

EFFECT OF CONTROL SYSTEMS ON BACTERIA COMMUNITY STRUCTURE IN SEQUENCING BATCH BIOFILM REACTORS TO TREAT WASTEWATER

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Abstract. This study aimed to evaluate the effect of control systems, including an intelligent control system (ICS) and a conventional timer control system (TCS), on bacteria community structure. Clone library methods for 16S rDNA were used in sequencing batch biofilm reactors (SBBRs) to treat wastewater. A total of 108 clones were randomly selected and individually sequenced from 16S rDNA libraries. Results indicated that the community structure was different in each SBBR under different controlled systems. The dominant bacterial communities and their contents showed significant differences. The dominant bacterial community Bacteroidetes has a proportion of 45%, whereas β -proteobacteria has 37%. The significant difference in the performance of nitrogen and phosphorus removal in ICS from TCS was the dominant bacterial community.

Keywords: *intelligent control system (ICS), sequencing batch biofilm reactor (SBBR), 16S rDNA library, bacterial community, denitrification*

Introduction

The most fundamental method for controlling water eutrophication and preventing water pollution is to treat pollution sources and control pollutant emissions. This approach will enable the nitrogen and phosphorus obtained from the wastewater treatment plants to meet emission standards (Zou et al., 2006). The wastewater discharge standard at present is increasingly becoming strict in all countries around the world. Many new and original wastewater treatment plants are facing the pressure of standard nitrogen and phosphorus emissions (Mielcarek et al., 2015; Ge, 2010; Wen et al., 2015).

Sequencing batch biofilm reactor (SBBR) is novel of composite biofilm reactor and a new technology for nitrogen and phosphorus removal that is being studied by scholars at home and abroad. SBBR utilizes time sequence. Construction cost is low because biological reaction proceeds in a single tank without physical partition (Yang et al., 2014). Given a large amount of biomass, the reactor can resist shock load (Goh et al., 2009; Fu et al., 2018). Sludge bulking is not a problem because of low sludge

Production (Amulya et al., 2015; Zhao et al., 2017) SBBR has been used in the research of nitrogen and phosphorus removal from urban wastewater (Choi et al., 2008; Zhang et al., 2009; Fu et al., 2010; Hosseini et al., 2012). Microorganisms in SBBR are attached to the carrier to form a biofilm with long sludge age and food chain. The

biofilm has certain thickness. Under aerobic conditions, the biofilm creates a hypoxic microenvironment where nitrification and denitrification can occur simultaneously; this condition leads to high nitrogen removal efficiency (Isanta et al., 2015). Changing the operating conditions of the reactor will change the environment where microorganisms depend on for survival, as well as the bacterial community structure of microorganisms, thus leading to the change of nitrogen and phosphorus removal efficiency (Aydin et al., 2015). Therefore, the present study on the effect of control systems on the bacterial community structure in SBBR system is important to optimize the bacterial community structure and improve the efficiency of nitrogen and phosphorus removal.

The 16S rDNA clone library method is used to amplify and analyze the full-length sequence of 16S rDNA to analyze the bacterial community structure in the environmental samples (Cantafio et al., 1996; Sanapareddy et al., 2009; Yu et al., 2010). Given its high speed, accuracy, good repeatability, and abundant information, this method has become the main approach used in the research of bacterial community structure and diversity in wastewater treatment systems. In the present paper, two SBBRs under different control systems were adopted to treat urban wastewater. SBBR under an intelligent control system (ICS) controls the process of wastewater treatment based on DO and temperature. By contrast, SBBR under a timer control system (TCS) changes the operation conditions based on time setting. The 16S rDNA clone library method was adopted to study the similarities and differences of the bacterial community structures of these two reaction systems and to determine the dominant bacterial communities. This approach identifies the effect of control systems on the bacterial community structure of the system.

Materials and methods

SBBRs configurations and operational conditions

Experiments were conducted in two SBBRs controlled by an ICS and a TCS. *Figure 1* shows that the acrylic reactors were composed of polymethyl methacrylate with a capacity of 30 L and a working volume of 20 L. Two SBBRs and operations conditions were described in an earlier publication (Jin et al., 2012).

