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Analysis of microbial characteristics and population difference between different compartments of the mixed system at low temperature

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ABSTRACT

In this study, activated sludge, sponge packing and pall ring packing was integrated into segmented feed multistage anoxic/oxic (A/O) reactor for treating low-temperature wastewater, the microbial characteristics were investigated under the stable removal efficiency. The sludge concentration and environmental scanning electron microscope images showed that the biofilm sludge concentration was lower than that of activated sludge, but the biofilm structure was denser. The microbial activity test results showed that the microbial activity of the mixed system was significantly higher than that of the single activated sludge system. High-throughout sequencing analysis revealed that the microbial community in the hybrid system was primarily dominated by *Proteobacteria* and *Bacteroidetes*, and there were obvious differences among the stages in the multistage A/O reactor on the biofilm, the biofilm populations of different compartments were also significantly different. The mixed system improves microbial diversity and was beneficial to the removal of pollutants from low-temperature wastewater.

Keywords: Multistage anoxic/oxic; Activated sludge; biofilm; Microbial community; Microbial population diversity

1. Introduction

Temperature is one of the important factors for the growth of microorganisms, which mainly affects the growth, reproduction and metabolic activity of microbial communities. Studies have shown that in the appropriate temperature range, the rate of chemical reaction usually increases as the temperature increases [1,2]. The degradation rate of pollutants in wastewater by microorganisms is mainly influenced by microbial enzyme catalysis, and the enzyme is highly sensitive to temperature, the activity of the enzyme has a significant effect on the efficiency of pollutant treatment [3]. Activated sludge treatment of low-temperature sewage, its microbial adsorption capacity, sedimentation, growth rate and metabolic capacity will be greatly affected, the efficiency of sewage treatment decreased significantly [1,4]. Northern China has a long winter, cold weather, the temperature of sewage generally maintained at about 10°C, the metabolism of microorganisms in the biological treatment system is weakened and the removal efficiency of pollutants is decreased.

The multistage anoxic/oxic (A/O) process is a post-denitrification process in principle, the sewage enters the anaerobic/anoxic stage, and the reflux sludge enters the

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anoxic stage from the secondary sedimentation tank to the first end of the system. The carbon source comes from the organic matter of sewage itself. Theoretically, the purpose of denitrification can be satisfied without internal reflux [5]. The reactor has a high average sludge concentration, complete degradation of pollutants and abundant biological population [6,7]. The multistage A/O process saves energy and has no additional carbon source, and has high denitrification efficiency [8,9].

Biofilm technology has strong applicability in low-temperature sewage treatment, biofilm on the carrier can improve sludge retention time, and is beneficial to the enrichment of bacteria with a longer generation time [10]. The mixed system of activated sludge and biofilm has many advantages over the single system, the activated sludge has a large specific surface area, sufficient contact with sewage, and a high utilization rate of dissolved oxygen, the biofilm system is beneficial to the growth of long mud-age organisms [11]. In the mixed system, biofilm can improve the sedimentation of suspended sludge, inhibit sludge expansion and improve the system operation stability.

This study focused on the microbial characteristics in the stages of multistage A/O reactor to treat low-temperature (10°C ± 1°C) wastewater. These include biomass, bioactivity, microbial structure, microbial community composition and inter-compartment population differences. The objective is to study the change of microbial activity and diversity in the mixed system after adding the ball filler and the characteristics of different compartments in the mixed system. The innovation of this paper is to analyze the biological changes between different compartments in the process of low-temperature sewage treatment to determine the difference of microbial population in a low-temperature environment. The influence of multi-stage operation mode on the diversity of low-temperature wastewater treatment population was analyzed by using various characteristic parameters of microorganisms. This study provides an important basis for analyzing the co-degradation mechanism of various microorganisms.

2. Materials and methods

2.1. Reactor description and samples collection

In this study, the three-stage A/O process was adopted (Fig. 1). Each stage of anoxic and aerobic compartments was divided into two compartments with the same volume, each compartment was labeled as Ax1/Ax2/Ox1/Ox2 (x stands for stages), and the effective volume ratio of each compartment was 3:3:4:4. Each compartment was filled with suspended activated sludge and displaced ball filler to form the activated sludit-biofilm mixing system. The displaced ball packing was internally filled with the same number of large-hole sponges and pall rings at a filling rate of 30%. The filling rate of the displaced ball packing in the reactor was 20%. The raw water flowed into the first anoxic compartment of each stage in the proportion of 3:2:1, reflux sludge from the bottom of the sedimentation tank to the A11 compartment. The aeration ratio of each aerobic section was 3:2:1, anoxic section adopts mechanical agitation.

