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Influence of temperature on sludge settleability and bacterial community structure in enhanced biological phosphorus removal systems

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ABSTRACT

In this study, the influence of temperature on sludge settleability and bacterial community structure was investigated in two EBPR systems for a better understanding of the seasonally variable settleability. The results indicated that settleability depended both on the filament content and on the non-soluble phosphorus (Pns) and non-volatile suspended solids (NVSS) contents at varying temperatures. When the temperature was increased, settleability was significantly improved because of the substantial reduction in the filaments. When the temperature was decreased, settleability changed slightly in the long run and was mainly determined by Pns/VSS and NVSS/VSS ($p < 0.05$). Canonical correspondence analysis results indicated that bacterial community structure was significantly correlated with temperature and settleability ($p < 0.01$). At a high temperature (25°C), 21 species mostly affiliated with *Proteobacteria*, followed by *Bacteroidetes*, were stimulated greatly. The proliferation of *Bacteroidetes* might have a close relation with the improvement of settleability. At a low temperature (15°C), 25 species which were different from that at a high temperature proliferated in the system. These species mostly affiliated with *Proteobacteria*, followed by *Actinobacteria*. The large population of *Actinobacteria* was closely correlated with poor settleability. This work provides a valuable basis for the control of seasonal sludge bulking in EBPR systems.

Keywords: EBPR; Temperature; Sludge settleability; Sludge density; Phosphorus content; Bacterial community

1. Introduction

Water eutrophication has emerged as a serious problem all over the world. Controlling phosphorous discharged from wastewater treatment plants is a key factor in preventing entrophication of receiving waters. Currently, phosphate removal is achieved largely by chemical precipitation, which is expensive and causes the increase in sludge volume. As an alternative method, enhanced biological phosphorus removal (EBPR) system has become more popular because of its simplicity, economy and various environmental benefits. However, sludge bulking is a common problem in EBPR systems, which results in low-quality effluent, decreased system capacity, and other problems [1]. Activated sludge settleability is normally measured by the sludge volume index (SVI), with

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high values indicative of poor settleability. Previous studies indicated that seasonal variations in settleability were frequently observed in EBPR systems, with maximum SVI values measured in winter/spring and minimum SVI values in summer/autumn [2,3]. Although sludge bulking can be induced by a variety of conditions, temperature tended to be a significant contributor to the seasonal variation in settleability.

Sludge bulking has been described to be associated with the proliferation of filaments. Andreasen and Nielsen demonstrated that filaments were inclined to grow at low temperatures rather than at high temperatures, thereby leading to poor settleability in winter and spring [4]. However, studies reported that the variation in settleability was not always concurrent with the changes in filament content [3,5]. Schuler et al. [6,7] used microspheres to adjust sludge density and proposed that when the floc structure and filament content were constant, the non-soluble phosphorus (Pns) and non-volatile suspended solids (NVSS) contents of sludge could increase sludge density, thereby improving settleability. To date, the influence of sludge physical properties on settleability remains unclear. Additionally, when discussing the factors affecting settleability, few studies took into account the filament content and sludge physical properties at the same time. Recently, Jassby et al. studied the combined effect of the filament content and sludge density (significantly affected by inorganic content of sludge) on settleability [8]. However, this study was carried out under relatively stable condition and the sludge density was adjusted artificially using microspheres. It did not reveal the influence of the operating parameters (such as temperature) on the bacteria and sludge density. In fact, when the temperature is changed in the EBPR system, the variations in filament content and sludge physical properties are much more complex than that in the aforementioned studies [9,10]. For instance, Pns and NVSS in the bacteria, which both increase sludge density, will change in different directions as the temperature decreases [11,12]. At low temperatures, filaments are inclined to grow, and NVSS will decrease, which both adversely affect settleability. However, Pns will increase because low temperatures are favorable for polyphosphate-accumulating organisms (PAOs), and thus might compensate the adverse effects of filaments and NVSS. Nevertheless, the overall influence of filaments, NVSS, and Pns on settleability is unclear. To date, few studies have concerned how filament content and sludge properties affect settleability at varying temperatures. Therefore, further research is very necessary.

The development of molecular biology techniques has led to a deep understanding of the bacterial community structure of activated sludge in EBPR systems [13]. Previous researchers used the fluorescence *in situ* hybridization (FISH) technique, and their results indicated that the growth of PAOs and glycogen-accumulating organisms (GAOs) was significantly influenced by temperature. Moreover, low temperatures are favorable for PAOs [11]. The enriched PAOs were demonstrated to increase easily the sludge Pns content, which led to the formation of dense clusters and significantly enhanced the settleability [6]. However, few studies focused on the relationship between bacterial community structure and settleability in EBPR systems at varying operating temperatures. Furthermore, model species based on the FISH method were mainly adopted in the previous studies [11]. Therefore, changes in abundances and diversity in closely related or unknown strains were not detected because the FISH-based approach relies on broad probes [14]. Compared with the FISH method, polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) analysis is an ideal technique for detecting unknown species with contents greater than 1%. Although this method suffers from biases associated with PCR, it can relatively quantify bacterial population in the system and thus provide valuable information of the microbial community. For instance, PCR–DGGE has been successfully used in microbial ecology to determine fine-scale changes in the overall microbial community structure [15,16]. Recently, using PCR–DGGE and canonical correspondence analysis (CCA), Rosenkranz et al. demonstrated that the phenol degradation performance in an anaerobic sequencing batch reactor was essentially driven by specific community traits [17]. In addition, Hesham et al. performed PCR–DGGE and showed that the changes in settleability may be correlated with the microbial community dynamics in the nutrient removal systems [18].

This study aims to investigate the changes in settleability and the bacteria community dynamics with changing temperatures by operating two continuous-flow EBPR systems. Statistical analysis was performed to determine the relationships among sludge physical properties, including settleability, Pns/VSS, NVSS/VSS, and density. PCR–DGGE analysis was performed to determine fine-scale changes in the overall microbial community structure during the entire experiment. CCA was performed to investigate the relationships among bacterial community structure, operating temperature, and sludge properties (phosphorus removal, filament abundance, and settleability). The effect and mechanism of temperature on settleability and bacterial community structure were discussed.

2. Materials and methods

2.1. System set-up and wastewater characteristics

Two sets of parallel-operated continuous-flow EBPR systems made of cylindrical Plexiglass were operated with a working volume of 6 L. The anaerobic and aerobic volume ratio of both systems was 1:3. The flow rates of the influent and return sludge were controlled by separated peristaltic pumps, and the recycle ratio of the return sludge was 100%. The influent flow rate was 18 L/d, and the hydraulic retention time of the systems was 8 h, including 2 h of anaerobic treatment time and 6 h of aeration time. The solid retention time was approximately 12 d. The operating temperature was set at 15, 20, and 25˚C to simulate the wastewater temperatures in winter, spring/autumn, and summer, respectively. First, both systems were inoculated with sludge from a steady-operated EBPR system in the laboratory and operated at 20˚C (spring /autumn, phase 1). After 34 d of operation, both systems reached their steady states. The concentration of mixed liquor suspended solids (MLSS) was approximately 3,000 mg/L, and the removal efficiencies of chemical oxygen demand (COD) and phosphorous were around 95 and 89%, respectively. The SVI value was approximately 260 mL/g. Then, the operating temperatures of the No. 1 and No. 2 Reactors were increased to 25˚C (summer) and decreased to 15˚C (winter), respectively (phase 2).