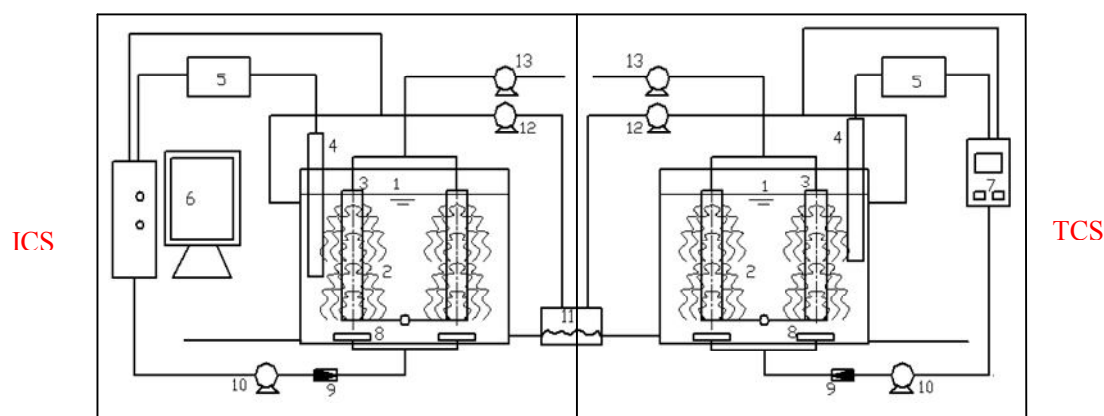


Figure 1. Schematic of two SBBRs. 1. Bioreactor. 2. Fiber threads carrier. 3. Cylinder. 4. DO sensor. 5. DO detector. 6. Computer. 7. Timer. 8. Porous stones. 9. Rotameter. 10. Air compressor. 11. Thermostatic basin. 12. Circulation pump. 13. Drawn pump

Synthetic wastewater

Synthetic wastewater was used as feed to evaluate SBBR performance. Wastewater was prepared based on the characteristics of Chinese municipal wastewater with the following composition (concentrations in mg/L): glucose (C₆H₁₂O₆) (332); NH₄Cl (213 at C/N ratio of around 5.0) l; KH₂PO₄ (21.95); ZnCl₂ (50), MgSO₄ (50); and a 1 ml trace element mixture consisting of CaCl₂, CuCl₂, NiCl₂, FeSO₄ and H₃BO₃. NaHCO₃ measuring 100 mg/L was used to maintain the pH of the wastewater at 6.8 to 7.2. Synthetic wastewater is characterized in *Table 1*. All chemicals and reagents were of analytical grade.

Table 1. Characteristics of synthetic wastewater during stable operation

Parameter	COD (mg/L)	NH ₄ ⁺ -N (mg/L)	TN (mg/L)	TP (mg/L)	pH
Values	326 ± 30	61 ± 10	65 ± 10	5 ± 1	7 ± 0.2

Experimental methods

Sample collection and DNA extraction

After about five weeks of domestication, the biofilm was successfully cultured in two reactors before being entered into the operating period. Through 30 days of sample analysis, the results showed that the reaction period was 6 h for ICS and 9 h for TCS. The operation results of a cycle were extracted as the example. The concentrations of COD, TN, NH₄⁺-N, and TP in inflow water were 309.5, 63.61, 60.28, and 5.16 mg/L, respectively. The removal rates of COD, TN, NH₄⁺-N, and TP were 93.4%, 85.6%, 100%, and 100% in ICS and 91.1%, 70.9%, 100%, and 100% in TCS, respectively. Thus, TN removal rate in ICS was 14.7% higher than that in TCS.