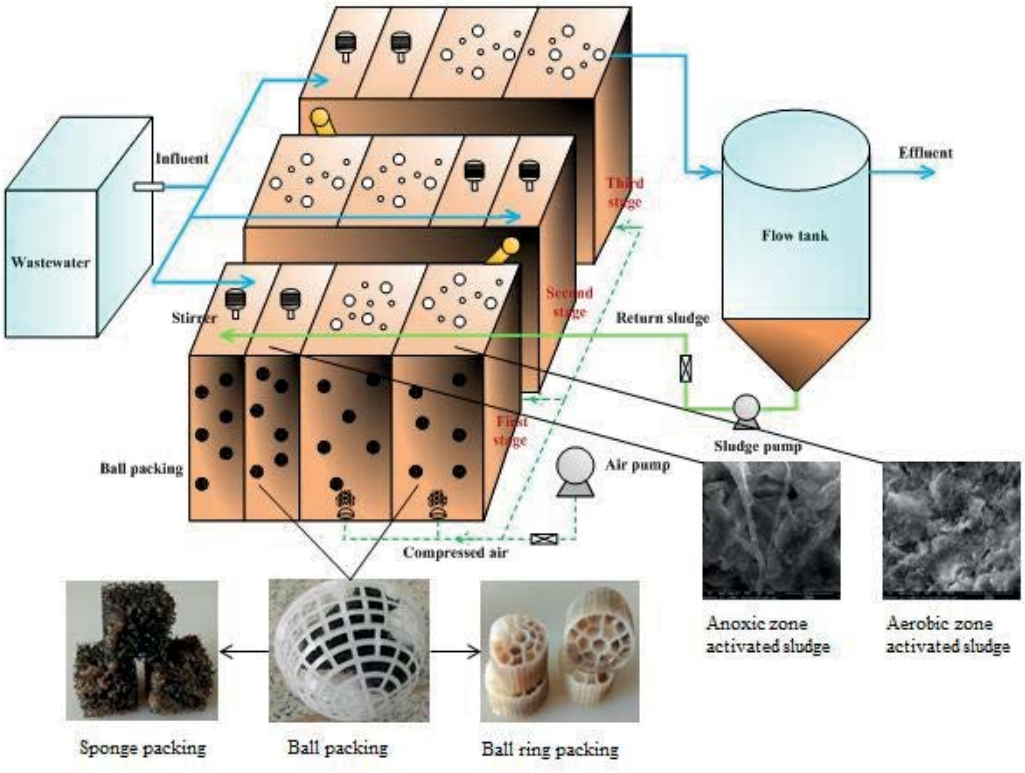

Fig. 1. Diagram of reactor structure and internal environment.

The reactor was operated in a constant temperature room, the temperature was controlled at 10°C ± 1°C and use refrigerating machine to control temperature constant.

The sewage used in this study was simulated municipal sewage in the laboratory, which was based on the contamination levels in domestic wastewater. Synthetic wastewater was prepared from tap water enriched with beef starch, extract, peptone, NH_4Cl and K_2HPO_4.

On the basis of stable pollutant removal efficiency, the corresponding compartments were sampled and tested, test items and corresponding compartments are shown in Table 1. Among them, environmental scanning electron microscope (ESEM) and microbial diversity studies were conducted on the second stage of the systems, the reason was that the second stage is less affected by sludge reflux and water volume impact, which can reflect the average level of microorganisms in the system.

2.2. Analysis methods

The concentrations of chemical oxygen demand (COD), NH_4^+–N, total nitrogen (TN) and total phosphorus (TP) were analyzed using quick-analysis apparatus (Lianhua Technology, China). Mixed liquor suspended solids and sludge volume index were analyzed according to the standard methods [12]. Test method for sludge concentration of biofilm on displaced ball packing was according to Bian et al. [13]. Sludge morphology was characterized by environmental scanning electron microscope (FEI Quanta 250FEG).

2.3. Microbial activity test

The extraction of tightly-bound extracellular polymeric substances (TB-EPS) and loosely-bound extracellular polymeric substances (LB-EPS) was according to Fish et al. [14]. The protein and polysaccharides were measured using the Coomassie Brilliant Blue G-250 method and anthrone-sulfuric acid method. The oxygen uptake rate (OUR) was according to Vincenzo et al. [15].

2.4. Microbial morphology and community structure analysis

The sample number of microbial community structure analysis is shown in Table 1, E.Z.N.A.® Mag-Bind® Soil DNA Kit (Omega, USA) was used for total genomic DNA extraction, and the integrity of DNA was analyzed through the agarose gel method (Gel imaging system from UPV, America). Bacterial sequencing primers were 341F:CCCTACACGACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCWGCAG and 805R (GACTGGAGTTCC T T G G C A C C C G A G A A T T C C A G A C T A C H V G G GTATCTAATCC) and the target region were V3-V4 (MiSeq sequencing platform). MiSeq sequencing raw data was stored in the NCBI (National Center for Biotechnology Information) Sequence Read Archive database, and the NCBI Sequence Read Archive database accession number was "PRJNA646614".

3. Results

3.1. Pollutant removal

The activated sludge and biofilm mixing system was in operation for 200 d, after 120 d of operation, the system reaches stability. At that time, the removal rates of various pollutants are shown in Table 2: COD and TP removal rate reached 85%, NH_4^+–N removal rate more than 95%, basically achieve complete nitrification. In addition, the TN removal rate was about 75%. The mixed system has high removal efficiency of pollutants from low-temperature sewage and remarkable effect of nitrification and denitrification, it was of great significance to study the microbial characteristics of the mixed system under this condition.

