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Effects of poly (1,4-butanediol succinate) carrier on the nitrogen removal performance and microbial community of sequencing batch reactors

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ABSTRACT

The C/N ratio and carrier type strongly affect aerobic denitrification and are critical for nitrogen removal by sequencing batch reactor (SBR) processes. Poly (1,4-butanediol succinate) (1,4-PBS) is a biodegradable material that provides electrons to denitrifying microorganisms. The effect of carrier on the aerobic denitrifying microorganisms is worth further studying. In this study, aerobic denitrifying microorganisms were combined with 1,4-PBS to evaluate the effects of microbial biomass on the biodegradable carriers and nitrogen removal efficiency. High-throughput sequencing results indicated that after 45 days of the operation, the Shannon index of bacteria in the 1,4-PBS group increased from 4.48 (phase I, C/N = 5 at 1−8 days) to 5.74 (phase II, C/N = 10 at 9−20 days) and continuously increased to 6.68 in phase III (C/N = 20, 21−45 days). The peak total nitrogen (TN), NH_4^+-N and total organic carbon (TOC) removal efficiencies were 56.4%, 75.49% and 59.56% respectively at high C/N of 20. This result indicated that PBS significantly increased microbial diversity and indirectly changed the structure of microbial communities. Moreover, principal component analysis (PCA) showed that 1,4-PBS might change the microbial community. Specifically, 1,4-PBS might increase microbial diversity and indirectly change the structure of microbial communities. Meanwhile, the content of extracellular polymeric substances increased to 416.33 g g^{-1} VSS. 3D-EEM spectra showed that protein-like and humic acid-like substances were the main components in SMPs, and the fluorescence intensity increased in the initial 31 days and decreased in the following 14 days. Therefore, 1,4-PBS can efficiently improve the nitrogen removal performance of microbial communities. This study offers a reliable reference for the effects of biodegradable carriers on the nitrogen removal performance of microorganisms.

1. Introduction

Biological methods are the main approach used for wastewater nitrogen removal, and then activated sludge process is the main biological wastewater treatment process in wastewater treatment plants (WWTPs) around the world (Chen et al., 2015). A wide variety of functional microorganisms, such as nitrifiers and denitrifiers, are related to nitrogen removal in this process (Zhao et al., 2018). Ammonia-oxidizing bacteria and nitrite-oxidizing bacteria are the main nitrifiers and transform ammonia into nitrate under aerobic conditions. (Gruber-Dorninger et al., 2015; Yamada et al., 2019; Chen et al., 2015). Denitrifying bacteria convert nitrate into nitrogen through denitrification with organic carbon as the electron donor. At the same time, the current 'Chinese Urban Wastewater Treatment Plant Pollutant Discharge Standards' (GB18918-2002) limit the total nitrogen (TN) concentration of effluent from WWTPs to 15 mg L^{-1}. Strict WWTP discharge standards require a stabler and highly efficient removal performance by activated sludge systems.

Heterotrophic denitrification is the key process for biological nitrogen removal, and it depends on the biodegradable carbon source content in the system (Shi et al., 2015). A limited amount of

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biodegradable carbon source would restrain the growth of relevant microbes and worsen the nitrogen removal performance of the system. As described by previous studies (Ge et al., 2017), several operational parameters, such as the ratio of the chemical oxygen demand (COD) to nitrogen (COD/N), are the main factors in denitrification performance. However, the organic content in actual wastewater generally does not reach suitable COD/N ratios (Wang and Chu., 2009). The performance of the activated sludge system would be affected, leading to effluent with characteristics below China standard. Therefore, a carbon source needs to be added to maintain its sufficient level in the wastewater. Aerobic denitrification has been proven to be one of the important nitrate metabolism pathways, because it can convert nitrate into nitrogen under oxygen conditions, which has attracted more and more attention (Ji et al., 2015). Different from the traditional denitrification process, aerobic denitrifying microorganisms contain a nap FDAGHBC gene cluster, which is not sensitive in the presence of oxygen (Zumft, 1997). Aerobic denitrification can be converted nitrate into nitrite by the enzyme encoded by *napA* gene. Subsequently, nitrite will be sequentially converted into nitrogen by the enzymes encoded by the genes *nirS*, *norB* and *nosZ* (Ruan et al., 2020), while the traditional denitrification process has higher requirements for the content of dissolved oxygen (for example, anoxic/aerobic process, A/O) and space (sequencing batch reactor, SBR) (Deng et al., 2019).

In many sewage treatment plants, liquid or soluble carbon sources (e.g., ethanol, glucose, acetic acid and citric acid) are often added to anoxic units to provide electron donors for denitrification (Aslan and Cakici, 2007; Chen et al., 2015; Shi et al., 2015; Mielcarek et al., 2017). However, the carbon sources mentioned above are easy to uptake, and the dosage is not controlled exactly; insufficient carbon sources lead to fewer electrons for denitrifying microorganisms and worse performance. Nevertheless, adding an excess of carbon source would increase the COD content in the effluent (Lee et al., 2006). On the other hand, solid carbon sources such as agricultural waste (e.g., stalks and timber) and biodegradable polymers (Lee and Wang, 2006) are applied as alternative carbon sources to solve this problem. (Wang and Wang, 2009). Biodegradable polymers can serve as both an organic carbon source and biofilm carrier for denitrification, which would do not caused starvation periods or overdosing of the carbon source. The solid carbon source must undergo a degradation process to become water-soluble, which could be caused by bacteria. It can provide dissolved organic carbon that can be utilized by heterotrophic bacteria (Abou-Zeid et al., 2001). As a biodegradable carbon source, agricultural wastes have been proven to promote denitrification performance (Feng et al., 2017). However, intense color in the effluent and a very low denitrification rate limit the wide application of the carbon source (Warneke et al., 2011; Xu et al., 2019). In comparison, polycaprolactone (PCL) and poly (butylene succinate) (PBS) are biodegradable and slowly release carbon into wastewater (Liu et al., 2018). These organic carbon sources are hydrolytically decomposed by enzymes produced by microorganisms, and the resulting monomers are released and further taken up by microorganisms for denitrification (Wu et al., 2012; Liu et al., 2018). PBS can be hydrolyzed by biological enzymes secreted by respective bacteria, and the hydrolysate product can support metabolic use of heterotrophic denitrifying bacteria (Wu et al., 2015). In addition, PBS use is not only economical but also less affected by environmental changes, such as pH, than the use of other carbon sources (Zhao et al., 2009). Furthermore, under hypoxic conditions, PBS can reduce the negative effects of oxygen on the heterotrophic denitrification process (Gutierrez-Wing et al., 2012). However, there are few studies on the application of poly (1,4-butanediol succinate) (1,4-PBS) as a solid carbon source for denitrification. To our knowledge, if 1,4-PBS is dosed as an organic carbon source, aerobic denitrification might be enhanced and the stability of reactor operation can be improved, so the operating cost of wastewater treatment would be greatly reduced (Wu et al., 2015).