Bacteria Sampling of the two reactors obtained according to the method of Hao et al. (2009). During the stable operation of two SBBRs, the biofilm samples were taken with sterile scissors and sterile brush from the fillers at different vertical depths and horizontal positions in the SBBRs under ICS and TCS. The samples were dissolved in 500 mL distilled water and fully shaken. The supernatants were removed after precipitation for 30 min. Then, 40 mL of the solutions was taken from the precipitation layers and placed in 50 mL centrifuge tubes. After 3 min of centrifugal separation (5147R centrifuge, Germany, Eppdorf) at the rate of 1000 r/min, 30 mL of sterile TE buffers (10 mmol/L, Tris-HCl, 1 mmol EDTA, adjusted to pH = 8, 121 °C and sterilized for 20 min) was added in the solutions. The supernatants were removed after re-centrifugation. Then, 15 mL of distilled water was added and fully shaken. Afterward, 5 mL of solution was taken for DNA extraction in the biofilms. The pretreated water samples were filtered (0.22 μm bacterial filter film). The filter films were soaked in 10 mL of sterilized saline water after filtration and then placed in an ultrasonic cleaning instrument (KH2200DB, Kunshan Hechuang in China) and shaken for 10 min. The solutions were then repeatedly beaten by the liquid drawing gun to enable the cells on the membrane to enter completely into the solutions. The solutions were placed in centrifuge tubes (1.5 mL) and underwent centrifugation for 3 min at the rate of 10000 r/min to facilitate bacteria collection. The metagenomics DNA extraction kits produced by Shanghai Sangon were adopted to extract ICS samples and TCS samples.

16S rDNA gene full-length amplification and carrier connection transformation

Bacteria 16S rDNA universal primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3', M = CorA) and 1492R (5'-CTACGGCTACCTTGTACGA-3', T = 3', Y = CorT) were adopted to amplify the target genes of two groups of samples (Masoud et al., 2011). The PCR reaction procedure (Hao et al., 2009) was predegenerated at 95 °C for 5 min, degenerated at 94 °C for 30 s; annealed at 52 °C for 30 s, and extended to 72 °C for 2 min for a total of 35 circulations. The procedure also involved extension at 72 °C for 10 min before preservation at 4 °C. PCR amplification products were detected by 1.2% agarose gel electrophoresis. The products were connected to PGEM-T clone by PGEM-T cloning (Promage Company) kits and heat-shock transformed into *Escherichia coli* DH5 α competent cells. The products were then coated with Amp/IPTG-Gal that contains LB culture mediums. After 16 h of culture at 37 °C, the samples were subjected to 4 °C color development process for 1 h. White-blue plaque selection and white plaque validation were conducted. The positive clones in each sample were randomly selected for sequencing by Shanghai Sangon Co., Ltd.

System development analysis

Chimera detection was conducted on the sequences using a Chimera detection program on the Ribosomal database project (RDP) website. The obtained sequences were then classified by Dutor software, and similar sequences were excluded. The remaining sequences were entered into the BLAST facility of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Comparative analysis was conducted on the remaining sequences and those existing in GenBank + EMBL + DDBJ + PDB. Sequences with the highest similarity and those with species that have high similarity were downloaded as reference. All sequences were compared by ClustalW program in BioEdit. The system development tree diagram was generated using MEGA 4.0.

Results and discussion

Bacterial diversity analysis of samples in ICS and TCS

The sequences were analyzed with Dutor software. After eliminating similar sequences, ICS obtained 30 genotypes and 32 for TCS. The sequence of each genotype in ICS and TCS was entered into the RDP website. The sample system group was determined by a classifier program. After analysis, 16S rDNA sequences of 108 positive clones of bacteria were identified in the ICS sample library, which belonged to 7 bacterial communities (as shown in *Figs. 2 and 3*). These sequences are *Bacteroidetes* (45%), *β -proteobacteria* (27%), *γ -proteobacteria* (16%), *α -proteobacteria* (3%), *Verrucomicrobia* (3%), *Chloroflexi* (3%), and unclassified *Proteobacteria* (3%). The dominant bacterial communities were *Bacteroidetes* (45%), *β -proteobacteria* (27%), and *γ -proteobacteria* (16%). The 16S rDNA sequences of 108 positive clones of bacteria in TCS sample library belonged to 9 bacterial communities (as shown in *Figs. 4 and 5*), namely, *β -proteobacteria* (37%), *γ -proteobacteria* (25%), *Bacteroidetes* (24%), *δ -proteobacteria* (3%), *Firmicutes* (3%), *Nitrospira* (3%), *Actinobacteria* (3%), *Acidobacteria* (3%), and unclassified *Proteobacteria* (5%). The dominant bacterial communities in ICS were *Bacteroidetes*, *β -proteobacteria*, and *γ -proteobacteria*, which account for 45%, 27%, and 16%, respectively. The dominant bacterial communities in