3.2. Biomass

The concentration of activated sludge and biofilm sludge in the reactor is shown in Fig. 2. The average sludge concentration of the three stages in the system was 6,400; 5,400 and 4,600 mg/L, respectively, the reflux sludge concentration was 11,200 mg/L, activated sludge concentration has an obvious gradient distribution, in line with the characteristics of the multistage A/O process. High sludge concentration helps to weaken the problems of low microbial activity and slow metabolism in the process of low-temperature sewage treatment, so as to improve the efficiency of low-temperature sewage treatment [5,16]. The biofilm sludge concentration in

Table 1
Test items and corresponding sampling compartments

Compartment test items	Stage one				Stage two				Stage three			
	A11	A12	O11	O12	A21	A22	O21	O22	A31	A32	O31	O32
Sludge concentration	o	o	o	o	o	o	o	o	o	o	o	o
ESEM	□	□	□	□	□	o	□	o	□	□	□	□
OUR	o	o	o	o	o	o	o	o	o	o	o	o
EPS	o	o	o	o	o	o	o	o	o	o	o	o
Microbial population structure	o	o	□	o	□	o	□	o	□	o	□	o
Microbial population difference	□	□	□	□	o	o	o	o	□	□	□	□
Sample includes activated sludge and biofilm					□	□	□	□	□	□	□	□
o Represents that the compartment has corresponding test item						□	□	□	□	□	□	□

Table 2
Contaminant removal efficiency at stable stage

Parameters	COD	NH_4^+–N	TN	TP
Inflow (mg/L)	186.60~269.40	13.97~18.52	23.52~34.96	5.58~7.50
Effluent (mg/L)	16.55~37.62	0.05~0.89	5.29~8.59	0~1.51
Removal rate (%)	79.84~93.86	94.27~99.72	69.87~83.71	78.79~100

COD – chemical oxygen demand; TN – total nitrogen; TP – total phosphorus.

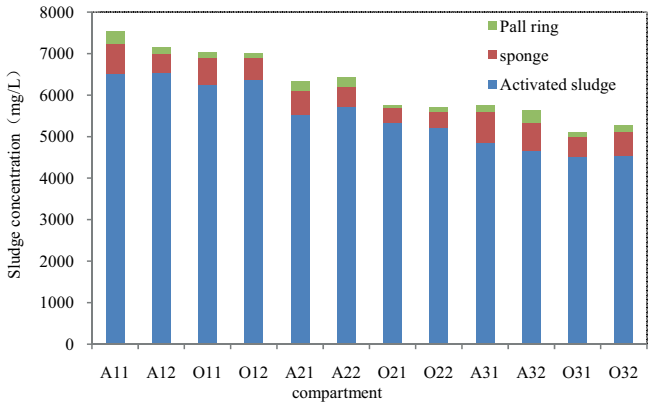

Fig. 2. Activated sludge and biofilm sludge concentration at the stable stage.

the reactor was between 400–800 mg/L, and the biofilm concentration gradually increased along the direction of water flow, this was contrary to the rule that the concentration of activated sludge decreased gradually. The reason was that the microorganisms in the stationary phase and suspended phase were in competition with each other in the mixed system [17]. It was also found that the concentration of biofilm in the anoxic segment was higher than that in the aerobic segment, which may be related to different shear forces of water flow.

3.3. Microbial structural

Figs. 3a and b show the ESEM photos of activated sludge in the anoxic and oxygenated section of the multistage A/O process. In the figure, the activated sludge in the anoxic zone was relatively loose and distinct, and the activated sludge in the aerobic zone is closely structured and cohesive. EPS as an activated sludge stem, meanwhile covered with a large number of bacteria of various forms, and there are obvious voids in activated sludge, which as a transport channel for substrates and nutrients [18]. Compared with activated sludge in the anoxic zone, activated sludge in the aerobic zone has a more compact structure and the number and species of microorganisms attached to EPS were more abundant, there were a lot of spheroids bacteria, short rod-shaped bacteria and filamentous bacteria on activated sludge.

The adsorption characteristics of solid substrate will change with the growth of solid surface biofilm adhesion, the amount of microorganisms and EPS on the biofilm had a direct effect on the growth and development of biofilm [19]. Figs. 3c and d show the ESEM photos of biofilm in the anoxic and oxygenated section of the multistage A/O process, the surface of biofilm was uneven and forms obvious multilayer structure, compared with anoxia segment, aerobic segment biofilms were fuller and denser, the biofilm skeletons crisscross and provide habitats for the attachment of microorganisms [20], then the area of microbial adhesion was increased, and the ability of microbial adhesion on the biofilm was improved to reduce the loss. A large number of bacterial communities were attached to the surface of the biofilm, the main microorganisms on the biofilm were coccus and bacillus, and a small number of filamentous bacteria and spiralis appeared.

3.4. Oxygen uptake rate analysis

Fig. 4 shows OUR test results of mixed system and sludge system, in the mixed system, the oxygen uptake rate of microorganisms in the anaerobic/aerobic compartment ranged from 58.34 to 16.44 mg/L, and in the sludge system between 30.37~7.68 mg/L. OUR value of the mixed system and sludge system decreases gradually along the movement path of sewage, this result was mainly related to substrate concentration. High substrate concentration can increase the metabolism, activity and respiration rate of microorganisms [21]. This result was consistent with the continuous decreasing trend of microbial OUR in the push-flow aeration pool [22].

