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Community diversity and biofilm characteristic response to low temperature and low C/N ratio in a suspended carrier biofilm reactor

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ABSTRACT

A modified anaerobic–anoxic–aerobic reactor was developed to investigate the influence of both a low carbon-to-nitrogen (C/N) ratio and low temperature on microbial community structure and biofilms. The thickness and density of the biofilm on the suspended carriers and production of extracellular polymeric substances decreased with temperature. The biofilm thickness was more obviously affected by temperature than was the biofilm density. The polysaccharide-to-protein ratio at 10°C was remarkably higher than at 18°C, which would suggest that microbes secrete more polysaccharides in low-temperature conditions. Filamentous bulking was observed at 10°C when SVI was above 170 mL/g; however, this phenomenon did not occur at other temperatures. Our experimental data support the hypothesis that low temperature was more likely to lead to sludge bulking than was nutrient limitation. Chao1 values of metagenomic sequencing at 26°C were higher than those at 10°C. *Haliscomenobacter*, a primary filamentous species, was found to be more numerous in biofilm than in activated sludge at the same temperatures. The bacterial communities in the biofilm were different from those in the activated sludge, suggesting that suspended carriers had a significant effect on microbial community structure and biomass. Additionally, the ability of suspended carriers to inhibit sludge bulking was more significant at low temperature. Achievement of stable operation of a suspended carrier biofilm reactor is highly beneficial in terms of lower carbon requirements to the removal of organics and nitrogen from domestic wastewater.

Keywords: Suspended carriers; Community diversity; Biofilm; EPS; Low temperature; Low C/N ratio

1. Introduction

Biological processing is an economical wastewater treatment method, but it is often limited to treating wastewater with a low carbon-to-nitrogen ratio (C/N) due to a shortage of carbon to release phosphorus in anaerobic conditions and to denitrification in anoxic conditions [1,2]. Biofilm processes, such as trickling filters, biological aerated filters (BAF), suspended carrier biofilm reactors (SCBR), and moving bed biofilm

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reactors (MBBR), are widely used in domestic and industrial water treatment [3]. The modified anaerobic–anoxic–aerobic (A^2O) process, which consists of adding a pre-anoxic tank to the basic A^2O process and combining suspended and attached growth processes by adding suspended carriers into the aeration tank, is a highly effective biofilm reactor and has attracted significant interest in the field of wastewater treatment. Zeng et al. studied the nitritation and denitritation of a modified A^2O process and found that the nitrite accumulation rate was about 90% and ammonia removal efficiency was over 95% [4]. A pre-anoxic tank in a modified A^2O system can reduce the effects of nitrate on releasing phosphorus under anaerobic conditions in returned sludge. More importantly, suspended carriers create a large surface area for colonization and maintain high specific biomass activity [5]. Rikmann et al. showed that sulfate reduction and ammonium oxidation were established in a MBBR at 20℃; however, the same process took place at 36℃ in an upflow anaerobic sludge blanket reactor (UASBR) [6]. Immobilization of biomass in the form of biofilms is an efficient method to alleviate the sludge retention time (SRT) conflict between nitrifiers and phosphorus-accumulating organisms operating in a single sludge process, and it combines the advantages of both activated sludge and biofilm processes [7,8]. In northeast and North China, one of the most important factors in the performance of biological wastewater treatment is the variation in the water temperature during the year, particularly in winter (water temperature can decrease to approximately 10℃). Low temperatures can decrease biological activity and prolong the generation time of micro-organisms, especially for nitrifying bacteria [9]. Additionally, the biofilm is thin and easily becomes detached at low temperature resulting in poor pollutant removal capacity.

Research has been conducted on the biofilm processes [3,10]. Zekker et al. developed an anaerobic ammonium oxidation (Anammox) process using MBBR for the removal of nitrogen from low C/N ratio (<1) wastewater [11]. However, this research was aimed at either low temperature or low C/N ratio. Additionally, much explanation is given of microbial community structure dynamics in biofilm processes, but little is presented on the comparison of micro-organism community structures between activated sludge and biological membranes. For community analysis, previous studies using conventional molecular techniques, such as PCR (polymerase chain reaction)–DGGE (denaturing gradient gel electrophoresis), FISH (fluorescence *in situ* hybridization), clone library, and T-RFLP (terminal restriction fragment length polymorphisms), have had difficulty obtaining a complete profile of the filaments. The ion torrent semiconductor sequencing platform has some principal advantages over traditional cultivation technologies and molecular methods for monitoring relative changes and the diversity of microbial communities [12].

In this paper, we developed a modified A^2O reactor to investigate the effects of low temperature and low C/N ratio on system performance and contrast the microbial community structures of biofilm and sludge. The purpose of installing the pre-anoxic zone was to reduce the nitrate concentration in the sludge returned from the settling tank to the anaerobic zone. Additionally, it would effectively mitigate or diminish the competition for carbon source between the denitrifying bacteria and phosphorus accumulating organisms (PAOs) in the anaerobic zone. The main objectives of this study are to assess how design and operation might improve pollutant removal in bioreactors and maintain the stability of biofilm structures on the suspended carriers, and in addition, to provide theoretical support for sewage treatment in the northeast and north areas of China.