The composition of the synthetic wastewater was as follows: Na-acetate, 172.69 mg/L; potato starch, 134.07 mg/L; peptone, 19.17 mg/L; milk powder, 70.93 mg/L; yeast, 57.41 mg/L; urea, 11.94 mg/L; $K_2HPO_4{\cdot}3H_2O$, 41.76 mg/L; $MgSO_4{\cdot}7H_2O$, 7.13 mg/L; $CaCl_2$, 5.46 mg/L; $NaHCO_3$, 27.50 mg/L; and $FeSO_4{\cdot}7H_2O$, 11.02 mg/L. The concentration of trace elements was as follows: KI, 60 μg/L; H_3BO_3, 300 μg/L; $MnSO_4{\cdot}H_2O$, 100 μg/L; $CoCl_2{\cdot}6H_2O$, 49.45 μg/L; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 105.34 μg/L; $CuSO_4{\cdot}5H_2O$, 702.99 μg/L; and $ZnSO_4{\cdot}7H_2O$, 379.78 μg/L. The COD, total nitrogen, and total phosphorus in the influent were 420, 18, and 12 mg/L, respectively.

2.2. Analytical methods

The COD, MLSS, volatile suspended solids (VSS), SVI, and soluble and total phosphorus were measured according to the standard method [19]. NVSS was the difference between MLSS and VSS. Pns, which included polyphosphate, was calculated by subtracting the soluble phosphorus from the total phosphorus [5]. On the basis of the method of Schuler and Jang [10], sludge density was determined by adopting Percoll as a separation medium. Sludge samples from both reactors were examined weekly under a microscope. Filaments were identified using the characteristics determined after staining with Gram, Neisser, and polyhydroxyalkanoates (PHA), morphologic observation, and other methods, following the method of Jenkin et al. [20]. Micrographs of Neisser staining were displayed in this study to illustrate the types of filaments. The filament content was determined based on the microscopic observation of the wet mount sludge samples. The overall filament abundance was evaluated according to the filament index scale from 0 (none) to 6 (excessive) described by Jenkin et al. [20].

2.3. Bacterial community analysis

PCR–DGGE analysis was performed to analyze the bacterial community structure [16,21]. Given that no noticeable difference was observed among microbial communities in both reactors during the first 34 d, the sludge samples from the No. 1 Reactor on days 23 and 34 were used to represent the bacterial community structure at the initial period. Other sludge samples were collected from each of the two systems on days 37, 60, 72, and 87 (Fig. 1(A)). These samples were stored at −20˚C until genomic DNA was extracted using a soil DNA kit (Omega Bio-tek Inc., USA). DNA was amplified by universal primers at the V3 region of the 16S rRNA gene for most bacteria and Archaea. The sequences of the primers were as follows: 341F (5′-CCTACGGGAGGCAGCAG -3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′) [16]. Primer 341F has an additional 40-nucleotide GC-rich sequence (GC clamp) at its 5′ end for optimal resolution of the DNA fragments. The sequence of the GC clamp was as follows: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG. A touchdown PCR program was applied to amplify the DNA [22]. The PCR products were verified using 1% agarose gel electrophoresis and separated by DGGE with polyacrylamide gel (8%, 37.5:1 acrylamide:bisacrylamide) with a linear gradient of 40% to 60% denaturant (Dcode™, Bio-Rad, USA) [15]. The DGGE gel stained by GelRed was placed on a UV transilluminator to obtain an image (GBoxEF, SYNGENE, UK). Quantity One (Bio-Rad, USA) was adopted to analyze the relative intensity of each DGGE band, which was considered a single species, to evaluate the microbial communities in each system [23]. The relative intensity of bands in the DGGE profiles was confirmed in replicate gels. A matrix containing the migration and intensity data of the bands was exported from Quantity One for CCA analysis (data shown in supplemental material, Table 3). DNA was recovered by cutting the target band from the DGGE gel. The DNA was reamplified and cloned into a