3.5. Extracellular polymeric substances analysis

EPS analysis can be divided into loosely-bound (LB-EPS) and tightly-bound (TB-EPS) according to its tight binding degree [23], according to the composition division mainly includes protein, humus and polysaccharide and so on [24,25]. Microbial activity decreased at low temperature, in the multistage A/O process, the substrate concentration, dissolved oxygen environment and intermediate products were constantly changing in different compartments, the production of EPS was affected by these external environments, then affect the activity and function of microorganisms [26,27].

Fig. 5 shows protein and polysaccharide content in activated sludge and biofilm, the protein was the main component in activated sludge EPS (Figs. 5a and c), TB-EPS was the main component, this result was consistent with the research conclusion of Wilén et al. [28]. However, the content of polysaccharides in the biofilm gradually increased along the direction of water flow, meanwhile, the main components were transformed from TB-EPS to LB-EPS

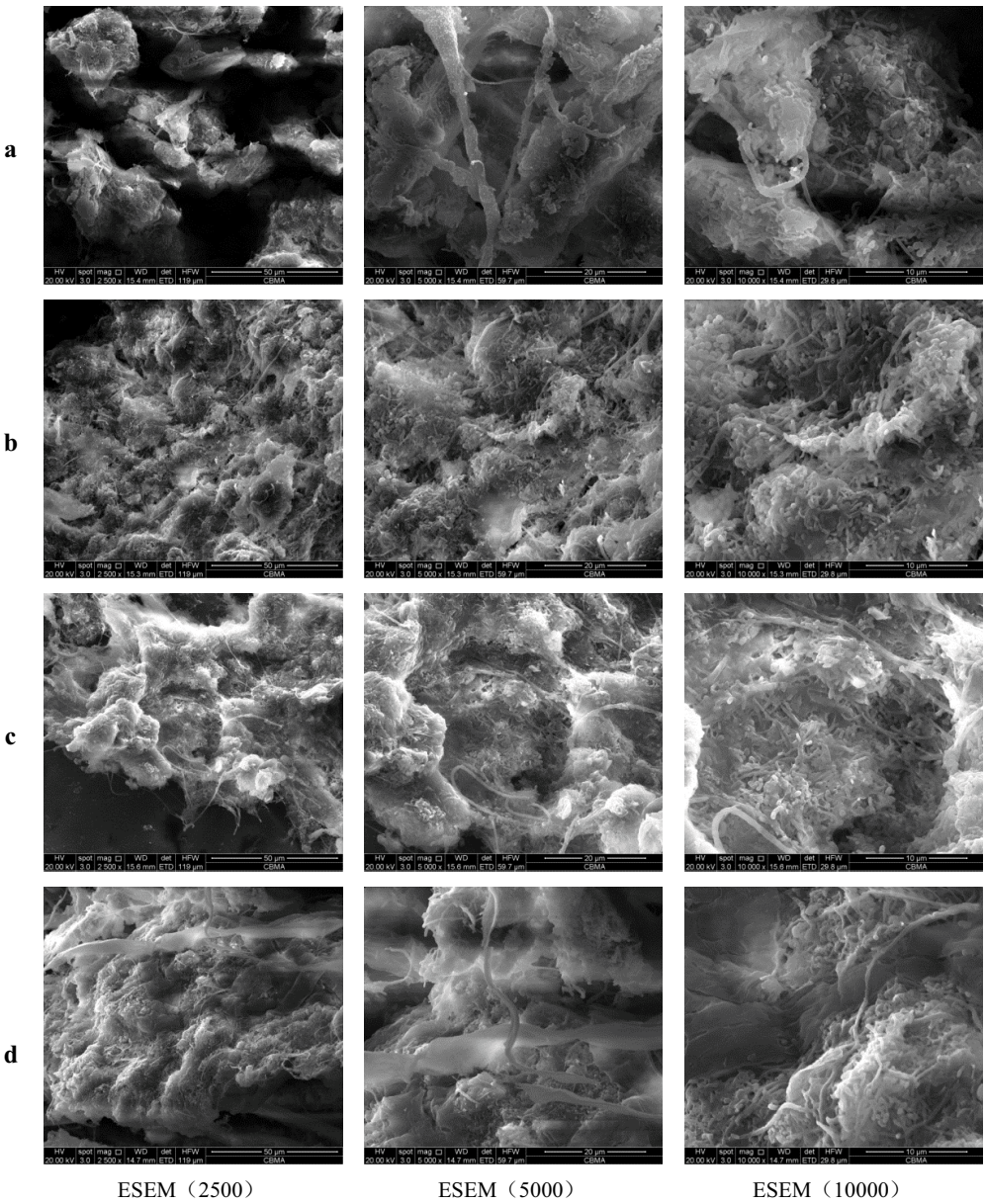

Fig. 3. Photos of anaerobic activated sludge (a) biofilm, (c) ESEM and aerobic activated sludge, (b) biofilm, and (d) ESEM.

