Monitoring microbial community structure and succession of an A/O SBR during start-up period using PCR-DGGE

WANG Xiuheng*, ZHANG Kun, REN Nanqi, LI Nan, REN Lijiao

State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin 150090, China. E-mail: xiuheng@hit.edu.cn

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Abstract

Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) protocol was employed for revealing microbial community structure and succession in a sequential anaerobic and aerobic reactor performing enhanced biological phosphorus removal (EBPR) during start-up period. High phosphorus removal was achieved after 15 d. On day 30, phosphorus removal efficiency reached to 83.2% and the start-up was finished. DGGE profiles of periodical sludge samples showed that dominant microbial species were 19 OTUs (operational taxonomy units). Unweighted pair-group method using arithmetic averages (UPGMA) clustering analysis revealed that rapid community succession correlated to low phosphorus removal rate and high phosphorus removal efficiency reflected on steady community structure. Sequencing results indicated that determined sequences (12 OTUs) belonged to Proteobacterium, Actinobacteria, Gemmatimonadales and unailiated group. Proteobacterium, Tetrasphaera elongata and Gemmatimonas aurantiaca may act important roles in phosphorus removal. With little amount as known glycogen accumulating organisms, Candidatus Competibacter phosphatis still at accumulating-phase had limited effect on microbial community structure. When climax community was obtained, dominant microbes were 14 OTUs. Microbes in a large amount were uncultured bacterium Thauera sp., uncultured γ-Proteobacterium and Tetrasphaera elongata.

Key words: biological phosphorus removal; polyphosphate accumulating organisms; glycogen accumulating organisms

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Introduction

Because of an increased eutrophication of lakes and reservoirs, the enhanced biological phosphorus removal (EBPR) process is widely implemented in wastewater treatment plants to remove phosphorus, a limiting nutrient in algal blooms. In activated sludge polyphosphate accumulating organisms (PAOs) are capable of storing orthophosphate as intracellular polyphosphate, leading to phosphorus removal via biomass wasting. However, in deteriorated EBPR systems, glycogen accumulating organisms (GAOs) are often observed in a large amount (Thomas et al., 2003). The biochemistry pathway of GAOs is similar to PAOs except for performing anaerobic phosphorus release or aerobic phosphorus uptake. Thus GAOs do not contribute to phosphorus removal but increase the anaerobic VFA (volatile fatty acid) requirements (Saunders et al., 2003). Studies have found that a high COD/P ratio (e.g., >50 mg/mg) in the wastewater feed tends to favor the growth of GAOs instead of PAOs (Mino et al., 1998). Micrococcus phosphovorus, Lampropedia spp. etc., were ever considered as functional PAOs and then denied because of their disagreement with biochemistry pathway of PAOs (Nakamura et al., 1995; Santos et al., 1999; Stante et al., 1997; Seviour et al., 2003).

Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) was firstly applied to molecular ecology by Muyzer et al. (1993). It is particularly effective in analyzing the microbial community structure of non-culturable environmental samples such as microorganisms responsible for EBPR. They showed that a high diversity of phylogenetic groups presented in lab- and full-scale EBPR sludges (Seviour et al., 2003) which verified EBPR system is a complex microbial community. γ-Proteobacteria, β-Proteobacteria and Actinomyces sp. etc.
were reported to be responsible for EBPR (Onuki et al., 2002; Schuler et al., 2002; Nielsen et al., 1999; Yu et al., 2005).

Since process performance is likely to be strongly effected by the microbial composition, it is recommended that further studies be devoted to linking operational factors and phosphorus removal performance with microbial community structure predicting microbial population dynamics in EBPR system. The function of various microbes in forming a steady microbial community for better phosphorus removal is not clear. Furthermore, the succession of microbial community has not been well described, from which the information about adaptability of PAOs switched into high phosphorus content environment will be obtained. For this, an anaerobic-aerobic sequencing batch reactor (A/O SBR) to achieve better phosphorus removal with acetate as the carbon source was used. PCR-DGGE protocol was employed for revealing microbial community structure and succession during start-up period. A medium COD/P ratio (30 mg/mg) was investigated to determine the microbial competition of PAOs with GAOs.