TCS were β -proteobacteria, γ -proteobacteria, and *Bacteroidetes*, accounting 37%, 25%, and 24%, respectively. Different operation modes and different growth environments for microorganism between ICS and TCS led to different dominant bacterial communities and different community structures. In ICS, the growth environment for biofilm microorganisms was (aerobic/hypoxic)ⁿ. *Bacteroidetes* was the dominant bacterial community, which has a proportion of 45%. In TCS, the growth environment for biofilm microorganisms was anaerobic/aerobic, in which β -proteobacteria was the dominant bacterial community with a proportion of 37%. The community structure of ICS was more stable than that of TCS, and the communities in ICS were less than those in TCS.

System development analysis of bacteria in samples in ICS and TCS

To further understand the system development of bacteria in biofilm samples in ICS and TCS, the genotypes that represent different sequences were ordered in accordance with the principle of maximum homology. This approach was adopted to construct the system development tree diagrams of Proteobacteria and other communities in ICS and TCS gene libraries, as shown in *Figures 4, 5, 6, and 7*. *Figures 4 and 5* show the system development trees of Proteobacteria and other communities based on 16S rDNA sequence in ICS system. *Figures 6 and 7* show the system development trees of Proteobacteria and other communities based on 16S rDNA sequence in TCS system.

System development analysis of bacterial in ICS

In ICS, *Bacteroidetes* was the dominant community with a proportion of 45%. This finding means that nearly half of bacteria in the biofilm samples in ICS were *Bacteroidetes*. The most similar structure of this sequence is from eutrophic lakes (Cantafio et al., 1996), which include 12 genotypes of uncultured bacteria. The bacteria in *Bacteroidetes* community were bacillus of different sizes. These bacteria were reported only in the phosphorus removal system, but were barely investigated in the activated sludge and nitrogen removal system (Amann et al., 1995). ICS did not adopt anaerobic phosphorus release, but it showed significant phosphorus removal effect. Moreover, the performance of ICS in TN removal was obviously better than that of TCS. Thus, we can conclude the presence of microorganisms with functions of anoxic phosphorus uptake and denitrifying phosphorus removal, which exist in the form of polyphosphate under the aerobic condition in *Bacteroidetes* (Liu et al., 1996). Therefore, new bacteria exist that are related to nitrogen and phosphorus removal in *Bacteroidetes*. These bacteria accounted for 27% of bacteria in the library, including 9 genotypes. The homology between Z-28 and *Thauera sp.* (CP001281) was as high as 99%. *Thauera sp.* was the denitrification bacterium that could remove NO₃⁻-N and PO₄³⁻-P from wastewater through denitrification (Manz et al., 1994; Amann et al., 1995). This bacterium played a very important role in denitrification and in the degradation process of organic matters (Ayara et al., 2011). In the anoxic or aerobic period, the exterior of the biofilm was the aerobic layer and the interior was the special structure for the anaerobic/anoxic layer. This structure provided favorable conditions for the existence of denitrifying bacteria and benefited nitrogen removal or denitrifying phosphorus removal in ICS. The other clones were uncultured bacteria. The γ -proteobacteria was the third largest bacterial community with the proportion of 16% in the library. This bacterium included three genotypes, among which the homology

between Z-15 and *Xanthomonas sp.* (AJ786786) was 98%. *Xanthomonas sp.* had strong degradation ability for organic carbon. Thus, it was beneficial for COD removal.