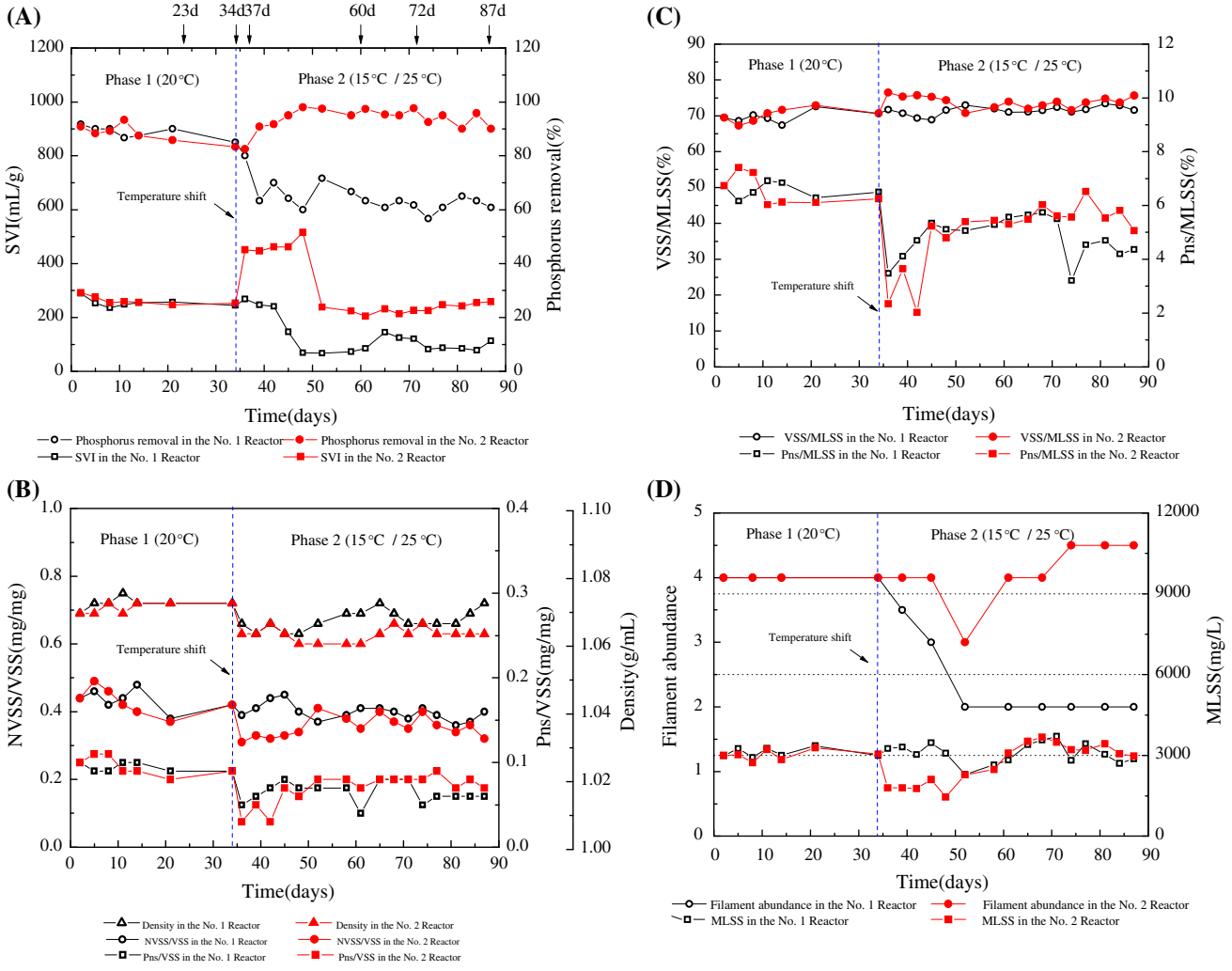

Fig. 1. Effect of temperature on sludge properties in the No. 1 and No. 2 Reactors. The arrows and numbers above the figure indicate the days when the sludge samples were collected to analyze the bacterial community structure. The blue lines show the day when the temperature in both reactors was changed. In phase 1, both reactors were operated at 20℃; in phase 2, the No. 1 and No. 2 Reactors were operated at 25 and 15℃, respectively.

pEASY-T vector and then sent to Sangon Biotech Co., Ltd (China) for sequencing. To determine the phylogenetic affiliation, BLAST was used for data retrieval and similarity comparison in the GenBank database, and the phylogenetic tree was established based on neighbor-joining method using MEGA 5.0 [24].

The sequence data have been submitted to the GenBank databases under accession numbers KF559164-KF559181 and KF728728-KF728746.

2.4. Statistical analysis

Statistical analysis was conducted using SPSS 16.0 for Windows (SPSS Inc, USA) to investigate the relationships among sludge physical properties [25]. The t-test was used to assess the changes in sludge properties in both reactors. Pearson's correlation coefficient (r_p) was used to estimate linearly the strength and direction of correlations between two parameters. The r_p is always between +1 and −1, where +1 means a perfect positive correlation, −1 indicates a perfect negative correlation, and 0 is the absence of a relationship. Correlations were considered statistically significant at a confidence interval ($p < 0.05$).

To investigate the relationships among bacterial community structure, operating temperature, and sludge properties (phosphorus removal, filament abundance, and settleability), CCA was performed using Canoco for windows version 4.5 [26], based on

the matrix exported from Quantity One. The CCA results were depicted on an ordination diagram wherein the first and second canonical axes were in the horizontal and vertical directions, respectively. The direction of the arrows represented the correlation between each variable and the canonical axes, and each other. The length of the arrows represented the relative contribution of the variables to the axes and the species–environment relationship. Monte Carlo permutation texts with 499 permutations were conducted to estimate the significance of the species–environment relationship.

3. Results and discussion

3.1. Influence of temperature on sludge properties

Fig. 1 illustrates the variations in sludge properties in both reactors, and Table 1 shows the sludge properties before and after temperature shift. The period after temperature shift (Days 34–87) was divided into two stages (the initial stage and the stable stage) for clarity because of the dramatic variation in sludge properties at the initial stage. As shown in Fig. 1 and Table 1, when both reactors were operated at 20˚C, no significant difference was observed. Whereas when the temperature changed, the sludge properties in the No. 1 and No. 2 Reactors changed in different directions.

In the No. 1 Reactor, the increase in temperature resulted in a notable decrease in phosphorus removal efficiency from 89.31 ± 1.86% to 63.11 ± 3.86% ($p < 0.01$). At the same time, sludge settleability improved substantially ($p < 0.01$) and filament abundance decreased to 2.0 ± 0.0. By contrast, in the No. 2 Reactor, the decrease in temperature led to a significant increase in phosphorus removal efficiency from 89.17 ± 2.63% to 90.22 ± 6.21% ($p < 0.01$). Sludge settleability was slightly improved ($p<0.01$), and filament abundance was insignificantly affect in the long run ($p = 0.741$).

At the initial stage (Days 34–51) after the temperature changed, sludge properties changed dramatically. In the No. 1 Reactor, the filament abundance decreased continuously till day 47, and MLSS changed slightly during the entire experiment. On the contrary, the filament abundance in the No. 2 Reactor rarely changed, but a serious sludge loss from the clarifier happened due to the abrupt deterioration of settleability till day 52. At the same time, MLSS in the No. 2 Reactor decreased continuously to approximately 2,282 ± 290 mg/L. The temperature shift also resulted in a substantial decrease in Pns/VSS, density, and Pns/MLSS in both reactors (Fig. 1, Table 1). It was possible that the temperature shift could have affected the microbial populations that influence biomass density, such as PAOs and GAOs. Large population of PAOs appeared to be washed out because they were incapable to adapt to the new environment.

From day 34 to day 51, the phosphorus removal rapidly increased from 89.31 ± 1.86% to 90.22 ± 6.21% (approximate 194.88 mg P/d) in the No. 2 Reactor. However, Pns/VSS of the sludge in this system decreased continuously. This phenomenon was resulted from the serious sludge loss aforementioned. The sludge loss from the clarifier resulted in a major loss of phosphorus from the clarifier because the sludge was rich in phosphorus. Meanwhile, the loss of PAOs due to their inability of adapting the new environment made the matters worse. Although the increase in phosphorus uptake from the influent was remarkable, it was not enough to offset the loss of phosphorus. Consequently, a significant decrease in Pns/VSS ($p < 0.01$), and Pns/MLSS ($p < 0.01$) was observed. When the stable stage was reached, a new balance was built, and the remaining PAOs survived and began to proliferate in the system. Then, sludge loss stopped and MLSS slowly increased. Because the phosphorus removal efficiency was high (94.64 ± 2.72%), the Pns content recovered quickly, and Pns/VSS and Pns/MLSS increased remarkably to 0.078 ± 0.006 mg/mg ($p < 0.01$) and 5.61 ± 0.39% ($p < 0.01$), respectively.

When both systems reached their steady states, phosphorus removal efficiency, Pns/VSS, Pns/MLSS, and VSS/MLSS were significantly lower in the No. 1 Reactor, whereas NVSS/VSS and sludge density were notable lower in the No. 2 Reactor (Table 1, $p < 0.05$). It was previously demonstrated that the degree of sludge mineralization was positively correlated with temperature [12,27]. Krzeminski et al. also found that the VSS to MLSS ratio of the membrane bioreactors sludge was lower during summer than during winter period [3]. In this study, VSS/MLSS ratio was used to measure the biomass mineralization (Fig. 1(C), Table 1). As shown in Table 1, the values of VSS/MLSS in the No. 1 and No. 2 Reactors were 71.93 ± 0.84 mg/mg and 73.19 ± 1.45 mg/mg, respectively ($p < 0.05$). Because high temperatures are disadvantage for the growth of PAOs [11], the Pns/MLSS was significantly lower in the No. 1 Reactor than that in the No. 2 Reactor (Table 1). This implied that the lower VSS/MLSS at a high temperature was not a consequence of the increase in Pns but because of the reduction of VSS. In other words, the mineralization in the No. 1 Reactor (25˚C) was higher than that in the No. 2 Reactor (15˚C).