1 Materials and methods

1.1 Reactor operation

A laboratory-scale A/O SBR with 2.5 L working volume was operated at (22 ± 1)°C to achieve EBPR. Seed sludge was taken from aerobic tank of Wenchang WWTP (waste water treatment plant) in Harbin, China. The running cycle of the reactor was 6 h consisting of a 90-min anaerobic period (8-min filling period included), a 240-min aerobic period, a 15-min sludge settling period and another 15-min for replacing supernatant. The components of synthetic wastewater (pH 7.0 ± 0.2) were as follows (per 50 L): NaAc·3H₂O 43.81 g, NH₄Cl 3.7 g, K₂HPO₄ 1.389 g, KH₂PO₄ 2.076 g, MgSO₄·7H₂O 2.5 g, CaCl₂·2H₂O 1.2 g, Peptone 1.25 g, trace elements liquid 15 mL. COD and PO₄³⁻-P were 300 and 10 mg/L, respectively.

1.2 Chemical analysis

COD (chemical oxygen demand), MLSS (mixed liquor suspended solids), MLVSS (mixed liquor volatile suspended solids) were analyzed by standard methods (APHA, 1995). The PO₄³⁻, NO₃⁻ and NO₂⁻ concentrations of the supernatant were measured by ion chromatography (DIONEX ICS-3000, USA) with an ion-pac AS11-HC column. TOC was measured by a TOC analyzer (TOC-VCPN, Shimadzu, Japan).

1.3 Microbial community analysis

1.3.1 Sampling, sample preparation and DNA extraction

The sludge samples of 0 (seed sludge), 6, 11, 15, 19, 26, and 30 d during start-up period were taken from the aerobic end, and stored in −20°C immediately. As all the sampling were finished, thawed the samples in 37°C water bath, then centrifuged at 9000 × g (Beckman 64R, J-25, USA) in 1.5 mL eppendorf tube for 5 min. The supernatant was removed and the sediment was cleaned up twice with PBS (phosphate buffered saline) solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 2 mmol/L KH₂PO₄, pH 7.4). The extraction of total genomic DNA was carried out by DNA Extraction Mini Kit (Watson, China) and detected by 0.8% (W/V) agarose gel electrophoresis.

1.3.2 PCR amplification

The region corresponding to positions 968 and 1401 in the 16S rDNA of Escherichia coli was PCR-amplified using the forward primer B129F (5’-CGCCTGCCGCGCCGCGCGCGCGG CGGGGGCGACGCGGGAACCGGAGAACTCTAC-3’) and reverse primer B850R (5’-CCGGCTCATTCC-TTTGAGTTC-3’) (Nuebel et al., 1996). The PCR amplification was conducted in an automated thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, USA). In order to improve the amplification specificity, Touchdown PCR was performed to provide sufficient DNA samples for DGGE, using the following protocol: initial denaturation of DNA for 5 min at 94°C; 30 cycles of 40 s at 94°C, 40 s at 55°C, and 30 s at 72°C; decreasing 0.1°C per cycle to 51.5°C, followed by a final extension for 8 min at 72°C. The PCR mixtures had a final volume of 30 μL which contained 3 μL of 10× Ex Taq buffer (containing Mg²⁺), 0.6 μL of each primer (20 pmol/L), 1.5 μL dNTP (2.5 mmol/L), 100 ng template and 0.2 μL Ex Taq (Takara, Japan). After PCR amplification, PCR products were electrophoresed in 0.8% (W/V) agarose gel for determining amplification effort.

1.3.3 Denaturing Gradient Gel Electrophoresis

DGGE was performed using the Dcode™ Universal Mutation Detection System (BioRad, USA). The PCR products were electrophoresed directly to 6% polyacrylamide gel in 1× TAE buffer containing a linear gradient ranging from 30% to 60% denaturant (denaturation of 100% corresponding to 7 mol/L urea and 40% (V/V) formamide). The gradient gel was cast with a gradient delivery system (Model 475, Bio-Rad, USA). Electrophoresis was run at 60°C with a constant voltage of 120 V. After 5 h of electrophoresis, the gel was stained with AgNO₃, and then visualized by a scanner (PowerLook1000, Umax, Taiwan, China).

