterial diversity, microbial community distribution, and relations between microbial group structure and nitrogen contamination through the analysis of microbial nucleic acid sequence fragment in CRI. The results further revealed the removal mechanism of contamination, which is essential for the improvement of wastewater treatment in CRI.
KEY WORDS: microbial molecular ecology techniques, Constructed Rapid Infiltration (CRI), bacterial diversity, microbial community distribution.

INTRODUCTION

Constructed Rapid Infiltration (CRI) system, developed from traditional rapid infiltration system, is a new wastewater treatment technology (He et al., 2002). In this system, natural river sand is used as infiltration medium instead of original soil, which can produce good penetrability, increase hydraulic loading (1.0–2.0 m/d), and decrease the space occupation. The operation mode of wetting and drying cycle is adopted to stabilize and improve the treatment performance. CRI is obviously effective in dealing with urban domestic wastewater, slightly polluted surface water, and some small towns' sewage.

In recent years, the technical design of CRI has attracted considerable attention of researchers due to its better effect in wastewater treatment, but less research concerning the bacteria aspect in the system, which plays a key role in biodegradation. Most of the methods applied in studying the bacteria in CRI were pure culture techniques, which could only be used to

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analyze countable bacteria (Wang et al., 2006) and could not reflect original state of bacteria (Amann et al., 1995); thus, the results are limited and some important processes that really happen in CRI are still not clear.

With the development of microbial molecular ecology techniques and its application, environmental microbiology was brought into a revolutionary new era (Moter and Gobel, 2000; Amann et al., 1995). This allowed us to study the bacterial diversity and microbial community distribution at different depths in CRI. In this article, molecular ecology techniques, including polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), construction of bacterial 16S rDNA gene clone library, and phylogenetic analysis, were used to study bacterial diversity, microbial community distribution of nitrifying bacteria (ammonium-oxidizing bacteria and nitrite-oxidizing bacteria), and anaerobic ammonium oxidation bacteria through the analysis of gene sequence (Darby et al., 2001). The results not only revealed the removal mechanism of contamination but also played an important role in improving the stabilization and efficiency of wastewater treatment in CRI.

MATERIALS AND METHODS

Collection of Samples

All sand samples were collected at different depths in CRI, which were at stable operation in Shenzhen. The inlet and outlet water was sampled from the outlet of sedimentation tank and CRI, respectively.

Experimental Methods

DNA extraction and purification

DNA extraction was carried out according to Kuske et al. (1997) and purified by using the UNIQ-10 PCR clean-up kit to prevent large amounts of humic acids and organic substances from interfering with PCR amplification and microscopic detection.

PCR-DGGE for microbial community distribution

PCR amplification was followed and heteroduplex was removed by reconditioning PCR, which means that the PCR products were ideal for the following DGGE. DGGE, based on the protocol of Muyzer (Muyzer et al., 1993), was performed using the Bio-Rad D Gene System (Bio-Rad). The PCR products were loaded onto 8% (w/v) polyacrylamide gels in 1× TAE. The polyacrylamide gels were made with denaturing gradient ranging from 35% to 60%, where 100% denaturant contains 7 mol/L urea and 40% formamide. Electrophoresis was run for 5 h at 60 ℃, 150 V. After electrophoresis, the gels were stained for 10 min in SYBR Green I nucleic acid gel stain. The stained gel was immediately photographed on an UV transillumination table with a Video Camera Module. The quantity and brightness of DGGE band could reflect the number and abundance of bacteria, and gene sequence analysis of DNA in each band could be compared with known sequence in Genbank to estimate the bacterial species (Liu et al., 2005; Thompson et al., 2002; Muyzer et al., 1993).

PCR-DGGE for community distribution of nitrifying bacteria

Nest PCR amplification was carried out as described by Kowalchuk et al. (1998), Ovreas et al. (1997), Boon (2002), and Regan et al*.* (2002). DGGE was also the same as before.

Construction of 16S rDNA gene clone library for bacterial diversity

PCR amplification of 16S rDNA was carried out as described by Di Cello et al. (1997). The reconditioning PCR products were purified by using the UNIQ-10 PCR clean-up kit (Gong Sheng, Shanghai) and embedded into a pMD19-T plasmid vector. Transformation into DH5α and clone was followed to construct clone libraries. Then, different clones that were separated from restriction enzymes *Csp*6 and *Hin*fI were sent to Gong Sheng for sequencing. The sequences were analyzed by software, such as DNA Man and Gene Tool, and compared with the known sequence in Genbank to estimate the bacterial species.