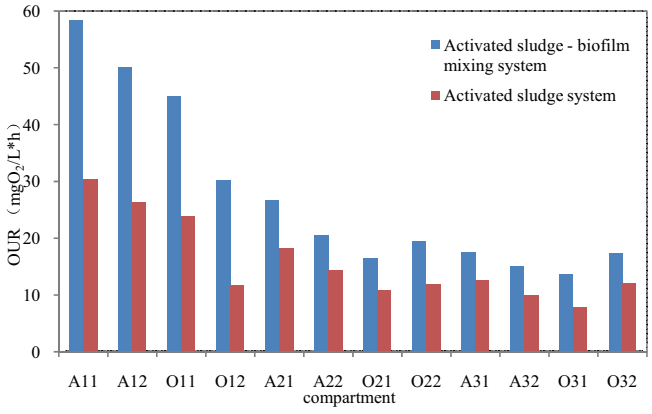

Fig. 4. OUR test results of mixed system and sludge system.

(Figs. 5b and d). This result was consistent with the research conclusion of Houghton and Stephenson [29]. This indicated that LB-EPS in biofilm accumulated gradually along the movement path of sewage and its composition changed, it was mainly related to the change of matrix environment.

3.6. Microbial population structure

Analysis of microbial population structure in the mixed system at the phylum level using high-throughput sequencing, Fig. 6a shows the distribution of microbial flora of reactor activated sludge at the phylum level. The relative abundance of *Proteobacteria* and *Bacteroidetes*, the main microorganisms on activated sludge was close to 80%. In addition, *Chloroflexi*, *Planctomycetes*, *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Parcubacteria*, *Nitrospirae* and

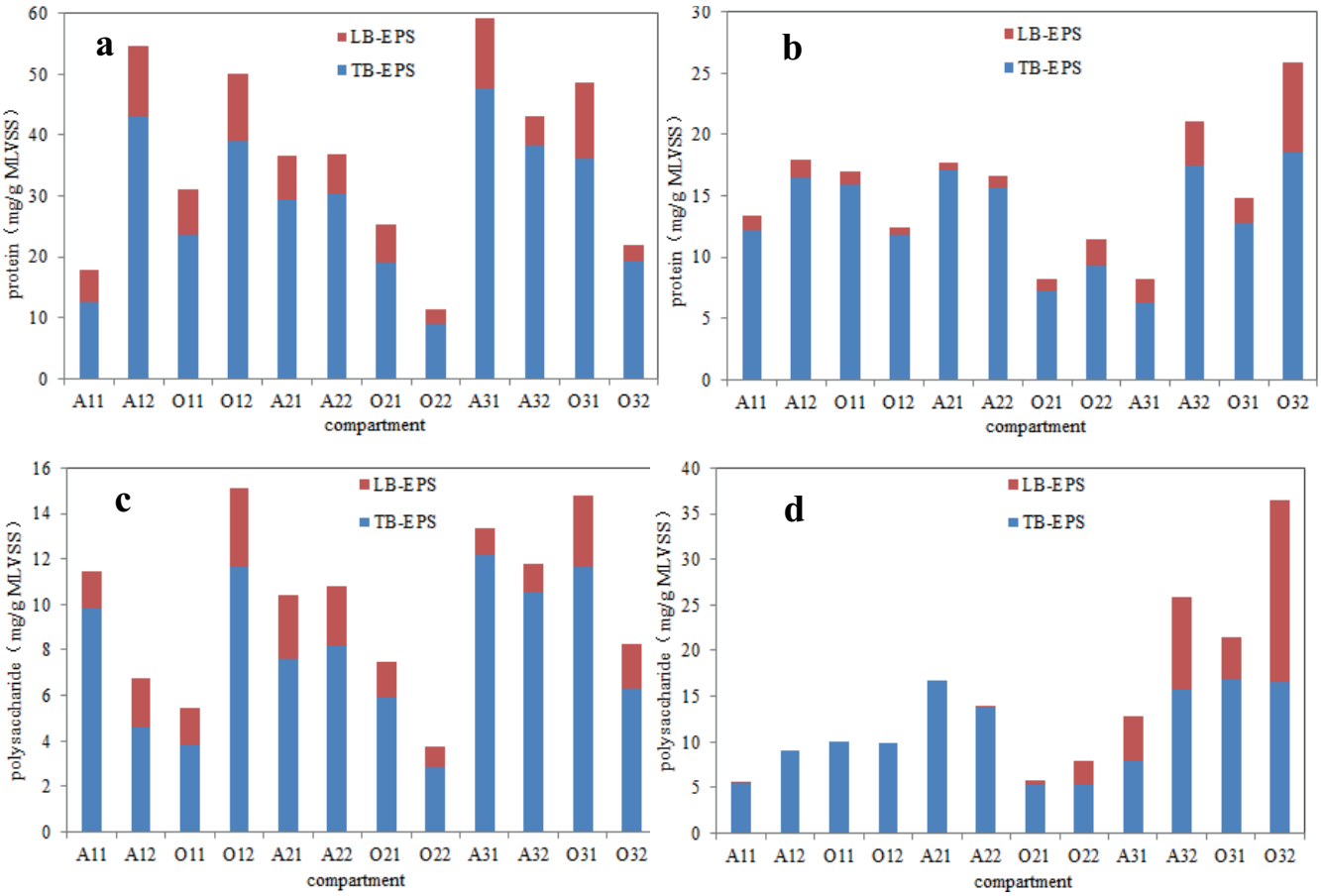

Fig. 5. Extracellular polymeric substances analysis of activated sludge and biofilm; activated sludge-protein content (a), biofilm-protein content (b), activated sludge-polysaccharide content (c), and biofilm-polysaccharide content (d).

other relatively abundant species have been found in activated sludge system, the research shows that these phylum of bacteria were common bacteria in low-temperature sewage treatment [30], among them, *Bacteroidetes* and *Chloroflexi* bacteria could promote the formation of the network structure of sludge on biological carriers [31].