2. Materials and method

2.1. Reactor operation

The experimental setup involved a modified A^2O reactor with an effective volume of 39 L, which consisted of a pre-anoxic zone (3 L), an anaerobic zone (6 L), an anoxic zone (12 L), and an aerobic zone (18 L) (Fig. 1). The modified A^2O was operated for 180 d at different temperatures in Harbin, China. Temperature was maintained by a thermostat (GDH-4006, SCIENTZ) with cool water circulating from 26, 22, 18℃, and 14 to 10℃. The system was operated under each temperature for four weeks. The pre-anoxic, anaerobic, and anoxic zones were equipped with magnetic stirrers. Air was supplied through diffusers placed at the bottom of the pre-anoxic, anoxic, and aerobic zones. The dissolved oxygen (DO) concentrations in the aerobic zone, pre-anoxic zone, and anoxic zone were maintained in the range of 2–4 mg/L, 0.2–0.5 mg/L, and 0.2–0.5 mg/L, respectively. The DO concentration was measured with a multi-parameter water quality digital analyzer (HQ30D, HACH). The aerobic zone was filled with suspended carriers (filling ratio approximately 40%). The suspended carriers were polypropylene sphere fillers with a diameter of 80 mm. The specific surface area was 350 m^2/m^3, and the specific weight was 8.3 kg/m^3. The average melting point of the suspended carrier was more than 120℃.

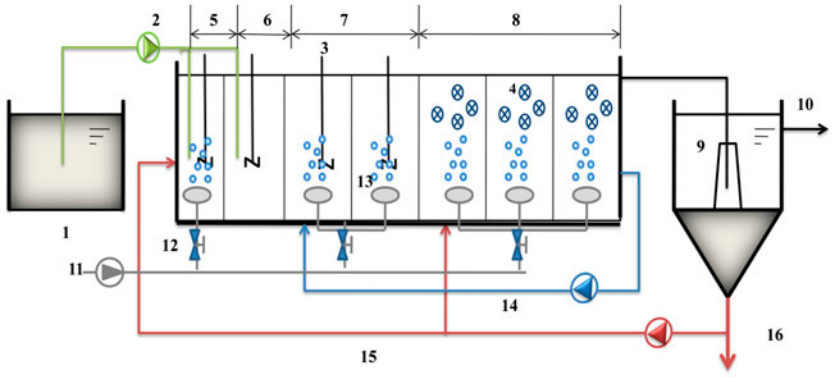

Fig. 1. Schematic diagram of the lab-scale modified A^2O reactor.
Notes: (1) influent tank; (2) pump; (3) stirrer; (4) suspended carrier; (5) pre-anoxic zone; (6) anaerobic zone; (7) anoxic zone; (8) aerobic zone; (9) settler; (10) effluent; (11) air pump; (12) airflow meter; (13) air diffuser; (14) internal recycle; (15) external recycle; (16) waste sludge.

The reactors were operated in continuous-flow mode with a hydraulic retention time (HRT) of 13 h. The influent flow rate was maintained at 3.0 L/h (pre-anoxic zone flow rate = 0.5 L/h; anaerobic zone flow rate = 2.5 L/h). The internal recycle ratio was 200%. The returned activated sludge (RAS) ratios to the pre-anoxic zone and aerobic zone were maintained at 35 and 15%, respectively. At different temperatures, the SRT of the reactor was changed from 15 to 20 d. The raw wastewater used for the experiment was taken from the drain well near our laboratory in Harbin, China. Seeding sludge used in this study was taken directly from the returned sludge of a settling tank at Harbin WenChang wastewater treatment plant (a full-scale plant located in the city of Harbin, China). The major characteristics of the wastewater and seeding sludge are described in Table 1.

Table 1
Characteristics of influent wastewater

	Parameter	Average
Wastewater	COD (mg/L)	205 ± 19.7
	BOD_5 (mg/L)	70 ± 13.2
	TN (mg/L)	49.7 ± 2.3
	TP (mg/L)	5.5 ± 0.38
	SS (mg/L)	150 ± 23.2
	PH	7.5 ± 0.02
	C/N	3.93 ± 0.43
Sludge	MLSS (mg/L)	9,300 ± 532
	MLVSS (mg/L)	7,200 ± 320
	SVI	77 ± 4.3

2.2. Analytical methods

Influent and effluent samples were collected daily for chemical oxygen demand (COD), ammonia nitrogen (NH_3-N), total nitrogen (TN), and total phosphorus (TP) analyses according to Standard Methods for the Examination of Water and Wastewater [13]. SVI, MLSS, and MLVSS were analyzed throughout the reactor once per two days also following Standard Methods [13]. Extracellular polymeric substances (EPS) was extracted using HCHO/NaOH following Malamis and Andreadakis [14]. Polysaccharide was quantified by the phenol–sulfuric acid method with glucose as the standard reference [15], whereas proteins were measured by the modified Lowry method [16] with bovine serum albumin as the standard reference. Every datum was measured three times to obtain the average value.

2.3. Preparation of biofilm samples

Biofilm samples were collected from the suspended carrier surface for analysis of thickness, density, composition, and EPS content after more than two weeks of operation. For this purpose, the suspended carrier was rinsed with deionized water three times to remove excess water. The biomass was removed manually, with a plastic spatula, from a specific length of carrier, and put into centrifuge tubes. The samples were stored at −20 ℃.

Biofilm thickness (T) was calculated based on the liquid volume displaced by the biomass (V) and the area scraped from the carrier surface was calculated according to $S = L \times B$, where S is the carrier area, L the carrier length, and B the carrier width. The biofilm thickness was calculated as $T = V/S$. Biofilm volumetric density (ρ) was obtained by determining mass (M) and volume (V) of the sample, and calculated as

$\rho = M/V$. Each parameter was measured three times and averaged.