Based on the above descriptions, a suitable carrier for denitrification communities is critical for nitrogen removal performance. The main purpose of this study was to evaluate the effects of 1,4-PBS carriers on nitrogen removal performance associated with changes in microbial community characteristics in a sequencing batch reactor (SBR). The total organic carbon (TOC), NH_4^+-N, NO_3^--N, NO_2^--N, TN and extracellular polymeric substance (EPS) concentrations were also evaluated. Furthermore, high-throughput sequencing was conducted to explore the differences in the structures of microbial communities. The microbial communities were collected from different carriers in the SBRs. Alpha diversity, LEfSe and phylogenetic analyses were conducted to elucidate the differences in the bacterial functional communities.

2. Materials and methods

2.1. Biocarrier

The physical characteristics of 1,4-PBS (Ningbo Tianan Biopolymer Co., Ltd., China) are as follows: density 1.18 kg L^{-1}, crystallinity 25−35%, tensile strength 38% and flexural strength 27 MPa. The 1,4-PBS was spherical with a size of 0.4 cm × 0.4 cm × 0.4 cm (width × length × height) and was washed by an ultrasonic cleaner (1 kW, 50 kHz) and dried at 60 °C. At the beginning of the experiment, 1,4-PBS carrier with a volume of 200 mL was added to the SBR reactor.

2.2. Seed microbial community

A seed microbial community was obtained from Zhejiang Taohuayuan Environmental Protection Technology Co., Ltd., and I-chip technology was used to isolate and obtain the main aerobic denitrifying microbial community. The microbial community was stably operated for 1 year at 25 ± 2 °C in an SBR. Before the microbial community was added to other reactors, the microbial liquid was mixed by a vortex mixer.

2.3. SBR setup and operation

Nitrogen removal capacity experiments were performed in two laboratory-scale SBRs. R1 was the control group (C-SBR) and had no added carrier. The R2 reactor received 1,4-PBS (1,4-PBS-SBR). The same volume of microbial liquid was added to reactors R1 and R2. In each SBR, the working volume was 7 L, and the cycle time was 24 h at ambient temperature (15 ± 2−30 ± 2 °C). The experiment ran for more than 45 days in total. The initial solid-liquid ratio of the bacterial liquid and the 1,4-PBS carrier in the R2 reactor is 1:1, which have reached to 700 ml L^{-1}1,4-PBS carrier was used as an extra carbon source for denitrification.

During the study, the operation strategy of SBR reactor including feeding for 20 min, anoxic conditions for 200 min, aeration for 960 min, settling for 240 min and decanting for 20 min. The exchange volumetric ratio was controlled at 60−70% to fully utilize the COD in simulated wastewater (Zhang et al., 2020). The effects of carriers on the nitrogen removal performance associated with changes in microbial community structure were evaluated. In this experiment, glucose was the carbon source. The different concentrations of COD, NH_4^+-N, and total phosphorus (TP) added into the influent. Additionally, trace elements were dosed to the influent. A trace element solution was prepared according to Sun et al. (2017). During operation, the influent COD/N ratio was increased by changing the COD content because the demand of denitrifying

bacteria for carbon sources gradually increased. The dissolved oxygen (DO) was controlled at 1.0–2.5 mg L^{-1} during the aeration phase by adjusting the air flow rate. TOC-L was used to detect the TOC released in the PBS-SBR reactor without microbial addition.

The reactors were operated for 45 days to investigate the TN removal capacity of the SBRs after 1,4-PBS carriers were added. Reactor operation was divided into three stages according to influent C/N ratio, i.e., the start-up stage (phase I: C/N = 5, 1–8 days), the low-COD/N stage (phase II: C/N = 10, 9–20 days) and the high-COD/N stage (phase III: C/N = 20, 21–45 days).

2.4. EPS extraction and characterization

EPS was extracted from biological samples of different reactors in different stages by thermal extraction (Cheng et al., 2019), and polysaccharide were determined by the anthrone method (Li and Yang, 2007). The modified Lowry method (Yin et al., 2015) was used to determine the protein content.

2.5. Microbial community analysis

To analyze the microbial community of sludge, biomass samples were collected at the end of each phase. The biological sample of R1 was composed of suspended sludge (Granular sludge was classified as a type of biofilm). Each R2 biological sample was composed of suspended sludge and a 1,4-PBS carrier sample. A Power Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) was used to extract microbial DNA. The microbial communities from sludge samples were analyzed by high-throughput sequencing. The high-throughput analysis method was according to Gao et al. (2020). The V3–V4 region of the bacterial 16 S ribosomal RNA gene was amplified by polymerase chain reaction (PCR) using the 515 F (5′-barcode-GTGCCAGCMGCCGCGG-3′) and 907 R (5′-CCGTCAATTCMTTTRAGTTT-3′) primers. High-throughput sequencing analysis was conducted by Personal Biotechnology Co., Ltd. (Shanghai, China). The Personal Biotechnology Co., Ltd. used an Illumina MiSeq platform to sequence the amplicons. Functional genes from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were obtained by the PICRUSt program (http://picrust.github.io/picrust/). According to Wang et al. (2020), The linear discriminant analysis (LDA) effect size (LEfSe) pipeline was used to identified the significantly discriminant taxa in each group. According to Bhat et al. (2020), used Principal component analysis (PCA) to analysis the relative band intensity and positions of bacterial community profiles.

2.6. Other analytical parameters

NH_4^+-N, NO_3^--N and NO_2^--N and TN concentrations were determined following standard methods (APHA, 2012). The TOC concentration was obtained using a TOC analyzer (TOC-L, Shimadzu, Kyoto, Japan). Excitation-emission matrix (EEM) fluorescence spectra were acquired using a fluorescence spectrophotometer (LS-55, Perkin-Elmer Co., USA).

3. Results and discussion

3.1. Nitrogen removal performances of the 1,4-PBS-SBR and the C-SBR

The reactors were operated for 45 days to investigate the TN removal capacity of the SBRs after 1,4-PBS carriers were added. Reactor operation was divided into three stages according to time, i.e., the start-up stage (phase I: C/N = 5, 1–8 days), the low-COD/N stage (phase II: C/N = 10, 9–20 days) and the high-COD/N stage (phase III: C/N = 20, 21–45 days). The amount of microorganisms in the SBR reactor gradually increased with the operating time of the reactor increased. The demand for nutrients also gradually increased. Hence, we have applied different TP concentration.

In phase I, the influent NH_4^+-N, TP, and COD concentrations were 15, 2, and 100 mg L^{-1}, respectively. The NH_4^+-N removal performances of the two reactors are shown in Fig. 1. Significant differences in the NH_4^+-N removal performances in the two reactors were observed. In phase I, the average removal efficiency of NH_4^+-N in the reactors were 58.82% (R1) and 51.34% (R2). Correspondingly, the concentrations of NO_2^--N and NO_3^--N in the effluent exhibited different trends, as shown in Fig. 1C–E and D-F, and the NO_3^--N and NO_2^--N concentrations in R1 remained at low levels. In contrast, the NO_2^--N concentration in R2 gradually increased, while the NO_3^--N concentration in R2 gradually decreased. The increase in NO_2^--N concentration and decrease in NO_3^--N concentration may be caused by the biofilm. Compared to C-MBR, microorganism was rapidly adhered onto the carriers, forming the coexistence of aerobic and anoxic micro-environment and providing the realization conditions for simultaneous nitrification-denitrification. According to Wang et al. (2018), the various functional microorganisms could coexisted in biofilm in the presence of an oxygen gradient. It is well reported that ammonia oxidation and nitrite oxidation mainly occurred in suspended sludge, and denitrification was the major contribution of biofilm (Lo et al., 2010).