Table 1
Sludge properties before and after temperature (*T*) shift

Parameters	Stable stage before *t* shift (D 1–33)				Initial stage after *T* shift (D 34–51)			
	*R*1 (20℃)	*R*2 (20℃)	*p*	Mean differences ($\frac{R2-R1}{R1} \times 100$, %)	*R*1 (25℃)	*R*2 (15℃)	*p*	Mean differences ($\frac{R2'-R1'}{R1'} \times 100$, %)
PR (%)	89.31 ± 1.86	89.17 ± 2.63	0.917	−0.16	70.42 ± 10.02	90.22 ± 6.21	0.003**	21.95
SVI (ml/g)	256 ± 18	264 ± 17	0.468	3.13	203 ± 78	432 ± 91	0.001**	53.01
NVSS/VSS (mg/mg)	0.44 ± 0.03	0.43 ± 0.04	0.773	−2.27	0.42 ± 0.02	0.34 ± 0.04	0.003**	−23.53
Pns/VSS (mg/mg)	0.095 ± 0.005	0.097 ± 0.012	0.765	2.11	0.070 ± 0.014	0.055 ± 0.023	0.216	−27.27
Density (g/ml)	1.072 ± 0.002	1.071 ± 0.002	0.175	−0.09	1.066 ± 0.004	1.065 ± 0.002	0.661	−0.09
Vss/mlss (%)	69.58 ± 1.74	70.08 ± 2.06	0.657	0.72	70.45 ± 1.15	74.61 ± 2.07	0.003**	5.58
Pns/MLSS (%)	6.57 ± 0.31	6.60 ± 0.61	0.903	0.46	4.87 ± 1.05	4.05 ± 1.67	0.334	−20.25
Filament abundance	4.0 ± 0.0	4.0 ± 0.0	/	0	3.5 ± 0.5	4.0 ± 0.0	0.225	12.5

Parameters	Stable stage after *T* shift (D 52–87)				Initial stage after *T* shift (D 34–51)		Stable stage after *T* shift (D 52–87)	
	*R*1 (25℃)	*R*2 (15℃)	*p*	Mean differences ($\frac{R2''-R1''}{R1''} \times 100$, %)	p_1	p_2	p_1	p_2
P removal (%)	63.11 ± 3.86	94.64 ± 2.72	0.000**	49.96	0.001**	0.713	0.045*	0.148
SVI (ml/g)	96 ± 25	233 ± 17	0.000**	142.71	0.132	0.006**	0.001**	0.000**
NVSS/VSS (mg/mg)	0.39 ± 0.02	0.37 ± 0.03	0.036*	−5.13	0.308	0.004**	0.030*	0.200
Pns/VSS (mg/mg)	0.065 ± 0.013	0.078 ± 0.006	0.005**	20	0.006**	0.005**	0.453	0.006**
Density (g/ml)	1.068 ± 0.002	1.063 ± 0.002	0.000**	−0.47	0.004**	0.014*	0.134	0.361
Vss/mlss (%)	71.93 ± 0.84	73.19 ± 1.45	0.024*	1.75	0.331	0.003**	0.024*	0.173
Pns/MLSS (%)	4.89 ± 0.78	5.61 ± 0.39	0.012*	14.72	0.009**	0.006**	0.972	0.008**
Filament abundance	2.0 ± 0.0	4.08 ± 0.58	0.000**	104	0.225	/	0.000**	0.741

Notes: Mean ± standard deviation. *R*1—the No. 1 Reactor operated at 20℃; *R*2—the No. 2 Reactor operated at 20℃; *R*1′—the No. 1 Reactor operated at 25℃ (initial stage); *R*2′—the No. 2 Reactor operated at 15℃ (initial stage); *R*1′′—the No. 1 Reactor operated at 25℃ (stable stage); *R*2′′—the No. 2 Reactor operated at 15℃ (stable stage); *P* removal—phosphorus removal; SVI—sludge volume index; NVSS—non-volatile suspended solids; VSS—volatile suspended solids; Pns—non-soluble phosphorus; MLSS—mixed liquor suspended solids; *p*—the levels of significance of the *t*-tests for equality means of the parameters in the No. 1 and No. 2 Reactors at each stage (two-tailed). p_1—the levels of significance of the *t*-tests for equality means of the parameters in the No. 1 Reactors in two contiguous stages; p_2—the levels of significance of the t-tests for equality means of the parameters in the No. 2 Reactors in two contiguous stages.

**Difference is significant at the 0.01 level (two-tailed).

*Difference is significant at the 0.05 level (two-tailed).

3.2. Changes in the floc structure and filaments

The changes in the floc structure and filaments were observed by microscopic examination with changing settleability. When both reactors were at 20℃, filaments proliferated in the systems and extended from the activated sludge flocs (Fig. 2(A)). The filament abundance was 4.0 ± 0.0, and the SVI values were 256 ± 18 mL/g and 264 ± 17 mL/g in the No. 1 and No. 2 Reactors, respectively. The dominant filaments in both reactors were *Thiothrix II* and Type 0041 (Fig. 2(B)). When the No. 1 Reactor was heated to 25℃, although no obvious change was found in settleability from day 34 to day 52, the population of the original filaments in the sludge was slowly washed out, accompanied by the continuous decrease in filament abundance (from 4.0 ± 0.0 to 2.0 ± 0.0). When the system gradually stabilized from day 52, sludge flocs became compact and only few Types 0914 and 0041 grew in the reactor (Fig. 2(C) and (D)). The filament abundance was 2.0 ± 0.0, and sludge settleability was improved significantly (SVI = 96 ± 25 mL/g). When the No. 2 Reactor was cooled to 15℃, no obvious change occurred in the filaments from day 34 to day 42, and the filament abundance maintained at 4.0 ± 0.0. However, the settleability deteriorated dramatically at this time. Then, *Thiothrix II* and Type 0041 were slowly washed out after one week, and filament abundance decreased from 4.0 ± 0.0 to 3.0 ± 0.0, accompanied with a notable improvement of settleability. At the stable stage from day 52, *Thiothrix II* began to proliferate and predominated in the system till the end of the experiment. In spite of the compact sludge flocs, abundant filaments extended from the flocs (Fig. 2(E) and (F)).