2.4. DNA extraction and sequence analysis for community composition

Genomic DNA was extracted from 0.2 g of both activated sludge and biofilm samples, in triplicate, using PowerSoil® DNA Isolation Kit for soil (MOBIO, USA) according to the manufacturer's instructions. Nucleic acid concentrations were measured spectrophotometrically by a NanoDrop Spectrophotometer 2000 (Thermo Scientific, USA). To compensate for variation among DNA in the extractions, the three extracts were mixed together before the DNA was analyzed [17]. Sequence analysis of the collected sludge samples based on V3 hypervariable region amplification of the bacterial 16S rDNA sequence was carried out on the ion torrent system using the universal primers BSF8 (5′-AGAGTTTGATCCTGGCTCAG-3′) and BSR534 (5′-ATTACCGCGGCTGCTGG-3′) with 10nt barcodes (Sangon, China).

The sequence reads were trimmed and assigned to each sample based on their barcodes. Sequences were clustered into operational taxonomic units (OTUs) at a 97% identity threshold. The aligned 16S rDNA sequences were used for a chimera check using the Uchime algorithm [18]. A diversity index was calculated, such as the Chao1 estimator of richness, observed species, and Shannon's diversity index [19].

2.5. Scanning electron microscopy

At the end of the batch process, a small piece of plastic sheet with biofilms was collected. The biofilm was fixed using 2.5% glutaraldehyde at 4℃ for 1.5 h. The sample was then washed three times, with 1.0 mM phosphate buffer solution (PBS, pH 7.2) for 10 min. The fixed sample was dehydrated sequentially with 30, 50, 70, 90, and 100% (v/v) ethanol and 100% ethanol: isoamyl acetate (1:1), for 15 min at each step. After fixing in pure isoamyl acetate for 15 min, the sample was dried in a drier for 8 h. The samples were then sputter coated with a thin 5-nm gold layer, and observed with a scanning electron microscope (SEM) (JEOL JSM-6480LV) in high vacuum mode (acceleration voltage 10e 15 kV, sample size 10 mm).

2.6. Statistical analysis

Pearson correlation analysis was conducted using SPSS software (version 17.0), false discovery rate corrections were made for all multiple correlations, and adjusted p-values were reported. There was a significant relationship when $p < 0.05$. ANOVAs was conducted to examine differences between various samples, and there was a significant difference when $p < 0.05$.

3. Results and discussion

3.1. Pollutant removal efficiencies in steady state

Pollutant removal of COD, NH_3-N, TN, and TP are described in Table 2. The COD of the influent was maintained at 205 ± 21.3 mg/L, and effluent COD ranged from 24.1 ± 11.4 to 29.8 ± 10.4 mg/L during operation. The average removal efficiency of COD decreased along with temperature from 26℃ (90.1 ± 2.1%) to 10℃ (84.5 ± 4.6%). Similarly, the removal efficiencies of NH_3-N and TP changed from

Table 2
Performance of the modified A^2O bioreactor

		26℃		22℃		18℃		14℃		10℃	
	IF (mg/L)	EF (mg/L)	RE (%)	EF (mg/L)	RE (%)	EF (mg/L)	RE (%)	EF (mg/L)	RE (%)	EF (mg/L)	RE (%)
COD	205 ±19.7	24.1 ±11.4	90.1 ±2.1	24.3 ±8.8	89.2 ±3.4	26.7 ±13.5	87.5 ±4.6	28.1 ±11.2	86.6 ±4.2	29.8 ±10.4	84.5 ±4.6
NH_3-N	45 ±1.8	3.1 ±1.3	93.1 ±1.4	3.2 ±1.3	92.9 ±1.1	3.4 ±1.3	92.4 ±1.4	3.7 ±1.5	92.3 ±1.6	3.9 ±1.4	91.3 ±2.1
TN	49.7 ±2.3	3.7 ±1.9	92.1 ±1.9	4.3 ±1.2	91.3 ±2.1	8.4 ±2.6	83.1 ±2.1	9.7 ±2.8	80.4 ±3.2	10.9 ±3.4	78.1 ±2.7
TP	5.5 ±0.4	0.34 ±0.1	93.8 ±1.3	0.37 ±0.1	93.3 ±1.2	0.4 ±0.1	92.7 ±1.4	0.41 ±0.1	92.1 ±1.2	0.45 ±0.1	91.8 ±1.8

Notes: IF = influent (mg/L); EF = effluent (mg/L); RE = removal efficiency (%).