Detection of anaerobic ammonium oxidation bacteria

PCR amplification was carried out with the specific primers Pla46rc and Amx820 as described by Luo and Hu (2005). The purified PCR products were

embedded into a pMD19-T plasmid vector, which was transformed into DH5α and cultured at 37 ℃ for 12 h. Then, blue-white selection was followed. One of the masculine clones, which were appraised by in-situ PCR, was sent to Sheng Gong for sequencing. The sequence was analyzed by software and compared with the known sequence in Genbank to estimate the bacterial species.

RESULTS AND DISCUSSION

Bacterial Diversity and Microbial Community Distribution in CRI

Because the experimental culture condition and culture medium were manually selected, the results analyzed by pure culture techniques could not reflect the in-situ state of bacteria (Jiang, 2007). On the contrary, PCR-DGGE method can analyze not only the countable bacteria but also the uncountable one; therefore, its results can reveal the bacterial diversity and microbial community distribution that were closed to the real system (Luo et al., 2003). DGGE could avoid the analytical warp in a traditional pure culture and was a powerful tool to analyze the diversity and changes in a complex microbial community (Xing et al., 2005). Moreover, sequence information determined from the analysis of DNA in each band could be used to estimate the bacterial species.

The bacterial 16S rDNA gene clone library was one of the classic tools in studying microbe constituent, which has been widely invoked for in water, soil, air, deposit, and wastewater treatment system samples. The results enriched the gene sequence database of environmental samples and could guide the separation and culture of bacteria (Baker et al., 2001; Brambilla et al., 2001).

Microbial community distribution in CRI analyzed by PCR-DGGE

DGGE profiles of different samples are shown in Fig. 1. The number of bank was different with different depth samples; the brightness and migration rate of the bank were also different. Generally speaking, the microbial community decreased from top to bottom in depth in CRI. In the upper 30 cm part, CRI was rich in microbial community (~18 species), which played some important roles in the biodegradation of COD

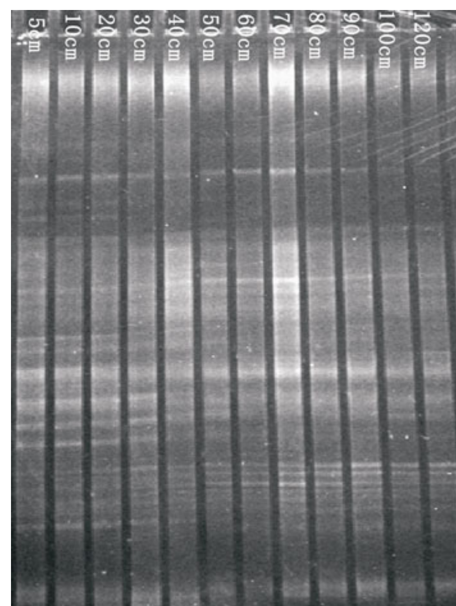

Figure 1. DGGE profiles of different samples.

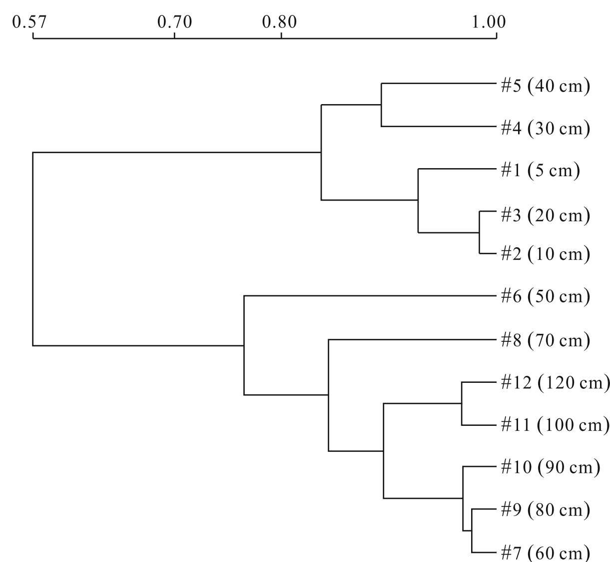

Figure 2. Cluster analysis of DGGE by UPGMA.

and NH_4-N, such as Firmicutes, α-proteobacterium. There were less than 12 species that existed below 40 cm, such as *Acidovorax*, *Nitrospira*, and *Clostridium* spp. The microbial diversity was prominently different spatially. *Clostridium* sp. was a kind of anaerobic bacteria, and this indicated the anaerobic microenvironment, which resulted from bad aeration process. However, the anaerobic microenvironment did not mean the high removal efficiency of total nitrogen (TN). Perhaps the activity of denitrifying bacteria was low or lacked some conditions, such as carbon source and alkalinity.