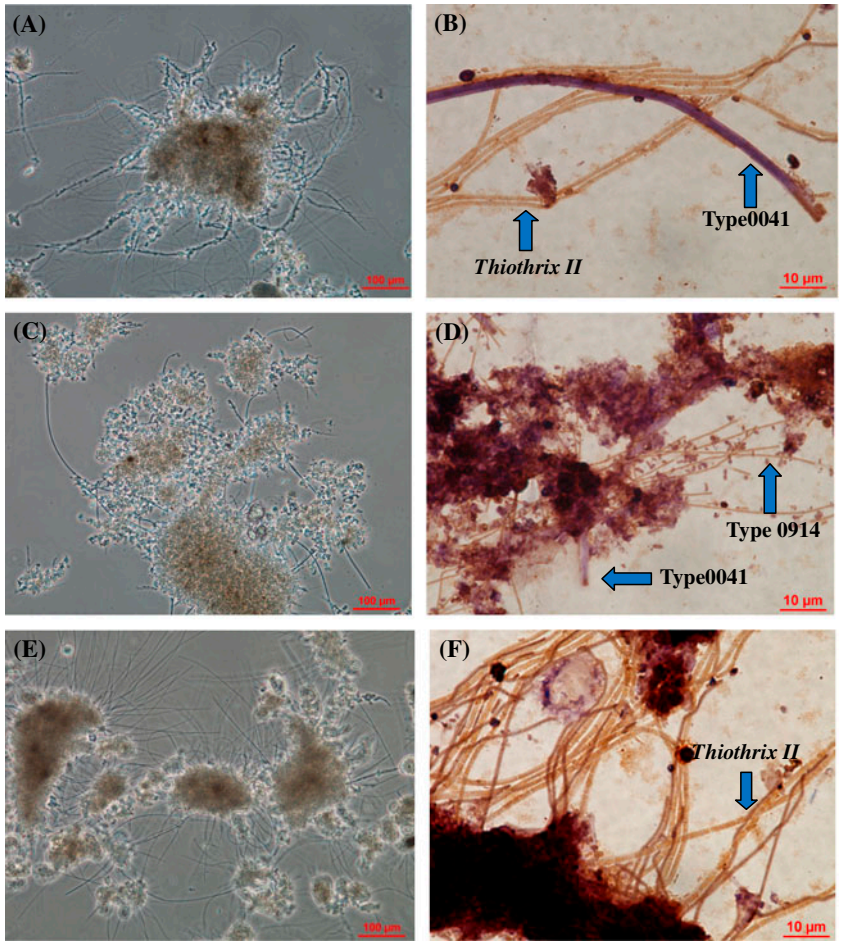

Fig. 2. Filament abundance categories using the subjective scoring system and micrographs of Neisser staining of filaments: (A) 4, very common (20℃), (B) Neisser-negative *Thiothrix II* and Neisser-positive Type 0,041 (20℃), (C) 2, some (25℃), (D) Neisser-negative type 0,914 and Neisser-positive type 0,041(25℃), (E) 4.5, between very common and abundant (15℃), and (F) Neisser-negative *Thiothrix II* (15℃).

The filament abundance increased slightly to 4.08 ±0.58, and the settleability was relatively poor (SVI = 233 ± 17 mL/g > 150 mL/g) although better than that at the initial stage after temperature shift (432 ± 91 mL/g). In general, high and low temperatures notably suppressed and stimulated filament growth, respectively.

A series of surveys indicated that the filaments population showed a seasonal variation in the full-scale wastewater treatment plants [2,3,28]. Most often the filament *Microthrix parvicella* were responsible for the bulking problem in winter/spring [28]. These filaments prefer the particulate substrates and usually proliferated at temperatures below 20˚C. However, in the current study, *Microthrix parvicella* did not appear during the entire experiment, despite the abundant particulate substrates in the influent and the low temperatures. Instead, Type 0041, which is the potential competitors for the particulate substrates [2], dominated at 20˚C in both reactors. Interestingly, *Thiothrix II*, the other important filament in this study, was greatly stimulated by the lower temperature (15˚C). *Thiothrix II* was reported to be favored by the septic conditions [3]. It appeared that the anaerobic (septic) zone in the EBPR systems might provide advantages for the growth of *Thiothrix II*, especially at low temperatures.

3.3. The combined effect of sludge properties and filaments on settleability

The relations among Pns/VSS, NVSS/VSS, and settleability were different in the reactors operated at different temperatures (Table 2). Given that the filament content in the No. 1 and No. 2 Reactors varied greatly at varying temperatures, the different relation might be caused by different filament contents. For the No. 1 Reactor, although high temperatures had adverse effects on Pns/VSS, the significant decrease in filament content tended to obscure the unfavorable effect of Pns/VSS on settleability. The improvement of settleability was mainly caused by the substantial reduction in filament content. By contrast, for the No. 2 Reactor, the population of filaments during the entire experiment slightly changed when the temperature was decreased. Therefore, settleability was mostly affected by Pns/VSS and NVSS/VSS, which was in line with previous studies [5,10].

During the initial period after the operating temperature was changed (from day 34 to day 42), settleability rarely changed in the No. 1 Reactor (25˚C) but dramatically deteriorated in the No. 2 Reactor (15˚C). It was likely that the population of filament, Pns/VSS, and NVSS/VSS were the major factors that affected settleability. During this period, large quantity

Table 2
Correlation analysis of sludge physical properties

Parameters	SVI_{R1}				SVI_{R2}			
	r_p	r^2	p	n	r_p	r^2	p	n
Pns/VSS	0.497	0.303	0.016*	23	−0.647	0.419	0.001**	23
NVSS/SS	0.611	0.373	0.002**	23	−0.463	0.214	0.026*	23
Density	0.378	0.143	0.076	23	−0.196	0.038	0.371	23
Parameters	$Density_{R1}$				$Density_{R2}$			
	r_p	r^2	p	n	r_p	r^2	p	n
Pns/VSS	0.629	0.307	0.001**	23	0.479	0.229	0.021*	23
NVSS/VSS	0.272	0.074	0.209	23	0.571	0.326	0.004**	23
Parameters	1# Pns/VSS_{R1}				2# Pns/VSS_{R2}			
	r_p	r^2	p	n	r_p	r^2	p	n
NVSS/VSS	0.571	0.272	0.004**	23	0.823	0.667	0.000**	23

Notes: SVI—sludge volume index; Pns—non-soluble phosphorus; VSS—volatile suspended solid; NVSS—non-volatile suspended solid; *R*1—the No. 1 Reactor; *R*2—the No. 2 Reactor; r_p—Pearson's correlation coefficient; r^2—the square of r; p—the levels of significance of the *t*-tests; n—sample size.

**Correlation is significant at the 0.01 level (two-tailed).

*Correlation is significant at the 0.05 level (two-tailed).

of Pns and NVSS was lost. In the No. 1 Reactor, although the substantial decrease in Pns/VSS adversely influenced the settleability, the effect might be compensated by the notable decrease in filament content and the increase in NVSS/VSS (Fig. 1(B) and (D)) [9]. However, in the No. 2 Reactor, when the operating temperature was decreased, the filament was still relatively high and changed slightly (Fig. 1(D)). The effect of the filament was negligible. As the decrease in Pns/VSS adversely affected the settleability, the decrease in NVSS/VSS would worsen the situation, thereby leading to dramatic sludge bulking. The poor settleability lasted for approximate 19 d until Pns/VSS and NVSS/VSS recovered at the stable stage. Then, settleability was improved and the SVI value decreased from 432 ± 91 mL/g to 233 ± 17 mL/g, which was close to the level before the temperature decreased (Table 1).