93.1 ± 1.4% to 91.3 ± 2.1% and 93.8 ± 1.3% to 91.8 ± 1.8%, respectively, with the decrease in temperature from 26 to 10℃. It was obvious that the temperature had little influence on carbonic substance degradation efficiency, ammonium nitrogen, and TP removal rate in the bioreactor. Adding suspended carriers in aerobic tanks benefited the performance of a target plant at low environmental temperature [3]. Lai et al. reported that using fiber polypropylene media in an A^2O process as a cost-effective strategy might significantly accelerate the efficacy of treatment of organics and nutrients [20]. However, compared with the removal of COD, NH_3-N, and TP, the efficiency of TN significantly decreased with temperature ($p < 0.05$) (Fig. 2). The total nitrogen loading rate (TNLR) fed into the modified A^2O reactor was approximately 90 mg N/L/d. The total nitrogen removal rate (TNRR) of 84.92 ± 3.20 mg N/L/d was achieved at 26℃, gradually decreasing the efficiency of TN from 92.1 ± 1.9% (26℃) to 78.1 ± 2.7% (10℃). The minimum TNRR achieved after four weeks of operation at 10℃ was approximately 71 mg N/L/d. The results may be attributed to incomplete nitrification caused by temperature decrease. In activated sludge systems, the decline of environmental temperature may significantly reduce the activity of nitrifying bacteria resulting in increased TN concentration [21]. After 180 d of operation, the biomass TNRR showed a specific TNRR of 74.67 ± 2.51 mg N/g VSS/h, which indicates a biomass activity increase by successful cultivation at a low temperature (10℃). The resence of nitrifying bacteria within the biomass might be beneficial when introducing suspended carriers to an aerobic zone. The suspended carriers had the potential to improve the adaptation of biomass to lower temperature.

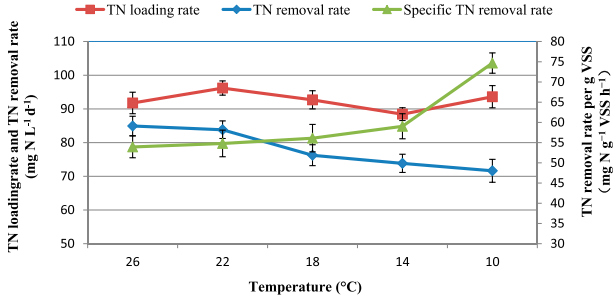

Fig. 2. Changes in the TNRRs, TNLRs, and specific TN removal rates in modified A^2O system. Error bars are defined as standard error of the mean ($n = 3$, technical replicates).

3.2. Impact of temperature on biofilm thickness and density

The biofilm thickness and density were a function of the exposure time of the biofilm to certain hydrodynamic conditions and environmental temperatures. Different temperature resulted in different thicknesses and compositions of the biofilm. In Fig. 3(a), the biofilm thicknesses significantly ($p = 0.001$) declined with decreasing temperature from >1,700 μm to approximately 1,200 μm. The greatest biofilm thickness reached 1,735 μm was observed at 26℃. Fluctuations in biofilm density during the testing period are also shown in Fig. 3(a). The average values seem to suggest that biofilm density decreased with decreasing temperature and reached a maximum value (530 g/L) at 26℃. At higher temperatures, micro-organisms exhibit higher activity, and this would in turn influence the biofilm density. One-way ANOVA was conducted to examine differences in biofilm thickness and density among different temperatures and adjusted p-values were reported. The biofilm thickness between 26 and 23℃ showed no significance difference ($p = 0.536$), whereas between 26 and 18℃, between 26 and 14℃, and between 26 and 10℃, the

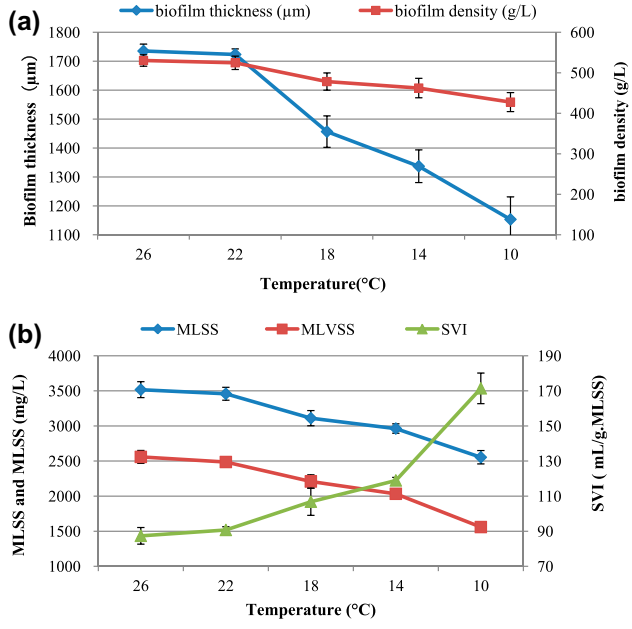

Fig. 3. Changes in biofilm characteristics for varying applied temperature in a modified A^2O reactor based on measurement of biofilm thickness and density (a) and sludge biomass concentration (b). Concentrations of sludge biomass were determined in terms of mixed liquor suspended solids (diamond), mixed liquor volatile suspended solids (square) and SVI. SVI = SV_{30}/MLSS. Error bars are defined as standard error of the mean ($n = 3$, technical replicates).

biofilm thickness differences were significant at $p \leq 0.001$. In contrast to the changes between 26 and 18℃, between 26 and 14℃, and between 26 and 10℃ ($p < 0.05$), the biofilm density declined between 26 and 23℃, but the difference was not significant ($p > 0.05$). Pearson correlation coefficients were determined for all multiple correlations and adjusted p-values were reported. Both biofilm thickness and density were strongly and positively correlated with temperature from 26 to 10℃ ($r = 0.760$, $p < 0.05$ for thickness; $r = 0.700$, $p < 0.05$ for density). The low level of biofilm thickness and density observed at 14 and 10℃ could be a consequence of the influence of temperature on microbial physiological activity. However, the decreased ratio of biofilm thickness (35.80 ± 4.38%) with temperature was greater than that of biofilm density (19.36 ± 2.34%), suggesting that the biofilm thickness was more obviously affected by temperature.