Bio-Rad Quantity One 4.5.1 was used to analyze

the profile of DGGE. The results showed that microbial community of the sand samples in the upper 40 cm part of CRI was similar to that of the below 50 cm (Fig. 2). However, the microbial communities were different from the upper part to the bottom, which resulted from the change of contamination concentration.

Bacterial diversity in CRI analyzed by 16S rDNA gene clone library

Because most bacteria were in the upper 30 cm part in CRI, a bacterial 16S rDNA gene clone library for sample at 10 cm depth was constructed to analyze bacteria diversity. Gene sequence was compared with known sequence in Genbank to estimate bacterial species, and the dominant group and the constituent ratio of bacteria were revealed.

One hundred and eight clones were picked stochastically and divided into 30 groups by restriction enzymes, such as *Csp*6 and *Hin*fI. One representative clone was sequenced from each group and the sequence was compared with the closest known relatives of the partial 16S rDNA segment in Genbank. If the compared results were the same, these clones were defined as an Operational Taxonomic Unit (OTU); therefore, 27 OTUs were obtained from the samples. The data of main OTUs in 16S rDNA clone library are shown in Table 1.

Table 1 Data of bacterial 16S rDNA clone library constructed with surface layer in CRI

OTU	No. & Percentage	Blast in GenBank (accession number, similarity)	Bacteria group
OTU1	18 (16.67%)	Uncultured bacterium clone (AY302113, 96%)	Uncultured bacterium
OTU2	3 (2.78%)	*Methylocystis* sp. (AY007196, 97%)	α-proteobacterium
OTU3	15 (13.89%)	Uncultured acidobacteria bacterium clone (AY921811, 97%)	Uncultured acidobacteria bacterium
OTU4	10 (9.26%)	Uncultured bacterium clone (EF516030, 98%)	Uncultured bacterium
OTU5	3 (2.78%)	Uncultured gamma proteobacterium clone (AY921861, 100%)	γ-proteobacterium
OTU6	2 (1.85%)	*Burkholderia thailandensis* (DQ388537, 95%)	β-proteobacterium
OTU7	13 (12.04%)	Uncultured acidobacteria bacterium clone (EF111087, 95%)	Uncultured acidobacteria bacterium
OTU8	2 (1.85%)	Uncultured bacterium clone (AF361091, 99%)	γ-proteobacterium
OTU9	2 (1.85%)	Uncultured planctomycetaceae bacterium (EF020316, 95%)	Uncultured planctomycete
OTU10	6 (5.56%)	Uncultured chloroflexi bacterium (AY922047, 96%)	Uncultured chloroflexi bacterium
OTU11	3 (2.78%)	Uncultured beta proteobacterium (AF204252, 96%)	β-proteobacterium
OTU12	2 (1.85%)	*Sphingomonadaceae bacterium* (AB220121, 96%)	α-proteobacterium
OTU14	7 (6.48%)	Uncultured planctomycete clone (DQ329794, 100%)	Uncultured planctomycete
OTU15	2 (1.85%)	*Acidovorax* sp. (AM084011, 96%)	β-proteobacterium
OTU16	2 (1.85%)	*Hyphomicrobium* sp. (AF279787, 96%)	α-proteobacterium
OTU17	6 (5.56%)	Uncultured planctomycete clone (AY922083, 98%)	Uncultured planctomycete
OTU18	2 (1.85%)	*Pseudomonas aeruginosa* (DQ464061, 100%)	γ-proteobacterium
OTU19	2 (1.85%)	*Chromobacterium* sp. (DQ985277, 100%)	β-proteobacterium

Twenty-seven OTUs in the clone library were divided into seven groups, including α-proteobacterium, β-proteobacterium, γ-proteobacterium, uncultured bacterium, uncultured Acidobacteria bacterium, uncultured planctomycete, and uncultured chloroflexi bacterium. Uncultured acidobacteria bacterium and uncultured bacterium were dominant in the clone library with the respective proportion of 39.8%, uncul-

tured planctomycete (13.89%), and β-proteobacterium (8.33%). The β-proteobacterium and bacteroidetes were predominant in wastewater treatment system as described by Wagner and Loy (2002), which has played an important role in biodegradation, removal of organism, and floccules formation. However, in CRI, uncultured acidobacteria bacterium and uncultured bacterium were dominant, which were caused by different contaminations and its concentration.