The relationship among settleability, filament content, and inorganic content of sludge has been studied by several researchers. However, most studies did not take into account the changes in filament content [3–7]. There was little information on how filament content and inorganic content of sludge combine to affect sludge settling in changing environment, such as the temperature. In the current study, the combined effect of filaments and inorganic content of sludge on settleability at varying temperature was investigated for the first time. The results indicated that filament content was the major factor affected settleability with changing temperature, and the influence of inorganic content would show up only when filament content changed slightly. Additionally, the abrupt changes in temperature or process disturbance might lead to significant changes in inorganic content of sludge even though the filament content changed slightly. This in turn could affect the sludge settling and even result in sludge bulking, and, in severe cases, would lead to washout.

3.4. Relationships among sludge density and other sludge properties

Sludge density has recently been demonstrated to be an important factor affecting sludge settling in both laboratory and full-scale systems [5–7,22]. However, in the current study, density had an insignificant correlation with settleability in both reactors. This result was inconsistent with previous study showing that sludge density was negatively correlated with SVI [5,6]. This phenomenon appeared to be resulted from the high density in both reactors. The density in the previous studies normally ranged from 1.02 to 1.07 g/mL [5,6], whereas the density in this study was relatively high and slightly varied, ranging from 1.06 to 1.07 g/mL. Because SVI was rarely affected by density as long as the density was within this range [10], an insignificant correlation was observed between density and SVI in the current study.

It was previously demonstrated that the increasing Pns/VSS and NVSS/VSS were concurrent with the increasing sludge density [5,6,10]. In the current study, both Pns/VSS and NVSS/VSS were positively correlated with sludge density in the No. 2 Reactor, whereas only Pns/VSS was positively correlated with density in the No. 1 Reactor. Table 2 indicates that the correlation between Pns/VSS and NVSS/VSS in the No. 2 Reactor (15˚C) was much stronger than that in the No. 1 Reactor (25˚C). It suggested that in the EBPR system with higher Pns content, Pns/VSS was the main factor affecting sludge density rather than NVSS/VSS. Furthermore, it should be noted that the sludge density was higher in the No. 1 Reactor with lower Pns/VSS than that in the No. 2 Reactor with higher Pns/VSS (Table 1). It was likely that aside from Pns/VSS, other parameters may be closely correlated with sludge density [29].

3.5. A hypothesis for the settling of sludge with high density

The settling of activated sludge is a very complex process, and a lot of research has been devoted to study the factors that affect settling [9]. The filament content is the best-known factor that can lead to dramatic sludge bulking. Recently, Schuler et al. demonstrated that sludge density was also an important factor affecting sludge settling [5–7]. It was inferred that sludge density could affect the settling rates because it was linearly correlated with the gravitational force that drove sedimentation. Through adjusting the sludge density with a range of filament contents, Jassby et al. investigated the combined effects of filament content and sludge density on settleability [8]. They found that SVI values increased linearly with increasing filament content, but decreased with increasing sludge density. Furthermore, high density sludge was less sensitive to changes in filament content than low-density sludge.

The sludge density in the current study was relatively high and slightly varied (approximately from 1.06 to 1.07 g/mL). Unlike the results in the previous study, sludge density was insignificantly correlated with SVI. Base on the study by Jassby et al., it was postulated that when the sludge density was high and slightly varied, the method to measure the sludge

density was not sensitive enough to reflect the small changes in gravitational force that drove sedimentation [5]. In this case, sludge density was not an ideal parameter to represent the factor that affected settling. In the current study, sludge density was correlated with Pns/VSS and NVSS/VSS, and there was a close relationship among settleability, Pns/VSS, and NVSS/VSS in both reactors. It was hypothesized that the small changes in gravitational force could be represented by the changes in Pns/VSS and NVSS/VSS. It implied that apart from filament content, Pns/VSS and NVSS/VSS rather than sludge density may be important factors that affected sludge settling in this case. This hypothesis appeared to be additional to the study by Jassby et al. and could well explain the phenomenon in the current study. Sludge density with high values and small variation had an insignificant correlation with settleability in both reactors. In the No. 1 Reactor, the improvement of settleability was mainly caused by the substantial reduction in filament content. The significant decrease in filament content obscured the effect of the Pns/VSS and NVSS/VSS on settleability. In the No. 2 Reactor, the population of filaments during the entire experiment rarely changed, and settleability was mainly determined by Pns/VSS and NVSS/VSS.

3.6. Bacterial community structure

Fig. 3 shows the DGGE profiles of the samples collected from both reactors. In spite of the different quantitative distributions of the bacteria in the reactors with different temperatures, the dominant species were similar. It appeared that there was a common core of bacterial phyla in the EBPR systems with different temperatures [30].

A total of 46 different bands were obtained from the DGGE gels. Only 37 bands yielded useful nucleotide sequence data. A phylogenetic tree was established to visualize the evolutionary relationships between the sequences retrieved in this study and the sequences of the closest relatives (Fig. 4). *Proteobacteria* (19 species) were the most prevalent followed by *Bacteroidetes* (8 species), *Actinobacteria* (5 species), *Firmicutes* (3 species), and *Chloroflexi* (1 species). Among the *Proteobacteria*, α-*Proteobacteria* were the most dominant (10 species) followed by γ-*Proterobacteria* (4 species), β-*Proterobacteria* (3 species), and δ-*Proterobacteria* (1 species). Most of the sequences showed 94–100% similarity with previously identified 16S rRNA gene sequences in the GenBank database, except the sequences of band 34 (data not shown).