The TN removal performances of the two reactors during operation are shown in Fig. S2. The average TN removal efficiency remained at 44.4 ± 2.3% (C-SBR) and 39.5 ± 1.5% (1,4-PBS-SBR). which might be attributed to the differences in denitrifying microbial activity. It was inferred that the coexistence of aerobic and anoxic microenvironments gradually formed with suspended microorganisms adhering to 1,4-PBS and provided better conditions for denitrification than R1 (Wang et al., 2018).

In phase II, NH_4^+-N (15 mg L^{-1}), COD (150 mg L^{-1}), and TP (2 mg L^{-1}) were added to the influent. The results indicated that the average removal efficiencies of NH_4^+-N in the reactors were 50.92% (R1) and 52.47% (R2). As shown in Fig. 1C–D and E-F, the NO_2^--N and NO_3^--N concentrations in R1 remained at low levels. In contrast, the NO_2^--N concentration in R2 increased sharply, while the NO_3^--N concentration increased slightly. The average TN removal efficiency in the C-SBR and 1,4-PBS-SBR remained at 36.7 ± 3.1% (C-SBR) and 47.3 ± 2.3% (1,4-PBS-SBR), respectively. At the same time, the average effluent NO_2^--N concentration in the 1,4-PBS-SBR remained at 1 mg L^{-1}. It is worth noting that the concentration of NO_2–N in the effluent remained at 1 mg L^{-1}, but the concentration of NO_3^--N increased slightly at the end of phase II. Because enough carbon source was provided throughout the operation, nitrogen was mainly removed by denitrification and biological nitrogen assimilation. (Gan et al., 2019).

In phase III, NH_4^+-N (15 mg L^{-1}), COD (300 mg L^{-1}), and TP (4 mg L^{-1}) were added to the influent, and the concentrations of these nutrients in the effluent were greatly reduced. The average removal rates of NH_4^+-N in the reactors were 40.58% (R1) and 62.05% (R2). The NO_2^--N concentration in R1 grew to a certain extent, with an average accumulation of approximately 1 mg L^{-1}. This behavior might have occurred because the microorganisms in the R1 reactor were in a free state and the microorganisms secreted EPS to protect themselves, causing the amount of carbon for denitrification to be insufficient. The resulting denitrification reaction stopped at nitrite (Ye et al., 2017). In R2, the maximum concentration of NO_2^--N was 2 mg L^{-1}. Compared to the range in phase II, the range of NO_3^--N concentrations was small. Simultaneously, the average TN removal efficiency in the C-SBR and 1,4-PBS-SBR remained at 40.1 ± 3.2% and 56.4 ± 4.1%, respectively. With increasing COD/N ratio, the TN removal rates of the two

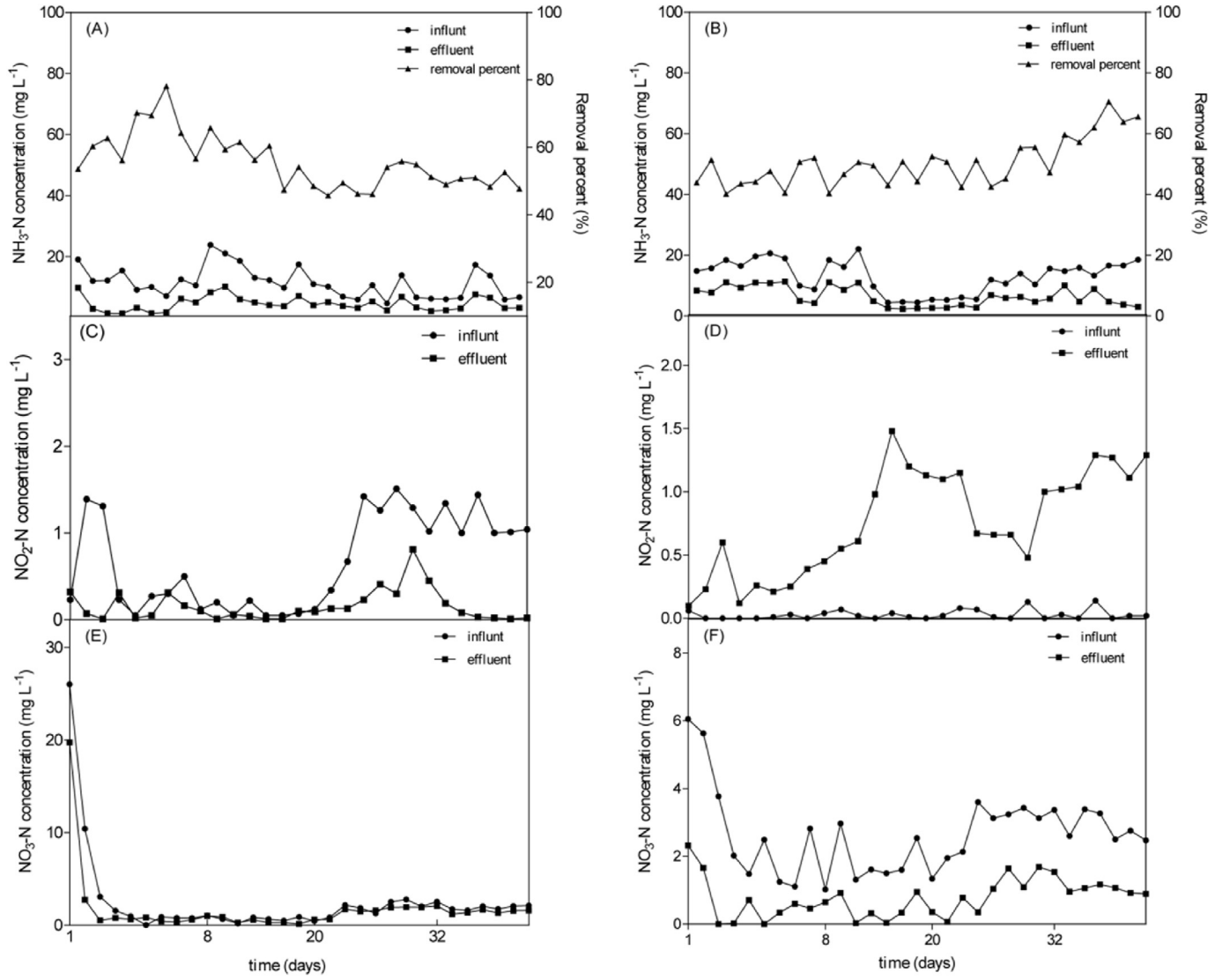

Fig. 1. Performances of the different reactors in a synthetic wastewater degradation experiment based on the NO_2—N, NO_3—N, and NH_3—N concentrations; (A, C, E) R1 (control) and (B, D, F) R2 (1,4-PBS).

reactors exhibited similar trends. The TN removal rate of the 1,4-PBS-SBR was higher than that of the C-SBR. This phenomenon might be caused by the carrier and additional carbon source in the 1,4-PBS-SBR reactor. This result indicated that the biofilms coexisting in R2 could play important role in denitrification. More importantly, the denitrification capacity of the reactor mainly comes from biofilms (Lo et al., 2010).