Chromobacterium, *Hyphomicrobium*, and *Pseudomonas* spp. also existed in clone library with a high percentage of 5.55% and were more than nitrite bacteria (0.925%), similar to the results of Yu et al. (2005).

It was noteworthy that uncountable bacteria were the dominant in the upper part of CRI. However, countable bacteria, such as *Pseudomonas aeruginosa* and *Chromobacterium* sp., were the dominant in pure culture. The results suggest that the culture techniques could not reflect the original state of bacteria, as described by Mobarry et al. (1996) and Jiang et al. (2008a, b).

Nitrogen Removal Bacteria in CRI

Nitrifying bacteria were composed of ammonium-oxidizing bacteria and nitrite-oxidizing bacteria, which are very important in the removal of NH_4-N. In CRI, NH_4-N is oxidized into NO_2-N by ammonia-oxidizing bacteria and then into NO_3-N by nitrite-oxidizing bacteria. As an electron acceptor, NO_3-N will be removed by reducing action in anaerobic system. There were no bacteria that could oxidize NH_4-N into NO_3-N directly without the intermediate product of NO_2-N.

Anaerobic ammonium oxidation (Anammox) was a recent discovery in the field of nitrogen removal. A better understanding of anaerobic ammonium oxidation bacteria would provide a new thought of technical improvement and the increase of denitrification for CRI.

Microbial community distribution of nitrifying bacteria in CRI analyzed by PCR-DGGE

In this article, PCR-DGGE was used to analyze microbial community distribution of nitrifying bacteria in different depths of CRI in order to find the removal mechanism of NH_4-N.

(1) Ammonium-oxidizing bacteria

DGGE profile (Fig. 3) shows that the microbial community increased and then decreased from top to bottom in CRI, which are controlled by change of contamination concentration. In the upper 10 cm part, there were only about five species, since the concentration of COD was high in the inlet. The autotrophic bacteria, such as nitrifying bacteria, were restrained by heterotrophic bacteria. From 20 to 60 cm, NH_4-N was adsorbed by sand and it was negatively electrically charged in wetting cycle and was oxidized into NO_2-N in the next drying cycle. Therefore, the CRI system was rich in microbial community by about 10 species. However, below 70 cm, most of NH_4-N was removed by oxidation with a removal efficiency of 89.2% (Ma et al., 2007), and the microbial community was only about three species.

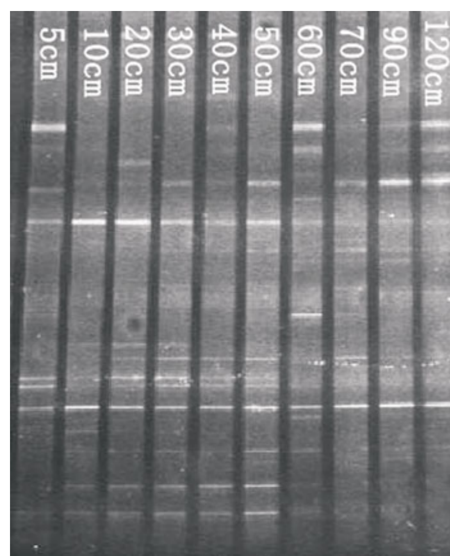

Figure 3. DGGE profiles of ammonium-oxidizing bacteria.

(2) Nitrite-oxidizing bacteria

DGGE profile is presented in Fig. 4. The results showed that bacterial diversity was relatively poor, and the microbial community increased from top to bottom. In the upper 50 cm, the amount of NO_2-N was less in the inlet and NO_2-N oxidized from NH_4-N would migrate downward. However, it was not adsorbed because of its negative electricity. As a result, the concentration of NO_2-N was low and the microbial community was less than two species. Then, the microbial community increased to about four species

because more and more NH_4-N was oxidized into NO_2-N in the process of infiltration and the bacteria were distributed into a stable and layered community correspondingly (Ma et al., 2008a, b).

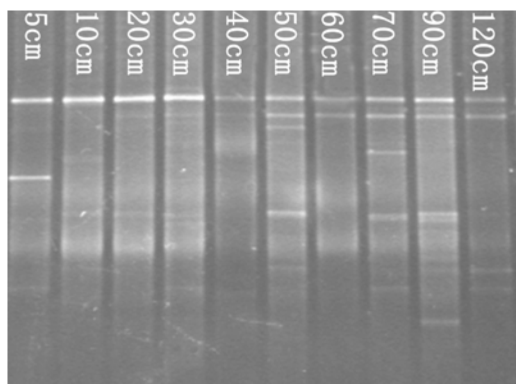

Figure 4. DGGE profiles of nitrite-oxidizing bacteria.