1.3.4 Sequencing and cluster analysis

Prominent DGGE bands were excised from DGGE polyacrylamide gel for 16S rDNA fragment sequencing. The reamplified DNA fragments were purified by Gel Recovery Purification Kit (Watson, China) and cloned by the pMD19-T plasmid vector system (TaKaRa, Japan). The DNA sequences were determined using the chain-termination method in an ABI 3730 stretch sequencing system by a commercial service (Sangon, China). Vector sequence was cut off and the rest was compared in GenBank using BLASTN program to obtain the most similar 16S rDNA fragments. Cluster analysis of DGGE patterns was performed with unweighted pair-group method using arithmetic averages (UPGMA) clustering algorithm.
2 Results and discussion

2.1 Reactor performance

The start-up period of reactor lasted for a month. The MLSS of the reactor increased from 1902 to 2315 mg/L as the microorganism growth. The COD removal efficiency was about 85% without large fluctuation and outlet COD concentration was less than 100 mg/L for all the time. Phosphorus removal and anaerobic phosphorus release performance in the start-up period can be divided into two stages, as shown in Fig. 1. In the former 15 d, good phosphorus removal efficiency was not achieved (only from 38.3% to 49.0%) and anaerobic phosphorus release was not notable. At the following 15 d, phosphorus removal performance and phosphorus release increased rapidly. On day 30, phosphorus removal efficiency and anaerobic outlet phosphorus concentration reached to 83.2% and 19.22 mg/L, respectively.

2.2 DGGE profiles and UPGMA clustering analysis

The DGGE profiles of sludge samples in start-up period are shown in Fig. 2. Since each band can be considered as an operational taxonomy unit (OTU), the DGGE profiles showed that dominant microbial species were 19 OTUs, which indicated a diverse microbial community. Microbial community structure was much complex on day 0 while most microbes existed in few quantities.

Since DGGE profiles can make a half-quantitative analysis of microbes, relative quantities of various microbes during start-up period are shown in the profiles. The quantities of some microbes increased (band 8, 10, 12, 15, 18 etc.), whereas others decreased (band 3, 13, etc.) or even clear away (band 4, 16, etc.). There are still some microbes keeping their quantities, such as band 5, 6, and 14. Community structure changed a lot in the former 15 d while kept almost the same from then on. With the enrichment of some functional microbes, community structure was simple and steady at the end which led to better phosphorus removal.

The UPGMA clustering analysis was used to analysis community similarity among different time (Fig. 3). It showed that microbial community structure in seed sludge had the lowest similarity. The rapid change of community structure at the first 6 d resulted from phosphorus shock load. Synthetic wastewater (10 mg P/L) was unfit for existence of some microbes original from municipal wastewater (about 3.2 mg P/L). PAOs responsible for phosphorus removal tended to be dominant in reactor with high phosphorus. In the following time, community succession rate declined. Community structure of 6 and 11 d, 19 and 26 d was almost the same. The community structure of former 15 d was obviously different from latter 15 d. When compared with phosphorus removal effect (Fig. 1), a correlation between community structure and phosphorus removal effect can be detected. In the former 15 d, rapid community succession correlated to lower phosphorus removal rate, whereas higher phosphorus removal efficiency reflected on steady community structure after then.

2.3 Sequencing results and functional analysis of microbes

16S rDNA fragments extracted from prominent DGGE bands were re-amplified, purified, cloned to PMD19-T vector, and sequenced. The closest relative strains available in the GenBank database were obtained using Blastn Program (Table 1). Among the 19 OTUs, the similarity between 12 OTUs and the closest sequences were more than 94%, which indicated that they may belong to the same genus. In these sequences, 6 bacteria clones belonged to Proteobacterium (1 to α-Proteobacterium; 2 to β-Proteobacterium; 3 to γ-Proteobacterium). Two bacteria clones were most similar to Actinobacteria and
Gemmatimonadales. There are other 4 bacteria clones that can not affiliate to putative group.

Recently, *Rhodocyclus* genus from β-Proteobacteria (related to *Candidatus Accumulibacter* phosphates) has gained increasing attention as the most possible PAOs candidates and a group of Actinobacteria is probably a new type of PAOs (Ahn et al., 2002; Lee et al., 2004). The previous publications showed that GAOs may belong to γ-Proteobacteria, such as *Candidatus Accumulibacter* phosphates, and α-Proteobacteria (Nielsen et al., 1999; Wong et al., 2004; Meyer et al., 2006).