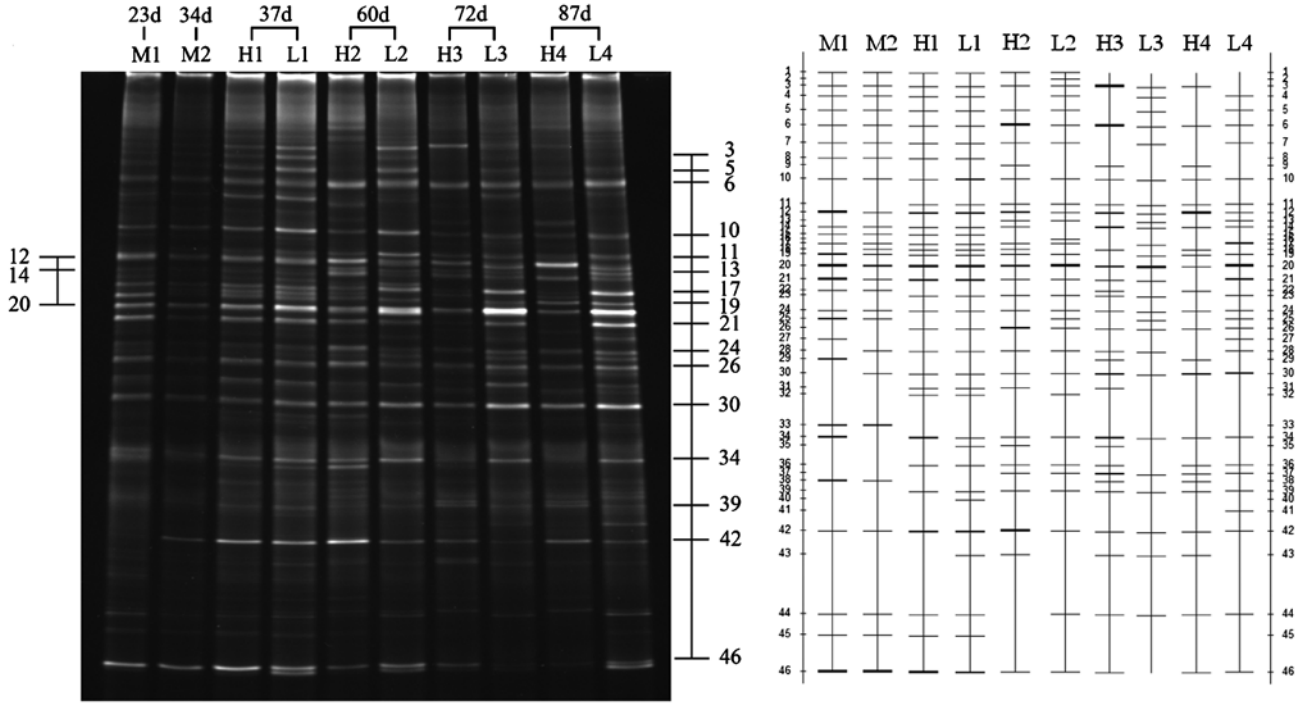

Fig. 3. DGGE profiles of samples collected from both reactors: H: high temperature (25˚C); M: medium temperature (20˚C); L: low temperature (15˚C).

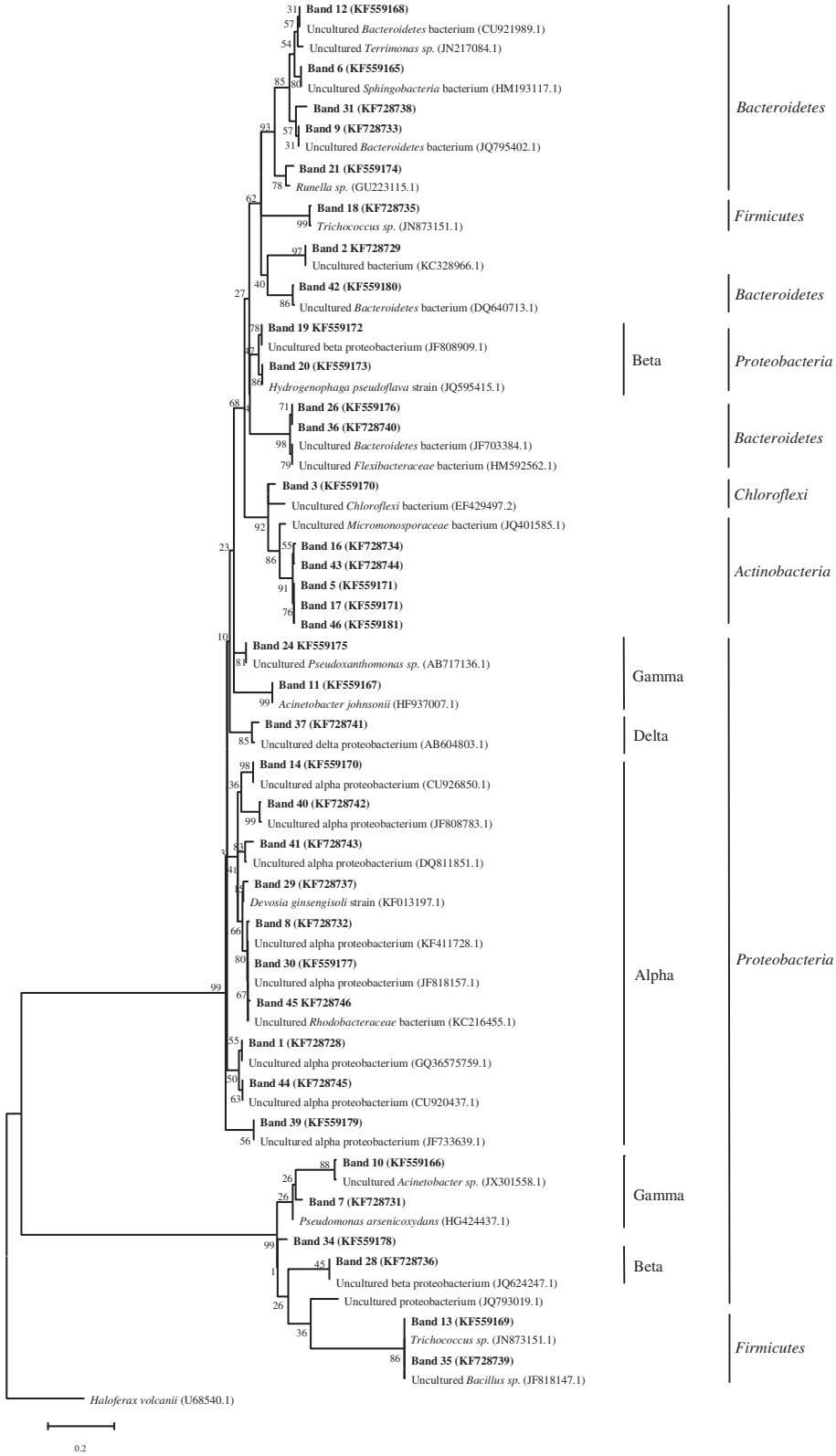

Fig. 4. Neighbor-joining phylogenetic tree of all the species in both reactors based on 16S rRNA V3 region sequences derived from DGGE band.

Fig. 5 illustrates the triplot diagram of CCA on sludge bacterial community, operating temperature, and sludge properties (phosphorus removal, filament abundance, and settleability) in both reactors. According to the CCA, the first four eigenvalues were 0.185, 0.135, 0.092, and 0.023. A Monte Carlo permutation test produced an *F*-ratio of 1.642 with a p-value of 0.002 for the first canonical axis, and an *F*-ratio of 1.738 with a *p*-value of 0.010 for all axes. The first two axes represented an estimated 73.5% of the observed variation in the composition of the bacterial community, and 100.0% was explained by the full four canonical axes. The first axis showed a high canonical correlation with the temperature, phosphorus removal, and filament abundance, while the second axis correlated with the SVI value.

The results of the CCA ordination diagram showed that most samples collected from the No. 1 (25˚C) and No. 2 (15˚C) Reactors clustered in the fourth and third quadrants, respectively, indicating the significant influence of temperature. Approximately 21 species of bacteria were positively correlated with temperature. Most of these species were affiliated with *Proterobacteria* (8 species), followed by *Bacteroidetes* (8 species), *Firmicutes* (2 species), *Actinobacteria* (1 species), and *Chloroflexi* (1 species). The top two bacteria species that grew to large populations at a high temperature were represented by bands 12 and 42. These species were affiliated with *Bacteriodetes*. A total of 25 different species of bacteria were negatively correlated with temperature, which were mostly affiliated with *Proteobacteria* (11 species), followed by *Actinobacteria* (4 species), *Bacteriodetes* (1 species), and *Firmicutes* (1 species). Among these bacteria, the species represented by bands17, 20, 21, and 30 proliferated at medium (20˚C) and low (15˚C) temperatures. A notable shift was also observed in the bacterial community structure along the arrow of SVI (Fig. 5). Approximately 21 bacteria species (mostly *Proterobacteria* and *Actinobacteria*) were positively correlated with SVI, whereas approximately 25 bacteria species (mostly *Proterobacteria* and *Bacteroidetes*) were negatively correlated with SVI.