In terms of sludge biomass, MLSS and MLVSS decreased from the initial stage and achieved a minimum at 10℃, with a decrease of 27.38 ± 3.02% in MLSS and 39.07 ± 4.91% in MLVSS ($p < 0.001$) (Fig. 3(b)). Compared with sludge biomass, large variations in the SVI were observed from 26℃ (87.36 ± 4.74) to 10℃ (171.41 ± 8.74) in operation. On several occasions, sludge between 26 and 14℃ experienced modest non-filamentous bulking (87 < SVI < 120 mL/g MLSS), but the bulking and dispersed growth phenomena were usually observed at 10℃ (200 > SVI > 170 mL/g MLSS). Nutrient limitation and low temperature have been identified as factors for the proliferation of filamentous bacteria in activated sludge [22,23]. This study shows that low temperature rather than low nutrient supply seems to stimulate filamentous bacteria growth.

3.3. Impact of temperature on EPS accumulation

EPS, complex high-molecular weight mixtures of polymers, are assumed to be another important component involved in biofilm formation and microbial flocculation properties. EPS is usually characterized and quantified based on polysaccharide and protein contents. The optimum ratio of proteins to polysaccharides can contribute to a balanced biofilm structure. The relationship between temperature and EPS accumulation per volatile solids concentration is shown in Fig. 4. Both proteins (PN) and polysaccharides (PS) showed a remarkable decrease in amount as the temperature began to decline (from 26 to 10℃: PS: 38.67 ± 3.51 vs. 25.33 ± 4.16 mg/g vss, $p < 0.05$; PN: 122.67 ± 9.50 vs. 82.67 ± 6.51 mg/g vss, $p < 0.001$). Statistical analyses of experimental data from this study indicate that the amount of protein ($r = -0.915$, $p < 0.05$) and total EPS ($r = -0.895$, $p < 0.05$) demonstrated a modest negative correlation with the SVI. However, no significant correlation existed between the polysaccharides content and the SVI. Inconsistent with the EPS profile, the polysaccharide to protein (PS/PN) ratio decreased from 26 to 18℃ (0.32 ± 0.05 vs. 0.28 ± 0.04, $p = 0.303$), followed by a slight increase in 18–10℃ transition, and remained at approximately 0.31 at 10℃ ($p = 0.394$). The PS/PN at 10℃ was higher than at 18℃, which suggested that the polysaccharides fraction of the total EPS concentration increased. In addition, the reactor was under limited filamentous bulking sludge at 10℃ (200 > SVI > 170 mL/g MLSS), demonstrating an interrelationship between the production of polysaccharides and activated sludge settleability. The EPS may form a dense gel that resists the expression of water from gel pores. Ultrastructural studies, using correlative microscopy, have revealed a complex and hydrated EPS within the floc matrix indicating a high capacity to retain water [24,25].

In this study, EPS production also showed a significant correlation with biofilm thickness and biofilm density (EPS, PS, and PN vs thickness: r > 0.965, $p < 0.01$; EPS, PS, and PN vs. density: $r > 0.967$, $p < 0.01$). The significant correlation between the total EPS content and the biofilm characteristics supports the notion that the presence of a large amount of EPS does have a positive effect on strengthening the stability of biofilm structure. This effect can be partially explained by steric forces arising from the EPS to form a matrix for bacteria to embed into [26,27]. Consequently, the content and composition of EPS played a crucial role in biological wastewater treatment. Decreasing temperature led to lower measured EPS

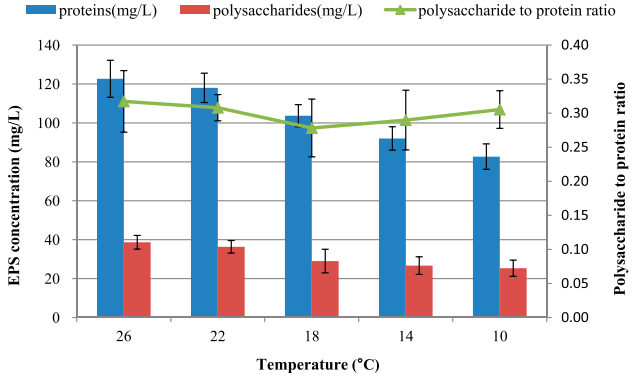

Fig. 4. EPS correlation with temperature. Total EPS was extracted from the sludge biomass and quantified for the extracellular polysaccharides (PS) and extracellular proteins (PN) components. Error bars are defined as standard error of the mean ($n = 3$, technical replicates).

content. A higher SVI along with a higher PS/PN ratio might increase the risk of sludge bulking due to low temperatures.

3.4. Microbial community structure analysis

Ion torrent semiconductor sequencing was used to analyze samples from activated sludge and biofilms to identify shifts in the microbial community in response to temperature. In total, 272,116 reads were generated in the study, leaving 204,739 reads after quality filtering and removal of chimeric sequences (QC). Most sequence lengths ranged from 400 to 500 bp, and the average was above 400 bp. Species richness, coverage, and diversity estimations were calculated for each data-set (Table 3). Shannon index and Chao1 index, measures of community richness and evenness, showed a community density change with temperature. Relatively good coverage revealed that these libraries represented the majority of the bacterial 16S rDNA sequences in each sample, with values ranging from 85.6 to 97.2%. Chao1 values clearly showed that the estimated bacterial richness in samples at 26˚C was higher than at 10˚C. Shannon indices among samples were significantly different ($p = 0.012$), ranging from 6.67 to 7.42. More interestingly, the biofilm's Shannon index and Chao1 index were slightly higher than that of the sludge with the decrease in temperature, which might indicate that suspended carriers result in high specific biomass activity by causing colonized growth, especially at low temperature.