3.2. TOC removal capacities of the 1,4-PBS-SBR and C-SBR

The TOC removal performances of the two reactors during the whole operation are shown in Fig. S1. Significant differences in the TOC removal efficiencies of the two reactors were observed. TOC consumption was mainly for microbe growth and denitrification. The average removal rates of TOC in the reactors were 28.47% (R1) and 42.78% (R2), respectively, in phase I. The poor TOC removal rate in the early stage (1–8 days) was attributable to cell oxidation, as the microbial community of the inoculum required time to adapt to the new environment. In this stage, the microorganisms were in a free state and easily oxidized, which resulted in the low activity of microorganisms (Chen et al., 2015; Zhang et al., 2019). From phase II to the final stage, the average removal rates of TOC in the reactors were 40.43% (R1) and 59.56% (R2), respectively. The TOC removal rate in R2 reached 59.56% and fluctuated as the organic matter fraction was released from the 1,4-PBS to the water. The average content of TOC released from 1,4-PBS was 18.3 ± 1.8 mg L^{-1} (Fig. S3). It might indirectly increase the COD content, thereby changed of the COD/N ratio. Organic matter was taken up by heterotrophic microorganisms for metabolism, and a high TOC removal rate was observed within a short time (Wang and Wang, 2009).

3.3. Biomass and EPS concentration variations in the 1,4-PBS-SBR and C-SBR

As shown in Fig. 2A, the increase in MLSS in the two reactors was mainly caused by microbial growth. From phase I to phase II, MLSS in the C-SBR and 1,4-PBS-SBR increased from 788.7 to 1645.9 mg L^{-1} and from 887.7 to 2030.7 mg L^{-1}, respectively. It is speculated that this phenomenon might be caused by extra carbon sources from 1,4-PBS. In phase III, MLSS in the C-SBR and 1,4-PBS-SBR were maintained at high levels with values greater than

2000 mg L^{-1}. This might be due to the increased COD/N ratio (Feng et al., 2019). In the 1,4-PBS-SBR, as the cells attached to the carrier surface, a biofilm was rapidly formed. The MLSS content in phase III was close to 3000 mg L^{-1}, greater than that in the C-SBR (2456 mg L^{-1}), which meant that 1,4-PBS was important for the microbial growth superiority in this reactor (Xu et al., 2018). Previous studies suggested that the bacteria loading rate could be an important factor in the stability and performance of the biofilm via influencing the microbial community (Hamza et al., 2018). At the same time, the MLSS content of the biofilm on the surface of 1,4-PBS was much higher than that of suspended sludge, indicating that 1,4-PBS could not only feed biomass but also improve the sludge attachment characteristics, reducing sludge washout (Wang and Chu, 2016; Li et al., 2019a,b). In this study, the microbes in 1,4PBS-SBR were affected by higher C/N ratio and carrier during the domestication, which may help to increase the enrichment of ammonia oxidizing and denitrifying bacteria. The enrichment of ammonia oxidizing and denitrifying bacteria would enhance the reactor performance.

According to Wang et al. (2014a), PS and PN were the main component of EPS. PN and PS content are helpful for the structural stability of activated sludge. Both of them were the one of the main materials for forms a three-dimensional scaffold structure with other biopolymers (Xiao et al., 2018). The main aim of this study was to explore the effect of carriers on the microbial community structure. According to Li et al. (2019a), changes in EPS content were mainly represented by changes in PS and PN content. The reactors were operated for more than 45 days to investigate the variations in EPS after the addition of carriers. The EPS in the different reactors was analyzed by thermal extraction and anthrone method. As shown in Fig. 2B, as the operation time of the SBRs increased, the EPS content first increased and then decreased. In phase I, the EPS content in R2 was 261.33 g g^{-} volatile suspended solids (VSS), higher than that in R1 (119 g g^{-1} VSS). The 1,4-PBS in R2 gradually released organic carbon into the wastewater (Wang and Wang, 2009). A sufficient amount of organic carbon increases the EPS content (He et al., 2018). In the low-C/N stage (phase II), the EPS content of both reactors increased more and accumulated at greater levels in R2 (416.33 g g^{-1} VSS) than in R1 (313 g g^{-1} VSS). This result indicates that at low-C/N ratios, a large amount of EPS was secreted to protect microbes and maintain higher microbial activity. The 1,4-PBS in R2 provides not only a carrier for microorganisms but also sufficient carbon for heterotrophic microorganisms to remove TN. It was speculated that the increase of EPS contents was due to more TOC content, which would promote the sludge aggregation and stable operation. In phase III, the EPS content of both reactors decreased. But EPS contents of 1,4-PBS-SBR was lower than that of C-SBR although the relative abundance of the main EPS secretion microbe. The EPS content was higher in R1 (208.67 g g^{-1} VSS) than in R2 (94.00 g g^{-1} VSS). It was inferred that the consumption of EPS was more powerful than in 1,4-PBS-SBR. On the other hand, according to Sheng et al. (2010), EPS can protect cells in harsh environments and act as a carbon source for starving cells. The growth of the biofilm increased with the increased of C/N ratio, which would cause the biofilm on the surface of the 1,4-PBS carrier too thickness. The thick biofilm would hinder the mass transfer of nutrients, which made the microorganisms inside of the biofilm cannot get enough nutrients. Although 1,4-PBS provided an amount of carbon source for the microorganisms inside the biofilm, it still cannot meet their growth. Therefore, the microorganisms on the biofilm will decompose EPS to provide their own growth and metabolism. Hence, EPS content might decrease (Wang et al., 2019b; Zhang et al., 2018).

3.4. EEM fluorescence spectral analysis of the variations in effluent SMPs

Soluble microbial products (SMPs), as secondary pollutants, account for an important percentage of COD residues in wastewater generated from biological wastewater treatment. Hence, this study used EEM fluorescence spectra to detect SMPs in the effluent of the two reactors at different stages. As shown in Fig. 3, two peaks can be identified in the EEM spectra. The first peak represented aromatic proteins (Ex/Em: 220–250/330-360 nm). The second peak represented SMP-like materials (Ex/Em: 250–300/330-360 nm) (Chen et al., 2003). As the operation time of the SBRs increased, the fluorescence intensity of SMPs first increased and then decreased. As shown in Fig. 3, there was a significant difference in the SMP fluorescence intensity of the effluent of the two reactors in phase I. The SMP fluorescence intensity was higher in R2 than in R1. In the R2 reactor, the 1,4-PBS carrier provided an additional carbon source for the microorganisms, so the EPS content was higher than in R1. Simultaneously, microorganisms used TOC for their own metabolism and denitrification, which gradually increased the fluorescence intensity of SMPs. (Wu et al., 2016). In phase II, after the reactors had been operated for 20 days, the concentrations of SMPs in the reactors increased differently. This result indicated that microbial activity may be different in the two reactors. Carriers can