The bacterial diversity of ammonium-oxidizing bacteria and nitrite-oxidizing bacteria decreased rapidly below 90 cm depth in CRI. This indicates that most of NH_4-N and NO_2-N had been oxidized, and TN was in the form of NO_3-N. Only a small quantity of NO_3-N was reduced in denitrification and others were discharged. The removal efficiency of TN was only 30.6%.

In order to increase removal efficiency of TN, the outlet was suggested to place up to 100 cm depth. Therefore, it was water-filled and anaerobic below 100 cm, which would strengthen the denitrification. Meanwhile, sawdust was suggested to be added as carbon source for denitrification because it was consisted of cellulose (Schipper and Vojvodic-Vukovic, 1998), which could be biodegraded to some organic matter. The longer time it was taken to biodegradation, the longer the life of sawdust as carbon source would be. The above removal mechanism of nitrogen contamination in CRI can be seen in Fig. 5.

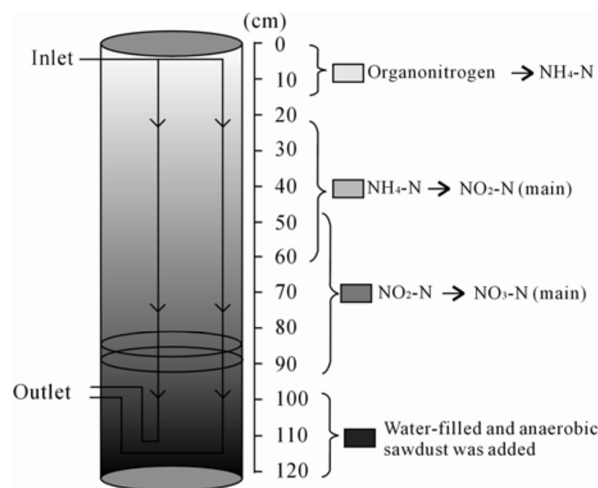

Figure 5. Removal mechanism of nitrogen contamination in CRI.

Detection of anaerobic ammonium oxidation bacteria by molecular ecology techniques

Shortcut nitrification-denitrification was carried out by anaerobic ammonium oxidation bacteria to a certain extent. If only shortcut nitrification-denitrification was done, 25% energy consumption would be saved and no other carbon source was needed (Verstraete and Philips, 1998). Specific primers of anaerobic ammonium oxidation bacteria were used for PCR amplification; then, cloning, sequencing, and phylogenetic analysis were performed.

The results showed that anaerobic ammonium oxidation bacteria existed at 50 cm depth in CRI, and the partial 16S rDNA sequence was 831 bp. Phylogenetic analysis indicated that this bacteria was close to anoxic biofilm clone Pla1-48, uncultured anoxic sludge bacterium KU2, and anaerobic ammonium-oxidizing planctomycete KOLL2a with the same function, and the DNA sequence similarity was above 97% (Jiang et al., 2008b). The existence of anaerobic ammonium oxidation bacteria provided a new thought of technical improvement and the increase of denitrification for CRI.

Three possible reasons can account for the existence of anaerobic ammonium oxidation bacteria at 50 cm depth in CRI. Firstly, there was anaerobic microenvironment below 50 cm since *Clostridium* sp. was detected in this area. Secondly, both NH_4-H and NO_2-N existed as substrate for biodegradation simultaneously, and NH_4-N as an electron donor would be removed below 50 cm. Finally, ammonium oxidation bacteria were the predominating population from 20 to 60 cm and were intergrowth with anaerobic ammonium oxidation bacteria. In one word, suitable living environment is essentially important for the existence of anaerobic ammonium oxidation bacteria.

CONCLUSION

In this article, a series of microbial molecular ecology techniques were applied in studying the bacteria in CRI. The microbial community distribution of bacteria was analyzed by PCR-DGGE qualitatively and a bacterial 16S rDNA gene clone library was constructed to analyze the bacterial diversity quantitatively. The anaerobic ammonium oxidation bacteria were proven to exist in CRI by phylogenetic analysis with a DNA sequence similarity of 97%. The relations between microbial groups' structure and nitrogen contamination and the removal mechanism of contamination were revealed. The basic theoretical finding in this article will be helpful to the stabilization and improvement of wastewater treatment efficiency in CRI.

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