From our filtered sequences, we identified a total of 30 bacterial phyla across all samples with RDP classifiers. The most prevalent phyla in all samples were: *Proteobacteria*, *Chloroflexi*, *TM7*, *Bacteroidetes*, *Planctomycetes*, *Actinobacteria*, *Firmicutes*, and *Acidobacteria*, comprising more than 85% of all reads (Fig. 5(a)). At the class level, sequencing reads could be assigned to 50 individual classes, of which 7 were present in all samples at a relative abundance of more than 1% (Fig. 5(b)). Dominant species were *β-Proteobacteria*,

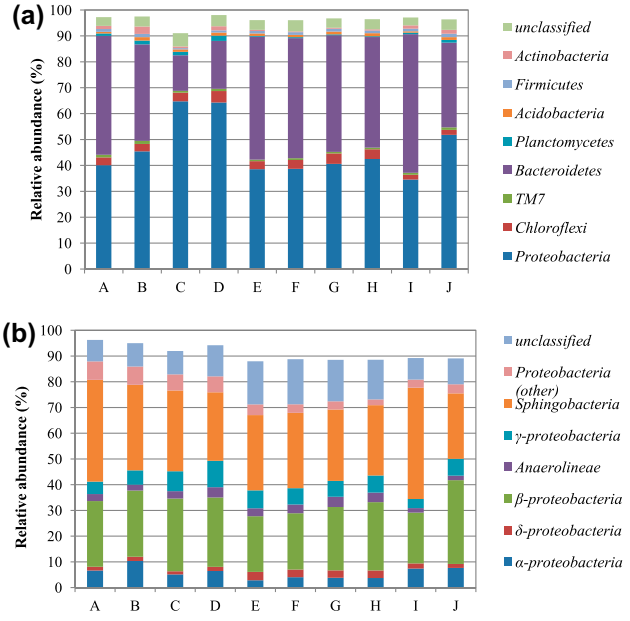

Fig. 5. Microbial community structures of the bioreactor in biofilm and activated sludge samples during the formation of biofilm at different temperature: (a) Phylum level and (b) class level. *A* = biofilm at 26˚C; *B* = sludge at 26˚C; *C* = biofilm at 22˚C; *D* = sludge at 22˚C; *E* = biofilm at 18˚C; *F* = sludge at 18˚C; *G* = biofilm at 14˚C; *H* = sludge at 14˚C; *I* = biofilm at 10˚C; *J* = sludge at 10˚C. Different colors represent different bacteria, including gray for unclassified sequence, namely the unclassified to sequence.

Table 3
OTU counts, richness, and diversity estimates for the bacteria in activated sludge and biofilm samples at different temperature

Sample ID[a]	Seq num[b]	OTU	Shannon index	Chao1 index	Coverage
26S	24,372	7,403	7.4195	24,131.45	0.9718
26B	20,853	6,688	7.4180	22,262.54	0.9138
22S	22,113	6,631	7.4034	23,610.21	0.9285
22B	21,934	7,340	7.3478	21,738.98	0.8829
18S	20,776	6,860	7.1068	24,207.67	0.9395
18B	17,175	5,907	7.0090	23,492.12	0.8556
14S	20,021	5,628	6.9093	20,803.22	0.8820
14B	18,442	5,920	6.9638	21,931.67	0.8688
10S	17,509	5,869	6.6687	18,347.09	0.8558
10B	21,544	7,533	6.8802	20,998.66	0.8655

[a]26 = 26˚C; 22 = 22˚C; 18 = 18˚C; 14 = 14˚C; 10 = 10˚C; S = sludge; B = biofilm.
[b]Seq num = sequence number; OTU = operational taxonomic unit number.

γ-Proteobacteria, *Sphingobacteria*, *Proteobacteria* (other), *α-Proteobacteria*, *δ-Proteobacteria*, and *Anaerolineae*. *Proteobacteria* was the dominant micro-organism accounting for approximately 35–52% (relative abundance) of the microbial community and including *Proteobacteria* (other) (unclassified *proteobacteria*, about 2.2–7.2%), *α-Proteobacteria* (approximately 2–10%), *β-Proteobacteria* (approximately 19–32%), *γ-Proteobacteria* (approximately 3.6–10.3%), and *δ-Proteobacteria* (approximately 1.5–3.3%). At the genus level, sequencing reads could be assigned to 621 individual genera, of which 24 were abundant community members (relative abundance > 0.1%) (Table 4) and the ratio of genera were calculated by Pearson correlation coefficients to state the changes in microbial species abundance to temperature decline (Table 5). Eight members showed strong correlations with temperature. *Brevundimonas*, *Haliscomenobacter*, *Dechloromonas*, *Thermomonas*, and *Terrimonas* presented a negative correlation with temperature in biofilm or sludge samples. Among these, *Haliscomenobacter* and *Zoogloeagenera* are of particular interest, as they are suspected to form bulking sludge in the activated sludge process. Strains of *Haliscomenobacter hyclrossis*, assigned to the genus *Haliscomenobacter*, have always been considered to be the primary filamentous species and found to be responsible for biomass bulking [28,29]. Filamentous bulking involves the unbalanced competition between *Zoogloea* bacteria and filamentous bacteria in the activated sludge. If the number of filamentous bacteria is higher than that of *Zoogloea* bacteria, flocculent settle ability will degrade. However, if filamentous bacteria are deficient, the activated sludge will be lacking in structure, which will lead to increased effluent turbidity. In short, it is important to keep a balance of both *Zoogloea* bacteria and filamentous bacteria in the system. As illustrated in Table 4, these genera remained relatively stable and the relative abundance of *Zoogloea* was greater than the abundance of *Haliscomenobacter* in all groups. At the same time, it was clear that the relative abundance of *Haliscomenobacter* in biofilm was higher than that in activated sludge at the same temperatures. It seems that filamentous bacteria are more likely to adhere to the surface of the suspended carrier, and this