Fig. 6b shows the species composition and relative abundance of microorganisms on the sponge biofilm at the phylum level. The main microorganisms on the biofilm were *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes* and *Acidobacteria*, and their relative abundance was over 80%. *Proteobacteria* accounts for the largest proportion, approaching or exceeding 50% of the relative abundance of the total samples.

Fig. 6c shows the species composition and relative abundance of microorganisms on the pall ring biofilm at the phylum level, The main microorganisms on the biofilm were *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Candidatus saccharibacteria* and *Acidobacteria*, and relative abundance was over 80%. *Proteobacteria* accounts for the largest proportion, approaching or exceeding 50% of the relative abundance of the total samples. The microbial community composition and abundance of the baur ring biofilm in the aerobic segment have little difference, which was related to the small filling amount of the baur ring.

4. Discussion

4.1. Enhanced microbial activity of the system

The oxygen consumption rate and oxygen demand in different compartments of the system are different in the multi-stage A/O coupled displacement biochemical process with segmented inlet water. Compare OUR values of mixed system and sludge system (Fig. 4), OUR value of a mixed system with displacement ball packing was obviously higher than that of activated sludge system, this results showed that adding displacement sphere filler changed the microbial growth environment, increased microbial activity, enhanced microbial respiration, and improved biological treatment efficiency.

Microbial activity was significantly correlated with EPS composition, including interspecific or intergeneric microbial interaction and biofilm production [32], and polysaccharides were important materials for biofilm structure and stability [33]. The flow rate in the back section of the multistage A/O process system was relatively larger, microorganisms produce large amounts of polysaccharides to increase the adhesion of biofilms, in order to adapt to the change of hydraulic environment and matrix concentration.

Compared with activated sludge, EPS content on biofilm increased gradually along the direction of water flow