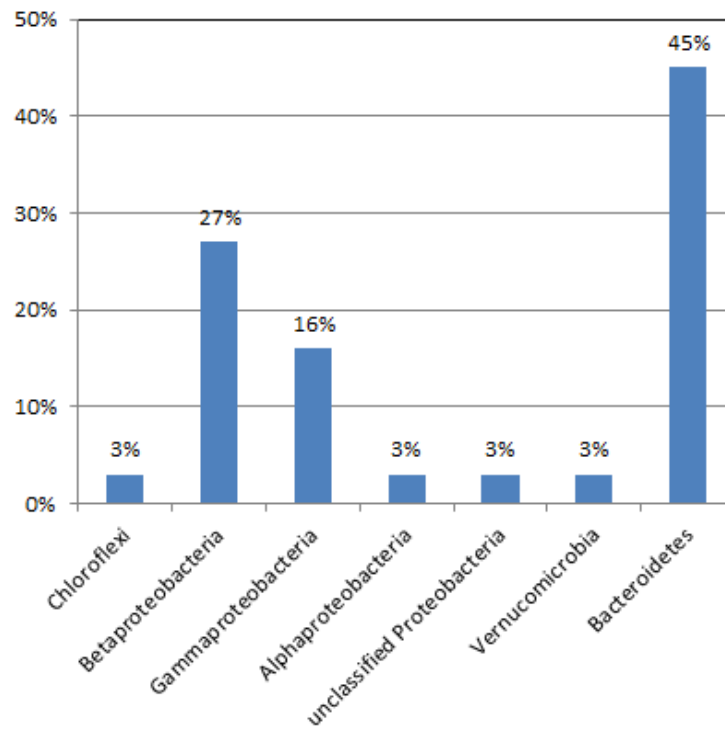


Figure 2. Proportion of phyla in the clone library from ICS

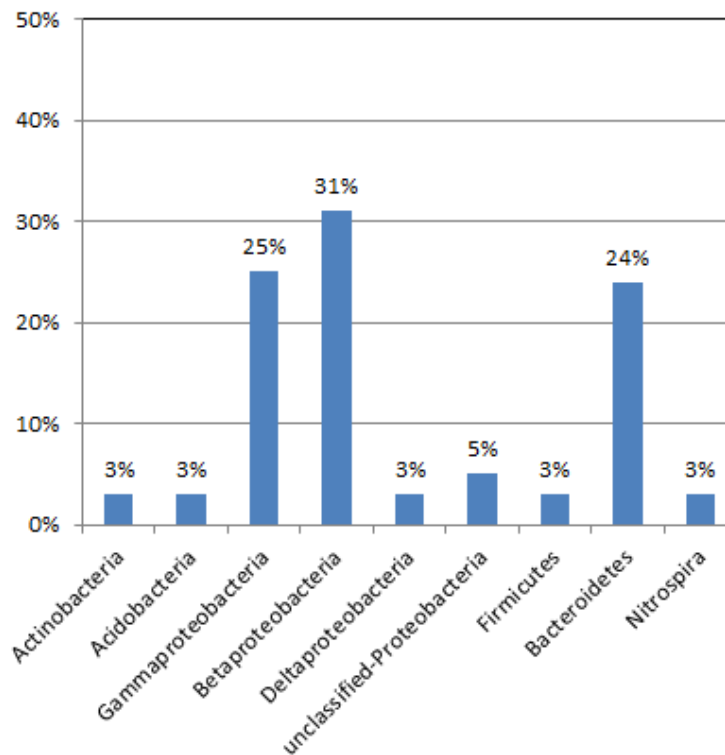


Figure 3. Proportion of phyla in the clone library from TCS

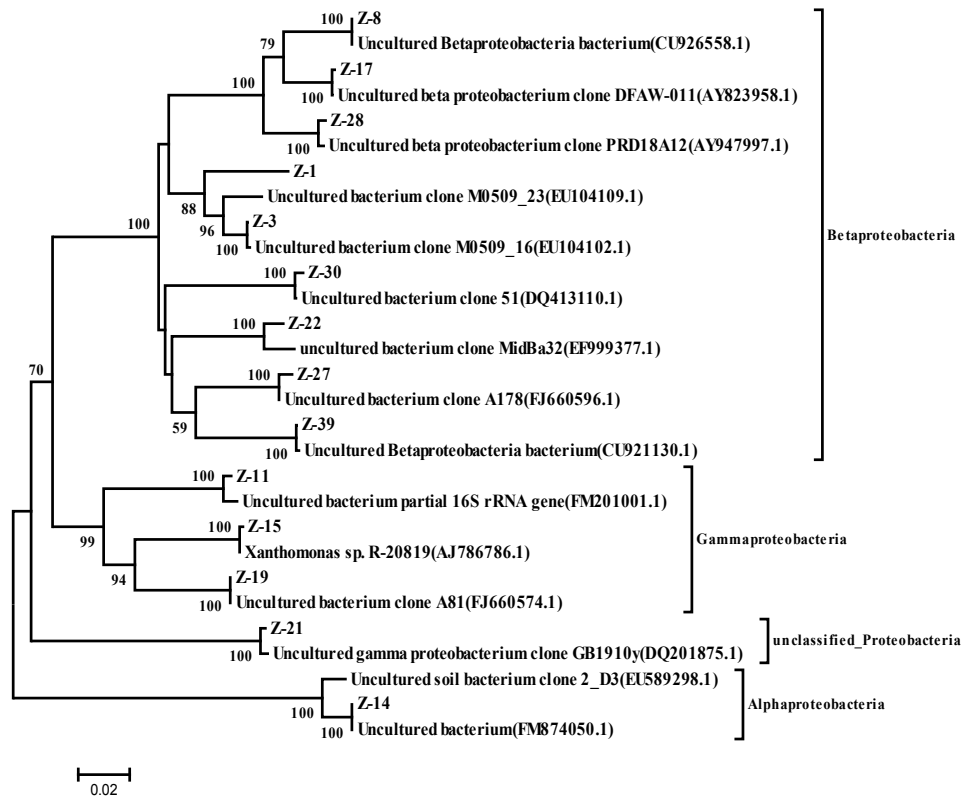