Table 4
The top 24 most abundant community members (relative abundance > 0.1%) atgenus level at different temperature

	26B[a]	26S	22B	22S	18B	18S	14B	14S	10B	10S
Brevundimonas	0.16	0.34	0.23	0.52	0.75	0.59	0.94	1.05	1.44	1.58
Ignavibacterium	0.28	0.34	0.25	0.26	0.35	0.29	0.19	0.23	0.38	0.47
Acidovorax	0.25	0.18	0.24	0.25	0.54	0.56	0.23	0.36	0.21	0.57
Nitrospira	5.52	4.64	4.35	4.34	4.16	4.11	3.13	2.33	2.59	2.23
Haliscomenobacter	1.58	1.35	1.76	1.38	1.90	1.41	2.13	1.62	2.91	2.56
Longilinea	2.13	2.29	2.66	2.45	1.17	1.21	1.41	2.02	1.22	1.86
Propionivibrio	0.41	0.39	0.47	0.58	0.66	1.46	0.49	0.14	0.30	0.55
Novosphingobium	0.11	0.23	0.27	0.25	0.17	0.43	0.77	1.05	0.91	0.37
Gemmatimonas	0.45	0.40	0.30	0.45	0.33	0.36	0.15	0.14	0.71	0.17
Dechloromonas	4.52	5.30	4.65	5.58	4.68	6.05	4.99	6.63	5.77	6.96
Azospira	0.38	0.46	0.53	0.56	0.74	1.09	0.97	0.44	0.81	1.68
TM7	0.69	0.59	0.64	0.61	0.95	0.76	2.49	1.24	0.63	0.54
Ferruginibacter	1.34	1.26	1.40	1.42	0.72	0.86	0.75	0.54	1.63	1.15
Thermomonas	0.32	0.28	0.34	0.37	0.49	0.54	1.18	1.52	1.43	1.60
Simplicispira	0.75	0.52	0.74	0.63	1.14	0.37	2.44	0.23	0.59	0.55
Caenimonas	0.26	0.55	0.47	0.53	1.34	1.05	1.32	1.29	1.6	1.62
Prosthecobacter	0.3	0.36	0.32	0.46	0.31	0.61	0.27	0.18	0.22	0.41
Filimonas	0.66	0.65	0.57	0.52	1.22	0.36	0.26	0.16	0.41	0.72
Terrimonas	0.49	0.6	0.64	0.70	0.77	1.12	1.67	3.91	4.2	4.23
Dokdonella	0.41	0.29	0.36	0.50	0.25	0.42	0.25	0.14	0.37	0.27
Bdellovibrio	1.29	1.27	1.19	1.11	0.71	0.78	0.58	0.66	0.45	0.28
Sulfuritalea	2.15	1.78	1.81	2.30	2.20	1.90	1.59	1.31	1.34	2.65
Zoogloea	12.59	13.65	11.89	11.97	11.67	10.50	10.49	9.35	9.99	9.24
Sediminibacterium	1.53	1.52	1.53	1.45	2.22	1.10	1.68	1.12	0.87	1.54
Unclassified	45.43	45.45	44.02	42.54	38.43	33.34	36.23	37.08	40.60	33.17

[a]26 = 26℃; 22 = 22℃; 18 = 18℃; 14 = 14℃; 10 = 10℃; S = sludge; B = biofilm.

Table 5
Pearson correlation of the top 24 community members and temperature

	Biofilm	Sludge
Brevundimonas[a]	$r = -0.977$; $p = 0.004$**	$r = -0.950$; $p = 0.013$
Nitrospira	$r = 0.986$; $p = 0.002$**	$r = 0.933$; $p = 0.02$
Haliscomenobacter[a]	$r = -0.925$; $p = 0.024$	–
Dechloromonas[a]	$r = -0.890$; $p = 0.043$	$r = -0.994$; $p = 0.001$**
Thermomonas[a]	$r = -0.936$; $p = 0.019$	$r = -0.930$; $p = 0.022$
Terrimonas	–	$r = -0.919$; $p = 0.027$
Bdellovibrio[a]	$r = 0.966$; $p = 0.007$**	$r = 0.988$; $p = 0.002$**
Zoogloea	$r = 0.982$; $p = 0.003$**	$r = 0.966$; $p = 0.007$**

[a]Negative correlation with temperature; significant differences are indicated as follows: **$p < 0.01$.

inspires us to make better use of biofilms on the suspended carrier to control sludge bulking. Furthermore, *Nitrospira*-like bacteria, e.g. *Nitrospira moscoviensis* sp. nov., which are considered to be associated with nitrification [30], were also observed during the study. The relative abundance of *Nitrospira* was positively related to temperature in the experimental groups.