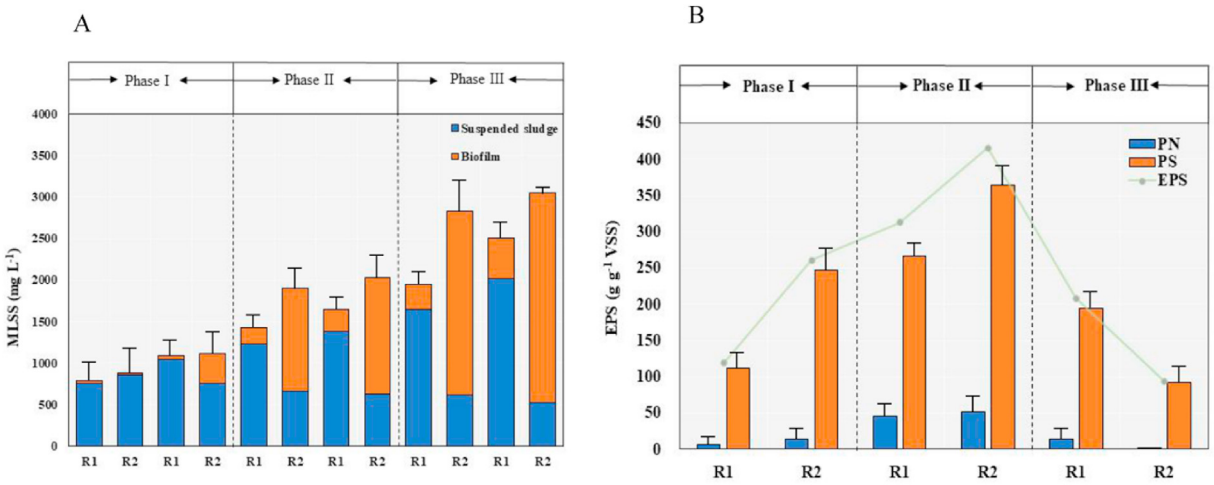

Fig. 2. MLSS (A) and EPS (B) concentration variations in the reactors during the experiments; R1 (control), R2 (1,4-PBS).

protect microorganisms (Lu et al., 2014). During the adhesion of microorganisms, free microorganisms swim in close proximity to the solid surface until they find a nutritious habitat for initial contact, then secreting EPS. The secretion of EPS might cause microorganisms to be more firmly adsorbed on the surface of the carrier. The uronic acid in EPS would exert its gelation properties and reduced the detachment of microorganisms. In the subsequent colonization of bacteria, the cells will produce more EPS. It would enhance the extent to which bacteria adsorb on solid surfaces, which helps the bacteria community adapt to the environment (Jia et al., 2017). Carriers can provide attachment points for microorganisms and promote the agglomeration of microorganisms. According to Jia et al. (2017), uronic acids are produced by microorganisms as they grow, resulting in the increased content of an unbranched exopolysaccharide composed of multiple uronic (Ferrentino et al., 2016). Eventually, the microbes stick to the carrier. These processes improved the resistance of microorganisms to environmental changes. In phase III (32 days), the fluorescence intensities of the effluent SMPs decreased gradually in both reactors. Independent of the SBR operation mode, the anoxic phase promoted SMP degradation in both reactors (Zhang et al., 2019). As a result, the SMP fluorescence intensity in the effluent gradually decreased.

3.5. Analysis of the microbial community

3.5.1. Diversity and richness of the microbial community

Values of the α-diversity indices (Chao, Shannon, Simpson, and ACE) are shown in Table 1. A large number of microorganisms were trapped in the pores of carriers; therefore, the resistance of

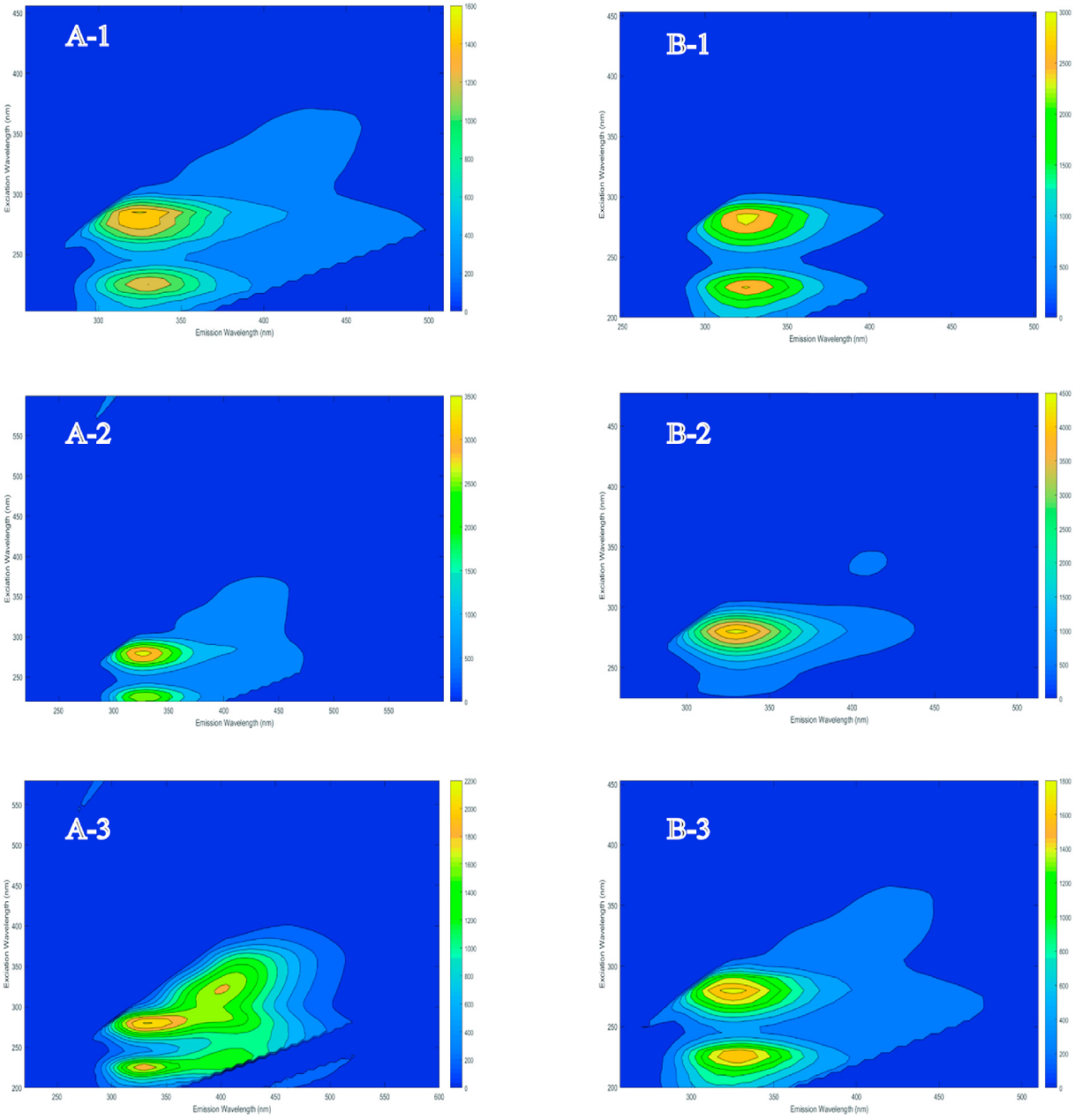

Fig. 3. EEM fluorescence spectra of SMPs from the different reactor effluents during the experiments; A-1 and B-1 - EEM fluorescence spectra for R1 and R2, respectively, in phase I; A-2 and B-2 - EEM fluorescence spectra for R1 and R2, respectively, in phase II; and A-3 and B-3 - EEM fluorescence spectra for R1 and R2, respectively, in phase III.

microorganisms to adverse environments gradually increased. The α-diversity indices for samples from the three phases were compared, and as the operation time of the SBR increased, the Shannon index, ACE and Chao values of R2 gradually increased from 4.48, 450 and 438 to 6.68, 810 and 789, respectively. At the same time, the Shannon index of R1 gradually decreased. At the end of the experiment, the Shannon index was highest in R2. The data presented in Table 1 indicates that the reactor with 1,4-PBS carrier had higher microbial richness and diversity.