Figure 4. Phylogenetic tree of proteobacteria isolated from ICS column based on 16S rDNA sequences

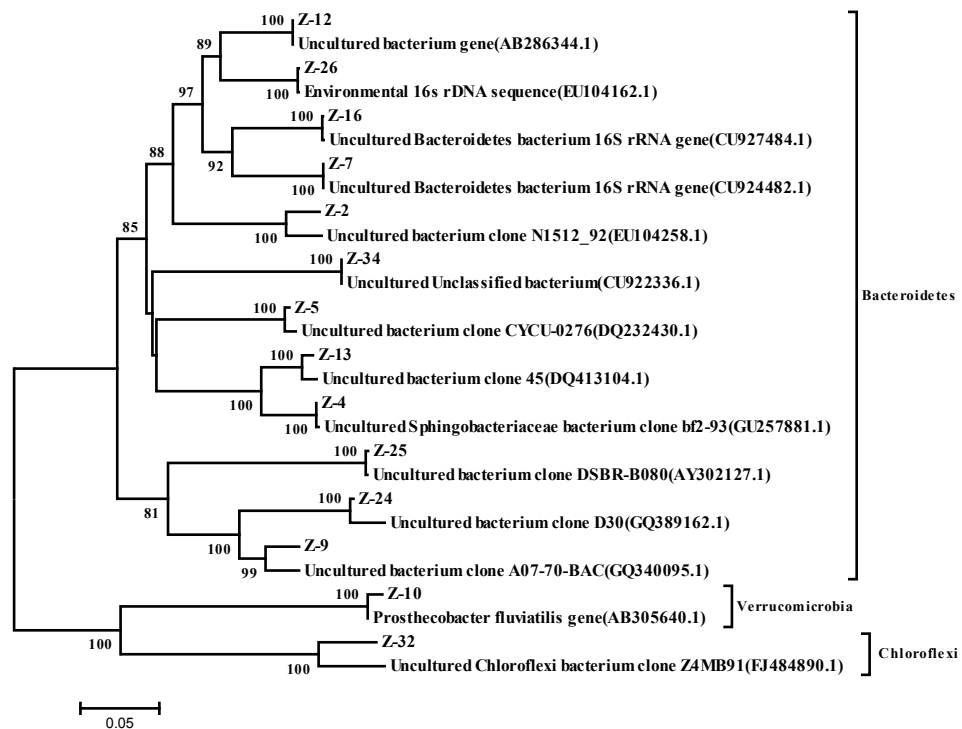


Figure 5. Phylogenetic tree of other bacteriophyta isolated from ICS column based on 16S rDNA sequences