Other genera related to bacterial community shifts have also been identified. *Bdellovibrio* is known to be a predator, with the ability to invade and consume other bacteria, penetrating their periplasm, and finally bursting the cell envelope [31]. Some studies showed that *Bdellovibrio* might easily survive on biofilm, because these organisms have been found in natural marine biofilms, but they were not always recovered from the surrounding water [32,33]. In conclusion, there were many differences in bacterial communities between the different types of biofilm and activated sludge samples, suggesting that the use of a suspended carrier played a more important role in structuring communities, than did temperature.

3.5. Biofilm growth and microorganism enrichment on suspended carrier

During the experiments, the biofilm covered the plastic sheet with a thin layer after each batch process, although some plastic could still be seen. The biofilm was thick and gray. This pattern of growth was similar at all temperatures. The average biofilm thickness attached to the suspended carrier was approximately 1.42 mm. However, higher temperatures resulted in thicker biofilm (Fig. 6).

Microbial population development and replenishment, before and after the system start-up, was investigated and compared using a SEM. In Fig. 6(a), bacteria on the suspended carrier were attached tightly to avoid being washed away by water, and presented different morphotypes (rods and cocci). Several researchers have shown that the predators of nitrifiers and denitrifiers in sewage processes play an important role in organics and nitrogen removal [34]. Additionally, Sich and Rijin showed that *Pseudomonas*-like and *Zoogloea*-like bacteria appeared to be the

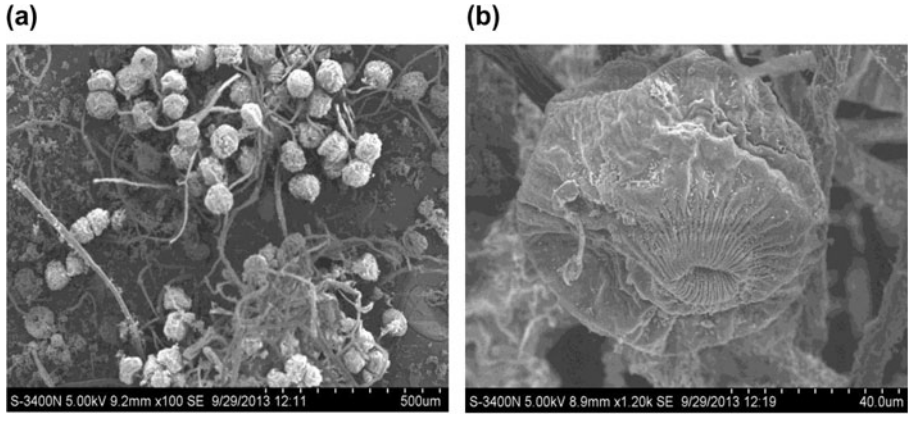

Fig. 6. SEM pictures of the biofilm of experiments with suspended carrier: (a) biofilm on suspended carrier and (b) partial enlarged image of (a).

dominant species after 20 d [35]. The results from SEM showed that suspended carriers maintain high specific biomass activity at low temperature.

4. Conclusions

Modification of the A^2O reactor benefited the performance of the plant at low environmental temperatures; however, the removal rate of TN was significantly influenced by temperature, from 92.1% (26°C) to 78.1% (10°C). Adding suspended carrier was an effective strategy to achieve high biomass. The biofilm thickness was more obviously affected by temperature than was biofilm density. The bulking and dispersed growth phenomena were usually observed at 10°C (200 > SVI > 170 mL/g MLSS), which might suggest that low temperature rather than low nutrient supplies stimulated the filamentous bacteria growth. The concentration of EPS decreased with temperature. Low temperature resulted in the increase in polysaccharides so that the PS/PN ratio at 10°C was higher than at 18°C. Sequencing analysis demonstrated that the use of a suspended carrier led to significant changes in the bacterial community structure. Some filamentous bacteria related to sludge bulking were more likely to stick on the carrier surface.

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Nomenclature

C/N	—	carbon-to-nitrogen
BAF	—	biological aerated filter
SCBR	—	suspended carrier biofilm reactor
MBBR	—	moving bed biofilm reactor
A^2O	—	anaerobic–anoxic–aerobic
UASBR	—	upflow anaerobic sludge blanket reactor
SRT	—	sludge retention time
Anammox	—	anaerobic ammonium oxidation
PCR	—	polymerase chain reaction
DGGE	—	denaturing gradient gel electrophoresis
FISH	—	fluorescence *in situ* hybridization
T-RFLP	—	terminal restriction fragment length polymorphisms
PAOs	—	phosphorus accumulating organisms
DO	—	dissolved oxygen
HRT	—	hydraulic retention time
RAS	—	returned activated sludge
MLSS	—	mixed liquor suspended solids
MLVSS	—	mixed liquor volatile suspended solids
SVI	—	sludge volume index
COD	—	chemical oxygen demand
NH_3-N	—	ammonia nitrogen
TN	—	total nitrogen
TP	—	total phosphorus
TNLR	—	total nitrogen loading rate
TNRR	—	total nitrogen removal rate
EPS	—	extracellular polymeric substances
T	—	biofilm thickness
V	—	liquid volume displaced by the biomass
S	—	carrier area
L	—	carrier length
B	—	carrier width
P	—	biofilm volumetric density
M	—	sample quality
OTUs	—	operational taxonomic units
PBS	—	phosphate buffer solution
SEM	—	scanning electron microscope
PN	—	proteins
PS	—	polysaccharides
QC	—	chimeric sequences

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