As shown in Fig. 4A, the number of operational taxonomic units (OTUs) shared among all the R1 and R2 samples was 4864, accounting for 85.48% of the total OTUs (5990), which indicated that there were differences in the bacterial community in each phase during the experiment. The results of PCA of six samples are shown in Fig. 4B. The contribution rates of components 1 and 2 to sample variation were 39.97% and 30.34%, respectively. The data indicated that the microbial community structures of the six samples were divided into four clusters: R2-1, R1-1, R2-3-R2−2 and R1-2−R1-3. To determine the key factors that led to changes in the microbial community structure, this study analyzed the effects of the carrier, COD/N ratio and substrate type. For substrate type, the control group used glucose as a carbon source, while the 1,4-PBS group used 1,4-PBS/glucose as an organic carbon source, which might cause differences in the microbial community structure.

3.5.2. Composition and diversity of the microbial community

As shown in Fig. 5A, there were differences in the relative abundances of the dominant phyla of the bacterial communities in different reactors. Samples from the three phases were compared, and the dominant phylum was *Proteobacteria* in both reactors, with relative abundances of 71.53% and 75.36%. The average relative abundance of *Bacteroidetes* was 20.43% as the second populous bacteria phylum in both systems. *Bacteroidetes* are involved in the hydrolysis of macromolecular substances under anaerobic or anoxic conditions, which is one of the important parts of denitrification (Lee et al., 2011; Pishgar et al., 2019). A difference in the relative abundances of *Bacteroidetes* made the nitrogen removal performance of the two reactors different. In addition, unique environments cultivate their own unique microorganisms, such as *Verrucomicrobia*. The bacteria in the phylum *Verrucomicrobia* is living in freshwater (Lemke et al., 2009) that can fix nitrogen (Khadem et al., 2010), oxidize methane (Lee et al., 2009) and degrade PS (Martinez-Garcia et al., 2012). *Verrucomicrobia* generally have a high relative abundance in oxic zones (Zhou et al., 2019). *Verrucomicrobia* was capable of degrading complex polysaccharides (Luo et al., 2014). This taxa potentially play an important role in the degradation of polysaccharides in aquatic ecosystems. And according to Feng et al. (2019), *Verrucomicrobia* could serve to act as a biofilter to reduce methane emissions into the atmosphere. This showed that the environment where *Verrucomicrobia* exists might be an environment where hypoxia and aerobic environment coexist. Hence, several genera of the *Verrucomicrobia* have been shown to possess clade II nosZ gene. Therefore, *Verrucomicrobia* can improve denitrification performance in a certain extent.

Table 1
Species richness and diversity indicators of the microbial communities of samples R1-R2.

		Simpson	Chao1	ACE	Shannon
Phase-1	R1	0.939	733	739	5.93
	R2	0.839	438	450	4.48
Phase-2	R1	0.893	644	641	5.48
	R2	0.928	569	572	5.74
Phase-3	R1	0.861	230	240	3.86
	R2	0.962	789	810	6.68

As shown in Fig. 5B, there were differences in the relative abundances of the dominant class of the bacterial communities in different reactors. *Gammaproteobacteria*, *Bacteroidia*, *Alphaproteobacteria*, *Actinobacteria*, *Deltaproteobacteria* and *Clostridia* were abundant classes in six samples, with relative abundances of 89.40%, 23.25%, 14.89%, 3.68%, 4.40% and 4.99%, respectively. However, the relative abundances of the above classes in the six samples varied significantly.

To get further insight into the bacteria structure, the bacteria abundance of the two reactors in genus level was investigated. As showed in Fig. 5 and Table 2, the relative abundances of *Cupriavidus* and *Azospira* in 1,4-PBS-SBR were increased from no detect (phase I) to 11.89% and 21.5% (phase III), respectively. But the relative abundance of these two bacteria in the C-SBR reactor were maintain at low level (below 1%) during the whole experiment. It is obvious that in 1,4-PBS-SBR, the abundance of *Cupriavidus* and *Azospira* was higher than that in C-SBR (no carrier), and the TN remove ratio of both reactor suggested the similar trend. This result might mainly owing to the carrier. Both of them have been detected in simultaneous nitrification and denitrification (SND) systems (Xie and Yokota, 2006). When the influent C/N ratio increased, the biofilm thickness gradually increased, which might caused the more thickness anaerobic area at carrier. This process brought about the abundance of *Cupriavidus* and *Azospira* increased. The increases in the relative abundances of *Cupriavidus* and *Azospira* can indirectly indicate that a stable SND process was established in the 1,4-PBS-SBR. This also indirectly proved that why the 1,4-PBS-SBR reactor has a stronger ability to remove TN than the C-SBR reactor. The abundance of *Flavobacterium* in the C-SBR was increased from 0.08% to 5.64%, and 1.48% in 1,4PBS-SBR. This result was connected with the increasing EPS content, which was consistent with the result of EPS. *Flavobacterium* species with hydrolytic function is important for the formation of sludge flocs because they easily combine with gel masses during growth (Du et al., 2017). It might be caused by adding carrier in the C-SBR. To resist changes in external environmental factors, microorganisms secrete EPS to protect themselves, resulting in the microorganisms gradually gathering. Therefore, the relative abundances of *Flavobacterium* as the skeleton of activated sludge might gradually increase. *Flavobacterium* might responsible for the part of MLSS increased in the C-SBR. Moreover, the changed trend of *Clostridium* were similar in C-SBR reactor and 1,4PBS-SBR reactor. In the phase II, the C-PBS contained more *Clostridium* than the 1,4PBS-SBR, with the percentage of 2.64% and 0.78% in C-PBS and 1,4PBS-SBR, respectively, which compared with phase I were decreased by 16.95% and 4.09%. At the low C/N ratio (phase I), microorganisms would secrete EPS (Qiu et al., 2016). This resulted in that the EPS content in phase I were highest among the three phases for both reactors. *Clostridium* included bacteria have the capacity to degrade biopolymers under anaerobic conditions. In addition, *Clostridium* mainly grows in an anaerobic environment, which might indirectly indicated that there were different ranges of anaerobic environments in the two reactors. Along with C/N ratio increased, the EPS content gradually decreased, the structure of the biofilm disintegrates to a certain extent, and the relative abundance of *Clostridium* decreased. This might be the reason for the fluctuation of the TN removal rate and NH_4^+-N removal rate of the both reactors. In the phase III, with the increase of the C/N ratio, the biofilm structure gradually stabilized in both reactors. The relative abundance of *Clostridium* gradually increased to 1.47% in R1 and 1.41% in R2, respectively. Simultaneously, the performance of the two reactors gradually stabilized. According to Chu and Wang., (2016), the degradation by-products of *Clostridium* can be used for denitrification through bacterial nitrification. This could infer that the