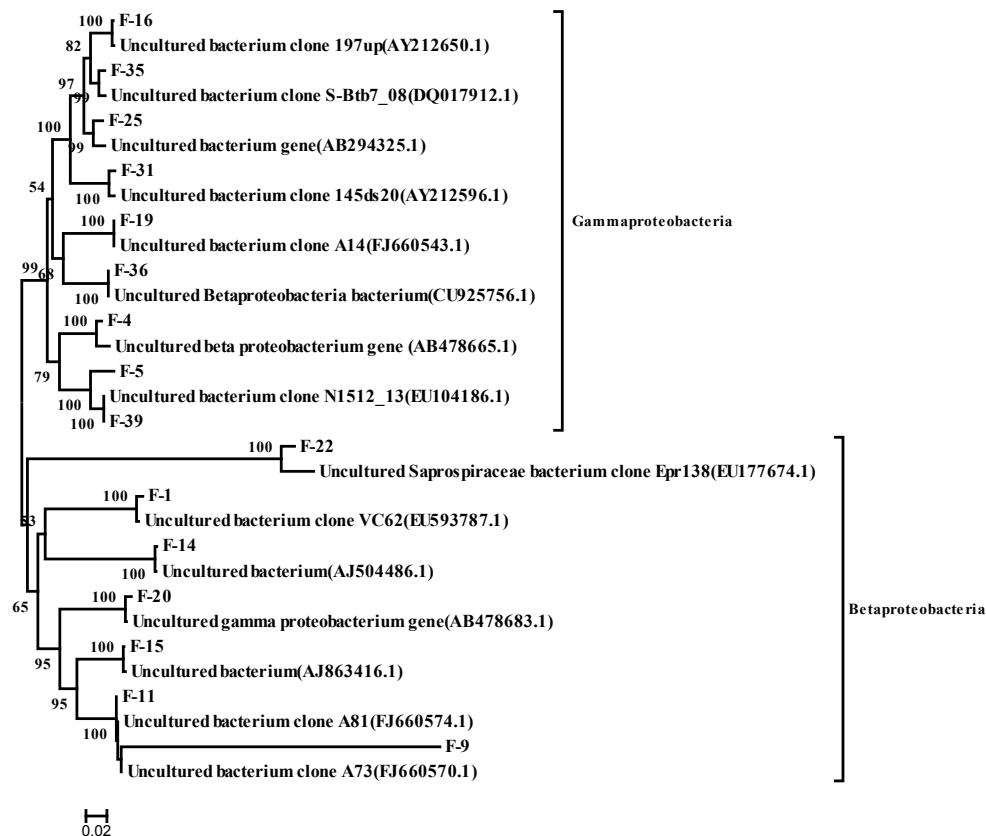


Figure 6. Phylogenetic tree of proteobacteria isolated from TCS column based on 16S rDNA sequences