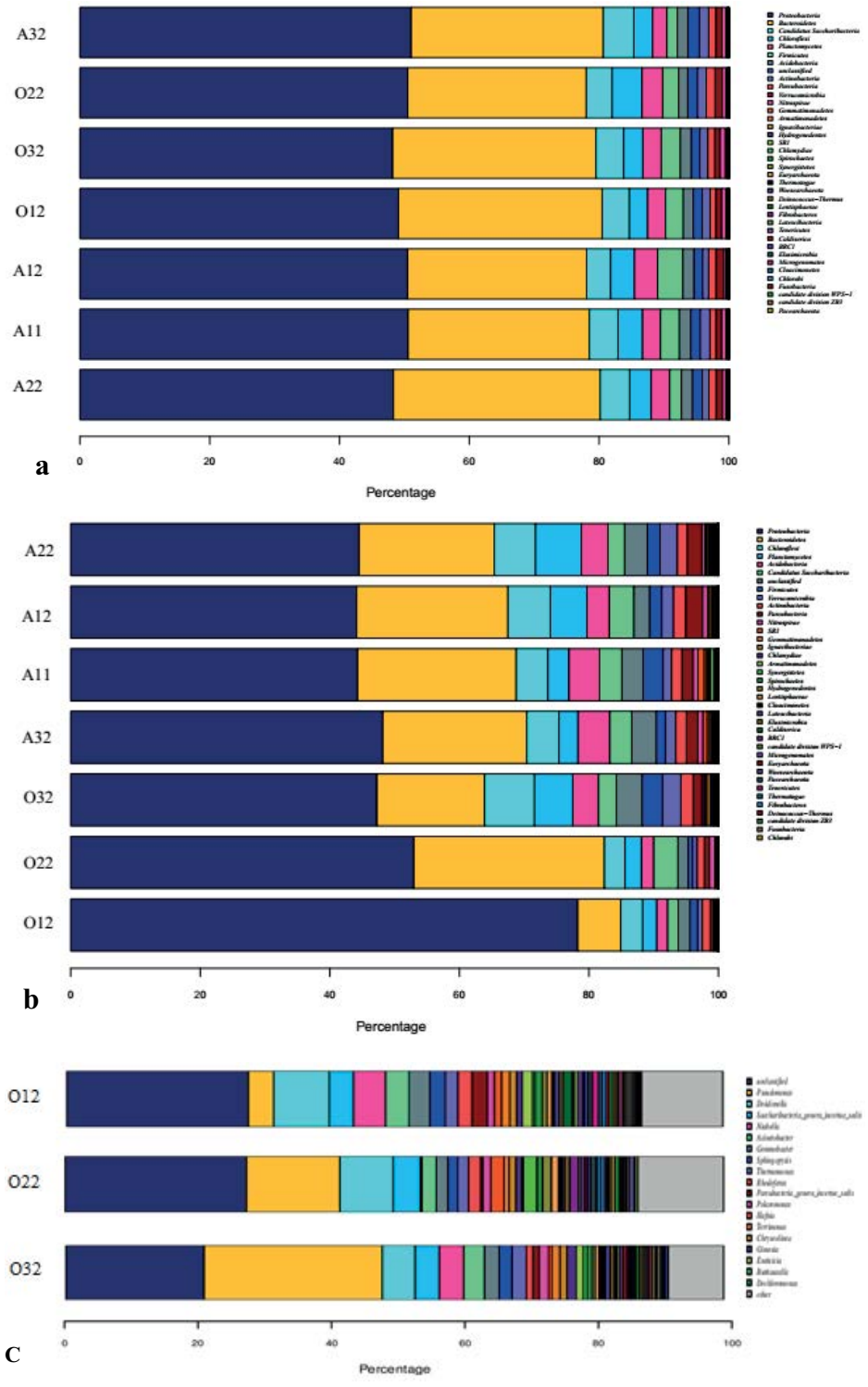

Fig. 6. The microflora composition and relative abundance of activated sludge (a), biofilms (b), and pall ring (c) at phylum classification level.

(Fig. 5). It has been pointed out that the formation of biofilm in mixed culture was greatly affected by the interaction of bacteria [34], competitive advantages of energy-requiring EPS synthesis compared to high cell growth, in the mixed culture system, the competitiveness of EPS strains will decline [35]. However in this study, the specific manifestation was biofilm EPS content increases gradually along the motion path, which indicates that the competitiveness of biofilm distributed along the movement path is gradually enhanced.

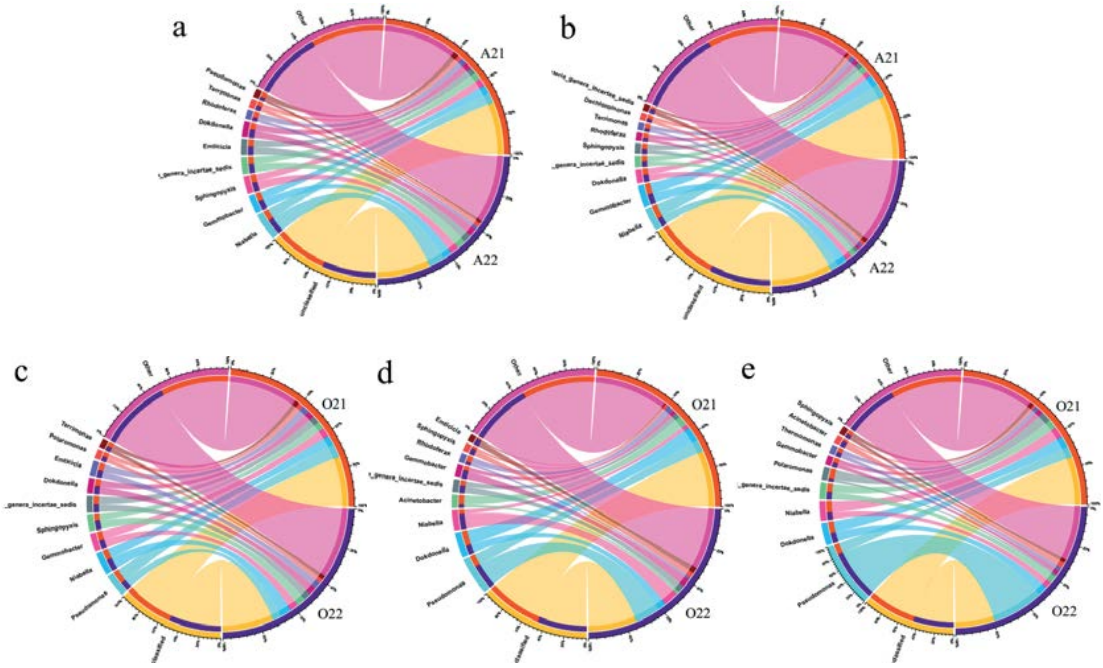

Fig. 7. Analysis on the difference of microbial species in genus level between different compartments with the same series; anoxic compartment activated sludge (a), biofilm on anoxic septum sponge (b), aerobic compartment activated sludge (c), biofilm on aerobic compartment sponge (d), pall ring biofilm in the aerobic compartment (e).

4.2. Improve the biodiversity of the system

The microbial structure in activated sludge was relatively loose, which means that activated sludge has a larger surface area and is more conducive to the rapid adsorption and degradation of pollutants. And in the biofilm, the microbial structure was relatively compact, rod-shaped bacteria and globular bacteria have the phenomenon of "bacterial cluster" growth, which means the same kind of bacteria "gather" together to grow and multiply [10], this characteristic creates superior conditions for the enrichment of slow-growing bacteria, such as nitrifying bacteria, so that the biofilm system has better biological conditions for nitrogen and phosphorus removal. In the mixed system, the microbial community with different structural characteristics improves the microbial diversity of the system and plays an important role in the performance and stability of the treatment system [36].

The results of phylum-level analysis of activated sludge microbial community showed that the relative abundance of *Proteobacteria* and *Bacteroidetes* was close to 50% higher than previous research results (the content of *Proteobacteria* and *Bacteroidetes* is about 30%) [37]. The activity of microorganisms decreases at low temperature, and microorganisms with low-temperature tolerance gradually become the dominant species [38], biodiversity decreased compared to normal temperature conditions. Analysis of microbial species level in system activated sludge, there was little difference in microbial species between the compartments, which was related to the better circulation and flow of activated sludge in the system.