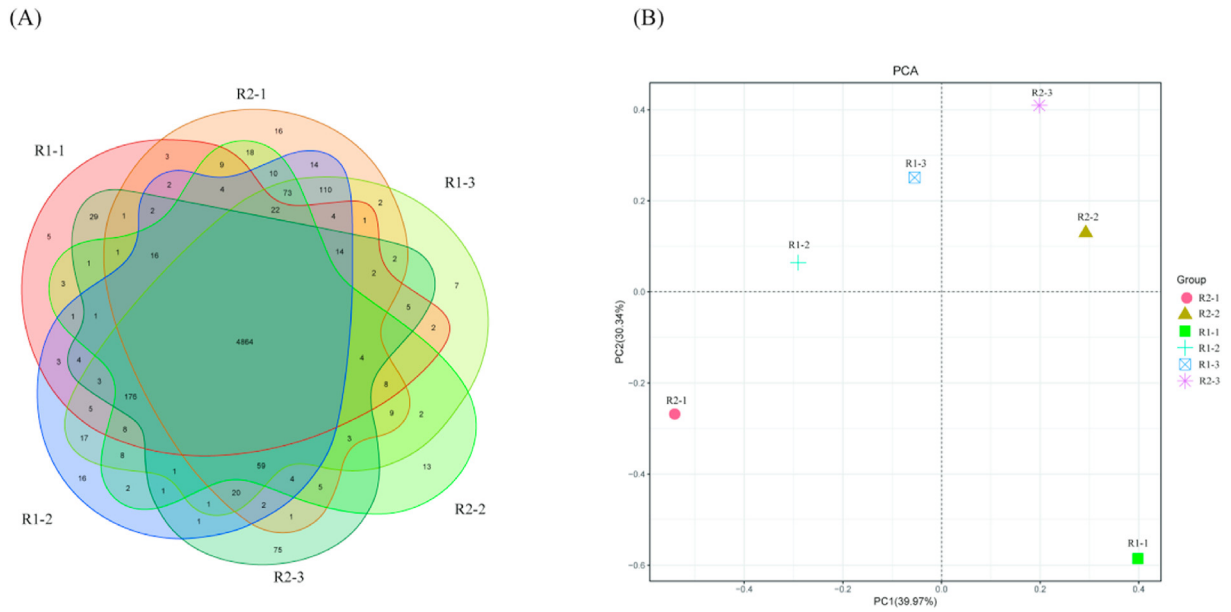

Fig. 4. Venn diagram of the microbial samples (A), principal component analysis (PCA) (B).

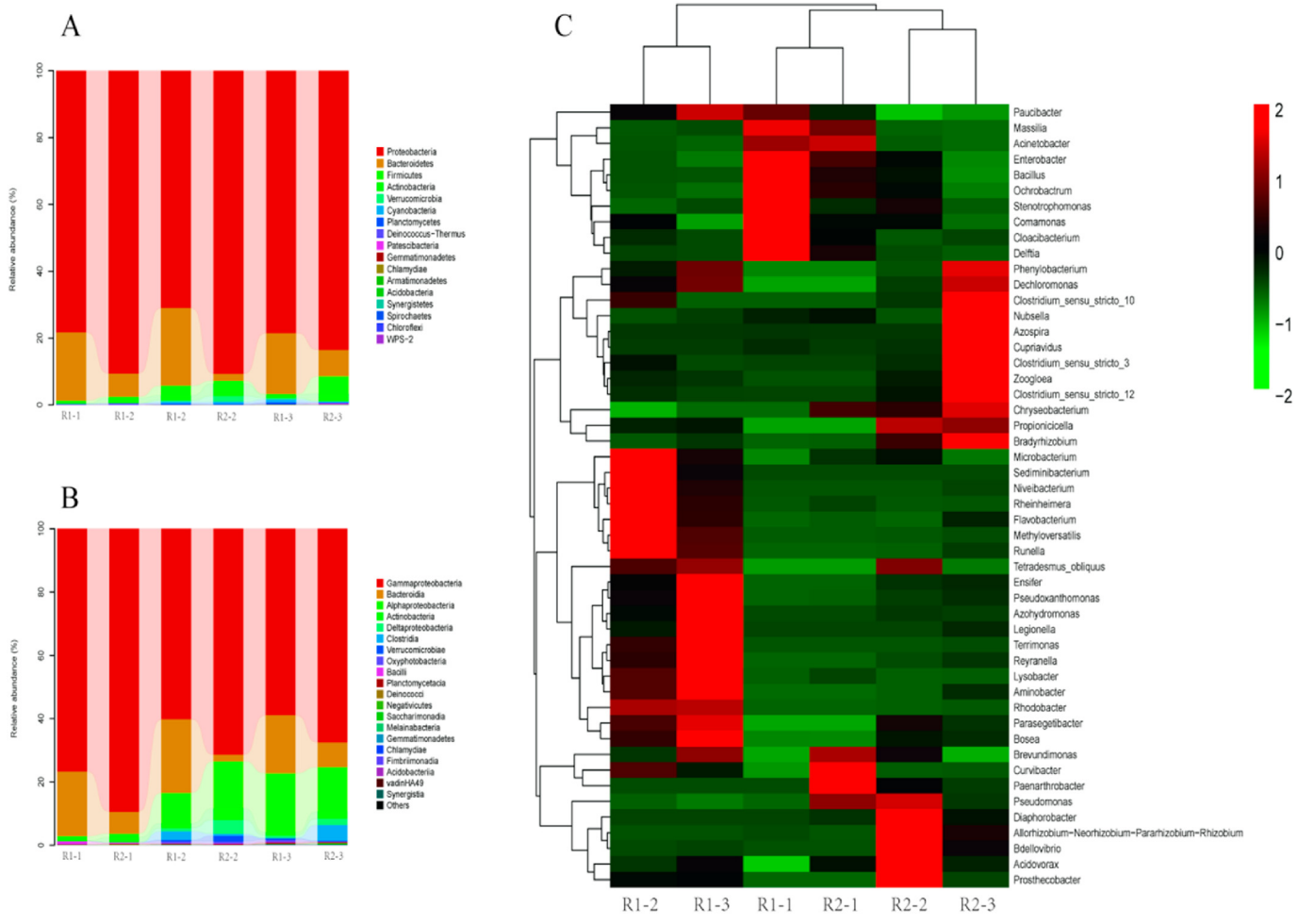

Fig. 5. Microbial population dynamics of the activated sludge samples in different phases at the phylum, class, and (C) genus levels.