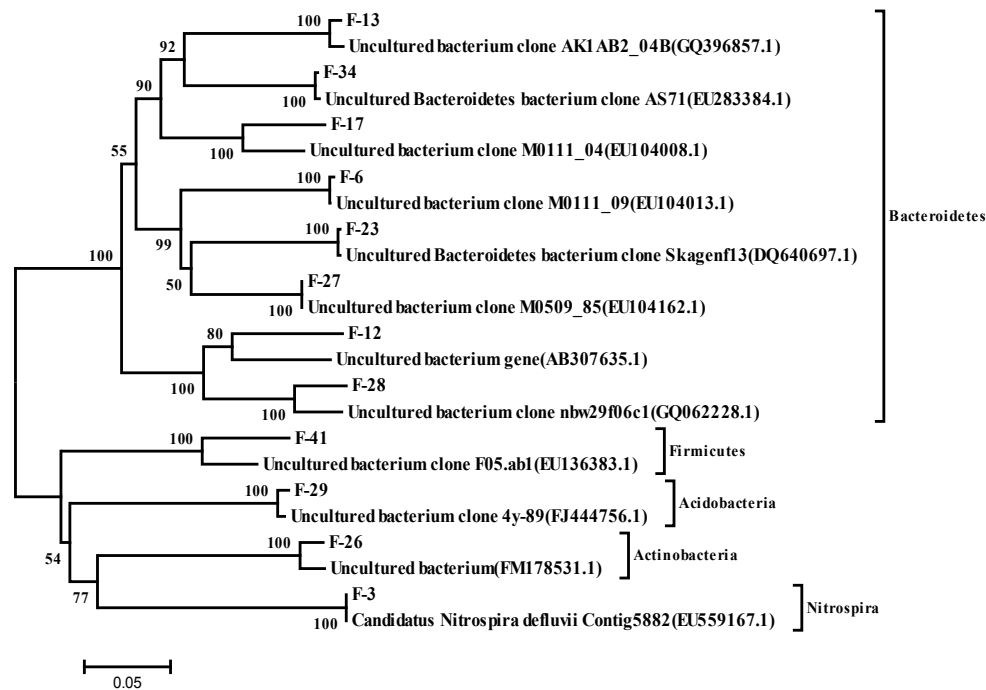


Figure 7. Phylogenetic tree of other bacteriophyta isolated from ICS column based on 16S rDNA sequences

System development analysis of bacterial in TCS

In TCS, *β-proteobacteria* was the dominant bacterial community with proportion of 37%. However, it was the second largest bacterium in ICS with proportion of 27%. This bacterium included 12 genotypes, in which the homology between F-2 and *Bacterium AEOC009* (AB208727) was 99% and that between F-42 and *Arabidopsis lyrata* was 99%. Other clones were uncultured bacteria. According to Amann et al.'s study of the bacterial community structure in the biological reactor, *β-proteobacteria* and *Bacteroidetes* were the most dominant communities in the wastewater treatment system. The former played an important role in organic matter degradation, nitrogen and phosphorus removal, and flocculation structure formation (Burrell et al., 1999). Second, *γ-proteobacteria*, *δ-proteobacteria*, and unclassified *Proteobacteria* accounted for 25%, 3%, and 5.5% of bacteria in the library, respectively. They were all *Proteobacteria*, which could efficiently remove organic matters, nitrogen, and phosphorus. *Bacteroidetes* was the third largest community in the library with proportion of 24%, but it was the dominant community in ICS. The bacteria in this community were related to nitrogen and phosphorus removal. Thus, ICS had better performance than TCS in nitrogen and phosphorus removal. *Nitrospira* (nitrification spirillum) accounted for 3% of bacteria in the library. The homology among F-3 and other two unnamed *Nitrospira* (EU55916 and DQ059545) was 99%. *Nitrospira* was an important NO₂⁻-N oxidation bacterium. The research of Burrell et al. (1999), Lee et al. (2002) and Benítez-Páez et al. (2016) showed that the bacteria in *Nitrospira* species could have played the role of NO₂⁻-N oxidation during nitrification rather than *nitrobacter*.

Conclusions

(1) The concentrations of COD, TN, NH₄ + -N, and TP in inflow water were 309.5, 63.61, 60.28, and 5.16 mg/L, respectively. The removal rates of COD, TN, NH₄ + -N, and TP were 93.4%, 85.6%, 100%, and 100% in ICS and 91.1%, 70.9%, 100%, and 100% in TCS, respectively. Thus, TN removal rate in ICS was 14.7% higher than that in TCS.

(2) Different control systems of SBBR will cause differences in bacterial community structures and their relative proportions. In the ICS sample library, 16S rDNA sequences by bacterial clones belonged to 7 bacterial communities, namely, *Bacteroidetes*, *β-proteobacteria*, *γ-proteobacteria*, *α-proteobacteria*, *Verrucomicrobia*, *Chloroflexi*, and unclassified *Proteobacteria*. In the TCS sample library, 16S rDNA sequences by bacterial clones belonged to 9 bacterial communities, namely, *β-proteobacteria*, *γ-proteobacteria*, *Bacteroidetes*, *δ-proteobacteria*, *Firmicutes*, *Nitrospira*, *Actinobacteria*, *Acidobacteria*, and unclassified *Proteobacteria*.

(3) The growth environment of the biofilm microorganisms in ICS was aerobic/hypoxic, in which *Bacteroidetes* was the dominant community with the proportion of 45%. The growth environment of the biofilm microorganisms in TCS was anaerobic/aerobic, in which *Betaproteobacteria* was the dominant community with the proportion of 37%.

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