In this study, bacteria clones of *Proteobacteria* can not be found in seed sludge and then accounted for larger proportion at the end of start-up period. *Thauera* sp. (band 10) and uncultured γ-Proteobacterium (band 12), performed an increasing trend especially from 19 d, and uncultured bacterium of β-Proteobacterium (band 8) from 11 d. Most of them were uncultured bacteria clone without fully understanding on their function for phosphorus metabolism. As quantities of *Proteobacteria* increased along with obvious improvement of phosphorus removal on day 11 and 19, they may have a positive effect in this reactor. At the end, *Proteobacterium* formed important part in microbial community structure with better phosphorus removal.

*Tetrasphaera elongata* from Actinobacteria represented by band 14 existed in large amount all over the start-up period, even in seed sludge. Hanada et al. (2002) isolated *T. elongata* by traditional method from an A/O SBR, exhibiting positive Neisser-staining and implying a role in the biological phosphorus-removal process. As the seed sludge performed phosphorus removal to some extend, the effect and perfect adaptation for both low and high phosphorus of *T. elongata*, which the only strain in seed sludge was proposed as PAOs, should not be neglected and require further study.

*Candidatus Accumulibacter* from Gemmatimonadales was firstly isolated by Zhang et al. (2003) and was proved of the ability to accumulate polyphosphate. *Gemmatimonas aurantiaca* (band 17) was not detected in seed sludge. It appeared on day 15 and grew better in the following time. Although *G. aurantiaca* always lived in little amount, its appearance corresponded to rapid increasing of phosphorus removal effect obviously. Therefore, it plays substantial role in the EBPR system but has a long adapting phase when converted to high phosphorus. The present results suggest that *G. aurantiaca* had stronger ability of phosphorus removal even at low quantities.

*Candidatus Competibacter* phosphates (band 19) appeared at the end of the start-up period (on day 26) with little amount. The organism belonging to the γ-Proteobacteria was identified as GAO phenotype and named by Crocetti et al. (2002). It has frequently dominated lab-scale cultures fed with acetate (Crocetti et al., 2002; Zeng et al., 2003; Oehmen et al., 2004). Better phosphorus removal here indicated that *Candidatus Competibacter* phosphates occurrence had not caused serious negative effect as mentioned by other researchers. This is mainly because all the previous studies have been conducted at an already deteriorated EBPR system. Little information on the beginning of GAO competition process and succession is available. The results showed that an accumulating-phase was necessary for GAO mature and a lag-stage existed when taking the performance of the reactor into consideration. It was also demonstrated that it is possible for an EBPR system to operate with an insignificant *Candidatus Competibacter* phosphates population.

For the bacteria clones which have the similarity more than 94% but represent unaffiliated group (4 OTUs), they all belonged to uncultured bacterium with less knowledge. Some of them are familiar in EBPR processes (band 2, 18), which may benefit phosphorus removing and stable community structure. Moreover, the similarity between some sequencing results and the closest sequences are less than 94% (7 OTUs), which indicated that their identities were uncertain.