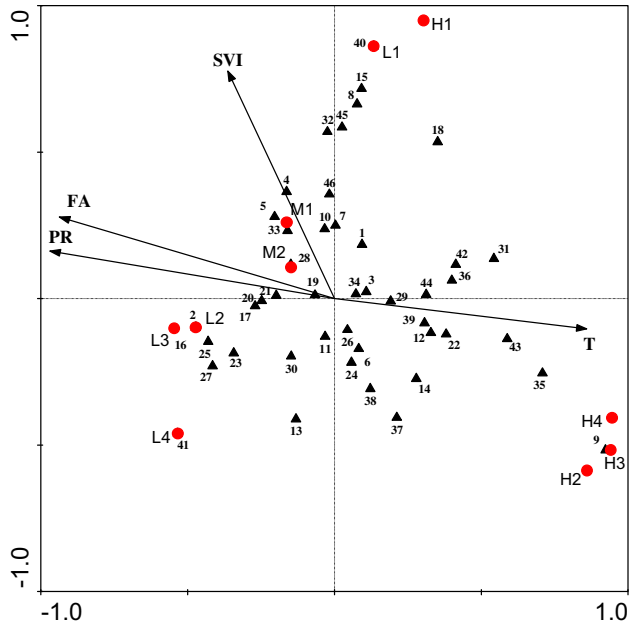

Fig. 5. Triplot diagram of CCA on sludge bacterial community in both reactors. The environmental variables, activated sludge samples, and order taxa were represented by arrow, red dot, and black triangle, respectively. PR: phosphorus removal; FA: filament abundance; T: Temperature; SVI: SVI. The combination of letters and numbers around the red dot represented the sludge samples from both reactors at different times. H: high temperature (25˚C); M: medium temperature (20˚C); L: low temperature (15˚C); the number indicated the different times. The number around the order taxa represented the different species (band 1 to band 46). The percentage variances explained were 42.5% (first axe), 31% (second axe), 21.3% (third axe), and 5.2% (forth axe).

3.7. Relationships among temperature, bacterial community structure, and settleability

In both reactors at different temperatures, pronounced differences were observed with regard to the quantitative distribution of the bacteria (Fig. 3) [31]. Many researchers have demonstrated that *Accumulibacter* (β-*Proterobacteria*) are important PAOs in both laboratory-scale and full-scale EBPR plants [30,31]. These bacteria can uptake volatile fatty acids (e.g., acetate and amino acids) and store them as intracellular PHA anaerobically. Besides *Accumulibacter*, *Tetrasphaera*-related organisms (*Actinobacteria*) are also PAOs that are abundant in many full-scale EBPR plants [32]. The *Tetrasphaera*-related organisms have a more versatile physiology than *Accumulibacter*. All the *Tetrasphaera* PAOs can consume amino acids and certain sublineages can uptake acetate. Additionally, most *Tetrasphaera* are able to hydrolyze starch and ferment glucose anaerobically [30]. Generally, the Actinobacterial genus *Tetrasphaera* is more abundant (25–30% of all bacteria) in EBPR plants than *Accumulibacter* (2–8% of all bacteria) [14]. In the current study, *Accumulibacter* did not appear during the entire experiment, whereas the bacterial species (band 17) similar to *Actinobacteria* tended to be spurred by the low temperature. The organic compounds in the influent were composed of Na-acetate, potato starch, peptone, milk powder, and yeast. Both Na-acetate and potato starch accounted for approximately a quarter of the total COD. Because

Na-acetate was not the sole carbon source, the amount of *Accumulibacter* might be too small to be detected by DGGE. However, the large quantity of starch and other particulate substrates seemed to stimulate the growth of *Actinobacteria* (probably *Tetrasphaera*-related organisms). Some researchers reported that EBPR may not be a primary consequence of the presence of *Actinobacteria*-related organisms. Notably, a diversity of organisms, including α-*Proteobacteria*, β-*Proterobacteria*, and γ-*Proteobacteria*, may be involved in phosphorus removal [33]. As shown in the current study, the three aforementioned *Proteobacteria* subdivisions (bands 20 and 30) tended to be closely associated with low temperature and high phosphorus removal. These bacteria were extrapolated to be PAOs or at least had a key function in the EBPR process. Additionally, band 21 was remarkably stimulated at a lower temperature but was suppressed at a higher temperature. These bacteria were similar to the *Runella* sp. affiliated with *Bacteroidetes*. *Runella* sp. was isolated from activated sludge in the EBPR system and was able to produce alkaline phosphatase and phosphohydrolase [34,35], which indicated that these bacteria might be putative PAOs.

The pronounced shift in bacterial community structure from *Actinobacteria* to *Bacteroidetes* was accompanied by a notable improvement in settleability. This result agreed well with a previous observation showing that the proliferation of *Actinobacteria* was concurrent with the low temperature and poor sludge settleability [18]. Therefore, besides the large quantity of filaments, the proliferation of *Actinobacteria* may greatly contribute to the poor settleability at medium and low temperatures. When the operating temperature was increased, the population of *Bacteroidetes* notably increased, which was concurrent with improved settleability. This phylum may have a possible metabolic advantage over filaments at higher temperatures and contribute to better settleability.

4. Conclusion

The influence of temperature on sludge settleability and bacterial community structure was investigated in two continuous-flow EBPR systems. Preliminary results indicated that settleability depended both on the filament content and on Pns/VSS and NVSS/VSS contents at varying temperature. In the No. 1 Reactor, when the temperature was increased from 20 to 25℃, settleability was significantly improved because of the substantial reduction in filament content, and the effect of Pns/VSS and NVSS/VSS on settleability was obscured by the decrease in filament content. On the other hand, when the temperature was decreased from 20 to 15℃ in the No. 2 Reactor, settleability changed slightly in the long run and was mainly determined by Pns/VSS and NVSS/VSS ($p < 0.05$). The effect of filaments on settleability can be disregarded because of the minor change in filament content. A common core of bacterial phyla was observed in the EBPR systems with different temperatures via PCR–DGGE analysis. The CCA results indicated that bacterial community structure was significantly affected by the temperature and sludge properties ($p < 0.01$). At a high temperature (25℃), 21 species mostly affiliated with *Proteobacteria*, followed by *Bacteroidetes*, were stimulated greatly. The proliferation of *Bacteroidetes* might have a close relation with the improvement of settleability. At a low temperature (15℃), 25 species which were different from that at a high temperature proliferated in the system. These species mostly affiliated with *Proteobacteria*, followed by *Actinobacteria*. The large population of *Actinobacteria* was closely correlated with poor settleability. This study provides insights to the sludge properties and bacterial community dynamics at varying temperatures and may help control sludge bulking and deteriorations in EBPR systems.

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