The microbial populations on the sponge biofilm showed great diversity. The relative abundance of microorganisms of the same phylum in anoxic compartment samples was less. However, the relative abundance of microorganisms of the same phylum varies greatly in aerobic samples, the relative abundance of *Proteobacteria* in O12 samples was the highest and accounting for nearly 80%, the relative abundance of *Proteobacteria* in the samples of O22 and O32 were 72% and 68%, respectively. This indicates that along the movement direction of water flow, the proportion of *Proteobacteria* of aerobic indoor microbial population decreased step by step, analysis of the reasons may be related to the segmented water intake model and the gradual reduction of water intake.

4.3. Microbial population difference

In this study, a three-stage A/O process was adopted, each stage contains two anoxic compartments and two aerobic compartments, the second level of the system was taken as the research object (according to Fig. 6, this level of microorganism has the greatest relative difference), the microbial community structure and abundance of A21, A22, O21 and O22 were studied, the result is shown in Fig. 7.

Figs. 7a and b respectively show the difference in the genus level of microbial on activated sludge and sponge carrier in the anoxic section, microbial communities are mainly different in *Niabella*, *Sphingopyxis*, *Saccharibacteria*, *Dokdonella*, *Emticicia*, *Rhodoferax*, *Pseudomonas*, *Proteocatella*, *Clostridium* and *Polaromonas*. Activated sludge bacteria, the relative abundance of *Pseudomonas* bacteria were 1.22% more than that of *Pseudomonas* bacteria in the A22 compartment. *Clostridium*, which produces volatile fatty acids, has 0.82% more relative abundance of A22 than A11 compartments. Biofilm microorganisms on sponge

packing, the relative abundance of *Pseudomonas* (bacteria with denitrification function) in compartment A22 was 7.92 times that of compartment A21; *Acinetobacter* which could remove phosphorus and nitrogen by denitrification, the relative abundance of A22 and A21 compartment samples were 1.36% and 0.55%, respectively. In Fig. 7b, the bacteria of the *Janthinobacterium* genus with oxidation and fermentation properties, which could reduce nitrate and nitrite, the relative abundance of A22 and A21 compartments were 0.77% and 0.03%, respectively. Some studies have suggested that *Janthinobacterium* has a denitrification function [20].

The above results showed that the diversity and abundance of activated sludge and microbial population on biofilm were opposite in hypoxic compartment, the difference of anoxic bacteria was *Pseudomonas* in two compartments. The abundance and diversity of the two anoxic compartments of the same stage showed opposite distribution on activated sludge and biofilm. The results of functional flora comparison showed that the abundance of functional flora in denitrification and phosphorus removal was higher in compartment A22 than A21, and the difference in biofilm was more obvious.

Figs. 7c–e show the difference in the genus level of microbial on activated sludge, sponge carrier and pall ring in the second aerobic segment of the system. The microbial community differences were mainly classified, *Niabella*, *Pseudomonas*, *Sphingopyxis*, *Polaromonas* and *Dechloromonas*. The relative abundance of aerobic activated sludge bacteria varied from 0.01% to 2.24%, there was little difference in the genus level. The relative abundance of the biofilm bacteria on the sponge was between 0.08% and 6.72%, there were four species of functional bacteria with great sexual difference: *Pseudomonas*, the bacteria abundance of *Pseudomonas* in O21 and O22 compartment were 6.17% and 12.88%, respectively. *Acinetobacter*, the relative abundance of *Acinetobacter* in O21 and O22 compartmentation were 5.09% and 1.51%, respectively. *Dokdonella*, the relative abundance of *Dokdonella* in O21 and O22 compartments were 7.82% and 4.51%, respectively. Studies have shown that *Dokdonella* was a kind of strictly aerobic heterotrophic bacteria, which did not reduce nitrate, but could be isolated from soil [39]. These bacteria play a certain role in protecting the anoxic ammox bacteria and denitrifying bacteria in the inner layer of the biofilm and consuming the dissolved oxygen in the water phase [40]. *Niabella*, the relative abundance of O21 and O22 isolates were 2.35% and 7.93%, respectively. The main genus of biofilm microorganisms on pall ring packing was *Pseudomonas*, the relative abundance of *Pseudomonas* bacteria in O22 and O21 compartments were 27.12% and 5.64%, respectively. Denitrifying bacteria in O22 compartment was significantly higher than O21 compartment.

5. Conclusions

An improved multistage A/O activated sludge coupled displacement ball biofilm process was used for treating low-temperature wastewater. The concentration of activated sludge and biofilm sludge in the mixed system has the opposite increasing rule. The addition of biofilm increased the activity of microorganisms and promoted the secretion of EPS. The biofilm populations of different compartments were significantly different, which enriched the biodiversity of the mixed system.

Contributions of each author

FW and JL conceived and designed research. ZN and XW conducted experiments. SA contributed new reagents or analytical tools. DJ and QR analyzed data. FW wrote the manuscript. All authors read and approved the manuscript.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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