### Table 1 Partial 16S rDNA sequence similarity of bands 1–19 on DGGE profiles

<table>
<thead>
<tr>
<th>Band</th>
<th>The closest sequences (GenBank number)</th>
<th>Similarity</th>
<th>Putative division</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uncultured α-Proteobacterium clone Ebpr9 (AF255630.1)</td>
<td>94%</td>
<td>α-Proteobacterium</td>
</tr>
<tr>
<td>2</td>
<td>Uncultured bacterium clone SBRQ157 (AF361092.1)</td>
<td>96%</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Type 0803 filamentous bacterium strain Beats5B (X86070.1)</td>
<td>90%</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td><em>Brevundimonas</em> sp. dcm7A (AF430126.1)</td>
<td>90%</td>
<td>α-Proteobacterium</td>
</tr>
<tr>
<td>5</td>
<td><em>Frigovigula</em> patogeniensis (AF450134.1)</td>
<td>90%</td>
<td>Clostridia</td>
</tr>
<tr>
<td>6</td>
<td><em>Thauera</em> sp. A7 (AY750693.1)</td>
<td>90%</td>
<td>β-Proteobacterium</td>
</tr>
<tr>
<td>7</td>
<td>Uncultured <em>Verrucomicrobiium</em> DEV055 (AJ401121)</td>
<td>96%</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>Uncultured bacterium FukuN108 (AJ299984.1)</td>
<td>94%</td>
<td>β-Proteobacterium</td>
</tr>
<tr>
<td>9</td>
<td><em>Dyella ginsengiol</em> strain LA-4 (EF191354.1)</td>
<td>97%</td>
<td>γ-Proteobacterium</td>
</tr>
<tr>
<td>10</td>
<td><em>Thauera</em> sp. R-24450 (AM231040.1)</td>
<td>99%</td>
<td>β-Proteobacterium</td>
</tr>
<tr>
<td>11</td>
<td><em>Nitrospira</em> sp. strain RC25 (Y14639.1)</td>
<td>91%</td>
<td>Nitrospirales</td>
</tr>
<tr>
<td>12</td>
<td>Uncultured γ-Proteobacterium clone AKYG1642 (AY921834.1)</td>
<td>99%</td>
<td>γ-Proteobacterium</td>
</tr>
<tr>
<td>13</td>
<td>Uncultured α-Proteobacterium strain C11-18 (AJ294357.1)</td>
<td>92%</td>
<td>α-Proteobacterium</td>
</tr>
<tr>
<td>14</td>
<td><em>Tetrasphaera</em> elongata strain ASP12 (AB051430.1)</td>
<td>98%</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>15</td>
<td>Uncultured <em>Myxobacteriaceae</em> bacterium clone Elyv.16S_1104 (EF019585.1)</td>
<td>91%</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>16</td>
<td>Uncultured bacterium clone YSK16S-27 (EF612990.1)</td>
<td>94%</td>
<td>–</td>
</tr>
<tr>
<td>17</td>
<td><em>Gemmatimonas aurantiaca</em> (AB072735.1)</td>
<td>100%</td>
<td>Gemmatimonadales</td>
</tr>
<tr>
<td>18</td>
<td>Uncultured bacterium clone FG-EBPR-61 (AY521685.1)</td>
<td>98%</td>
<td>–</td>
</tr>
<tr>
<td>19</td>
<td><em>Candidatus Competibacter</em> phosphates clone SBRQ22 (AY172162.1)</td>
<td>99%</td>
<td>γ-Proteobacterium</td>
</tr>
</tbody>
</table>

*: una affiliated group.
2.4 Stable microbial community structure at the end of start-up period

Figure 2 shows that the structure of microbial community and the relative quantity of various microbes tended to be stable from day 19. Corresponding to better phosphorus removal efficiency, a stable climax community was achieved at the end of start-up period.

DGGE profiles showed that dominant microbes in climax community were 14 OTUs. Microbes in a large amount were uncultured bacterium (band 8), Thauera sp. (band 10), uncultured γ-Proteobacterium (band 12), T. elongata (band 14) and uncultured bacterium (band 18). Among these microbes, T. elongata had been isolated and proved to have the ability of accumulating polyphosphate. Since the function of microbes may not be in proportion with their quantity, the action of microbes which account for little proportion should not be neglected, such as G. aurantiaca mentioned before. The function of other microbes, such as uncultured bacterium (band 8, 18), need further study.

3 Conclusions

A SBR reactor was operated under sequential anaerobic and aerobic conditions for phosphorus removal fed with acetate. The 30 d start-up period can be divided into two stages when analyzing the phosphorus removal efficiency. The 15 d lag phase followed by a rapid increasing of phosphorus removal efficiency which reached to 83.2%.

DGGE profiles of sludge samples showed that dominant microbial species were 19 OTUs during start-up period. UPGMA clustering analysis revealed that lower phosphorus removal rate correlated to rapid community succession and higher phosphorus removal efficiency reflected on steady community structure.

Sequencing results indicated that determined sequences (12 OTUs) belonged to Proteobacterium, Actinobacteria, Gemmatimonadetes and unaffiliated group. Proteobacterium may have a positive effect on a phosphorus removal microbial community structure. Tetraspheara elongata and G. aurantiaca may act important roles in phosphorus removal in this study condition. With insignificant amount as known GAOs, Candidatus Competibacter phosphatis still at accumulating-phase had a limited influence on phosphorus removal and microbial community structure.

A stable climax community was achieved at the end of start-up period. Dominant microbes in climax community were 14 OTUs. Microbes in a large amount were uncultured bacteriums, Thauera sp., uncultured γ-Proteobacterium and T. elongata. The action of microbes which account for little proportion should not be neglected, such as G. aurantiaca and Candidatus Competibacter phosphatis.

Acknowledgments

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