Table 2
Functional microorganisms and relative abundances (%) of the major genera in the C-SBR (R1) and 1,4-PBS-SBR (R2).

Genus	Phase I		Phase II		Phase III	
	R1	R2	R1	R2	R1	R2
Enterobacter	30.01	17.17	4.33	10.03	1.89	0.90
Comamonas	19.97	7.75	8.20	7.73	2.10	3.63
Cloacibacterium	19.59	4.87	2.64	0.78	1.41	1.47
Stenotrophomonas	10.81	1.75	0.36	4.00	1.02	0.55
Curvibacter	2.48	48.96	29.99	7.97	15.41	8.31
Pseudomonas	0.23	3.78	0.03	4.59	0.03	0.29
Flavobacterium	0.08	0.47	12.93	0.07	5.64	2.25
Rhizobiaceae	0.39	1.96	0.02	9.48	1.35	3.45
Azohydromonas	2.83	1.89	2.83	0.72	16.64	0.44
Ensifer	/	/	1.49	0.57	5.28	0.72
Azospira	/	/	0.01	11.12	0.01	21.50
Cupriavidus	/	/	0.02	0.46	0.00	11.89
Zoogloea	/	/	0.61	3.23	0.41	5.94
Diaphorobacter	0.03	0.09	0.03	27.25	4.05	0.16

increase in the relative abundance of *Clostridium* may enhance the denitrification performance of the reactor (Sun et al., 2019). According to Chen et al. (2020), during eight months of operation at natural conditions, pyrite based bioretention system (PBS) can adapt to low temperature and irregular wet and dry alternation, achieving the average $NH4^{+}$-N, TN and TP removal efficiency of 87.6%, 89.3% and 81.6%, respectively. The dominant genera in PBS were *Denitratisoma*, *Ellin6067* and *Thiobacillus*, which were highly related to autotrophic denitrification. In the up-flow solid-phase (PBS) denitrification biofilm reactor (Gao et al., 2020), after 100 days operation, the nitrate and COD removal efficiencies were high of 97% and 80%, respectively. *Simplicispira*, *Diaphorobacter*, *Hydrogenophaga*, *Pseudoxanthmonas* and *Stenotrophomonas* were the dominate genera in the reactor, which were responsible for the removal of nitrate, organics and degradation of solid carbon source, respectively. In general, the PBS carrier will change the microbial community structure and promote the enrichment of denitrifying bacteria.

3.5.3. LDA comparing bacterial communities in reactors with different carriers

In addition, LEfSe analysis was used to determine the significant differential distribution of microorganism groups in relationship to the carrier. As shown in Fig. 6, differential species of 2 phyla, 5 classes, and 13 genera were screened as indicator species in R2. *Proteobacteria* and *Bacteroidetes* were the most abundant phyla within R2. At the genus level, the microorganisms with significant differences were *Curvibacter*, *Enterobacter*, *Comamonas*, *Diaphorobacter*, and *Cloacibacterium*. These microbes had aerobic denitrification capabilities and required high amounts of carbon and nitrogen for aerobic denitrification. When the COD/N ratio increases, the denitrification activity of these bacteria increases. This enhanced the ability of the R2 reactor to remove TN. *Cloacibacterium* species can also perform denitrification and are important in the removal of nitrogen pollutants in wastewater treatment. The results indicate that the 1,4-PBS carrier increases the relative abundances of *Curvibacter*, *Enterobacter*, *Comamonas*, *Diaphorobacter* and *Cloacibacterium*, thereby enhancing the TN removal rate of the R2 reactor. Hence, the structure and function of the bacterial community may be markedly changed with 1,4-PBS carriers.

3.6. Prediction of functional genes

The microbial community played an important role in nutrient removal, and structures with an abundance of denitrifying bacteria were important for nitrogen removal. To date, detailed analyses of denitrifying bacterial variation are still limited in the 1,4-PBS-supported SND system. As shown in Fig. S4, clusters of orthologous group (COG) categories were applied to predict potential microbial metabolism based on marker gene data and a database of reference genomes. The potential microbial metabolism could provide useful insights into changes in microbial community structure (Zhang et al., 2017; Langille et al., 2013). The COG categories related to A (amino acid transport and metabolism) were more abundant in the 1,4-PBS-SBR than in the C-SBR. There were significant differences in the gene families of the six samples. The dominated genus, *Comamonas*, included the functional genes of denitrification, including *narH, narJ, narB, nirS* and *norB*, followed by the genus *Cupriavidus*, with 5 types of denitrification functional genes. *Stenotrophomonas* and *Flavobacterium* both had 3 types of denitrification functional genes, and the genus of *Diaphorobacter*, *Pseudomonas* and *Azohydromonas* all contained 2 denitrification-related genes. The relative abundances of these microorganisms were higher in the 1,4-PBS-SBR than in the C-SBR. The functional genes *narH, narJ, narB, nirS* and *norB* might play a major role in the PBS-SBR system. The functional gene abundance of the microbial communities indirectly indicated the nutrient removal potential. Furthermore, the 1,4-PBS carrier changed the community structure of microorganisms, which caused changes in the relative abundances of microbial functional genes, eventually causing changes in reactor performance.

3.7. Comparison of 1,4-PBS carriers with other carriers involved in denitrification

The above results indicated that 1,4-PBS carriers might be suitable for aerobic denitrifying bacteria. Compared with the C-SBR, systems with a 1,4-PBS carrier exhibited significantly increased nitrogen removal efficiency. Moreover, the efficient aerobic denitrification in 1,4-PBS systems was slightly affected by DO (3.5–4.0 mg L^{-1}), as described by previous studies (Meng et al., 2008). The pathways of microbial nitrogen removal were determined by the relative abundance and structure of functional microbes. Traditional denitrification and aerobic denitrification are the main denitrification pathways in this study (Lu et al., 2014). As shown in Fig. 1, high NO_2^--N accumulation in phase II of the 1,4-PBS-SBR operation facilitated partial nitrification and aerobic denitrification (Guo et al., 2009). The 1,4-PBS carriers might microscopically change the DO level in the system and influence the nitrogen conversion pathways. Generally, the presence of EPS is regarded to be helpful to SND, which is generally related to hydrophobicity. Therefore, under conditions of high DO (3.5–4.0 mg L^{-1}), the accumulation of NO_2^--N could be explained by the formation of microanaerobic zones through the pores and voids on the surface of the 1,4-PBS carrier according to the results of Guo et al. (2009). The microanaerobic zone was suitable as a habitat for anaerobic microorganisms.

As shown in Table S1, the microbial community influenced the relationship between microorganisms and the carrier. During biofilm formation, the proliferation rates of microorganisms attached to the biofilm may be mainly affected by the carrier and available carbon sources (Zhao et al., 2019). Therefore, in the aerobic denitrification system supported by the 1,4-PBS solid carbon source, the biofilm formation period (the adaptation period for aerobic denitrifying microorganisms) might be mainly determined by the carbon source and the carrier. In this study, 1,4-PBS promoted the cellular growth of *Proteobacteria* members such as *Curvibacter*, *Enterobacter*, *Comamonas*, *Diaphorobacter*, *Azospira* and *Cloacibacterium*. The increase in the relative abundances of

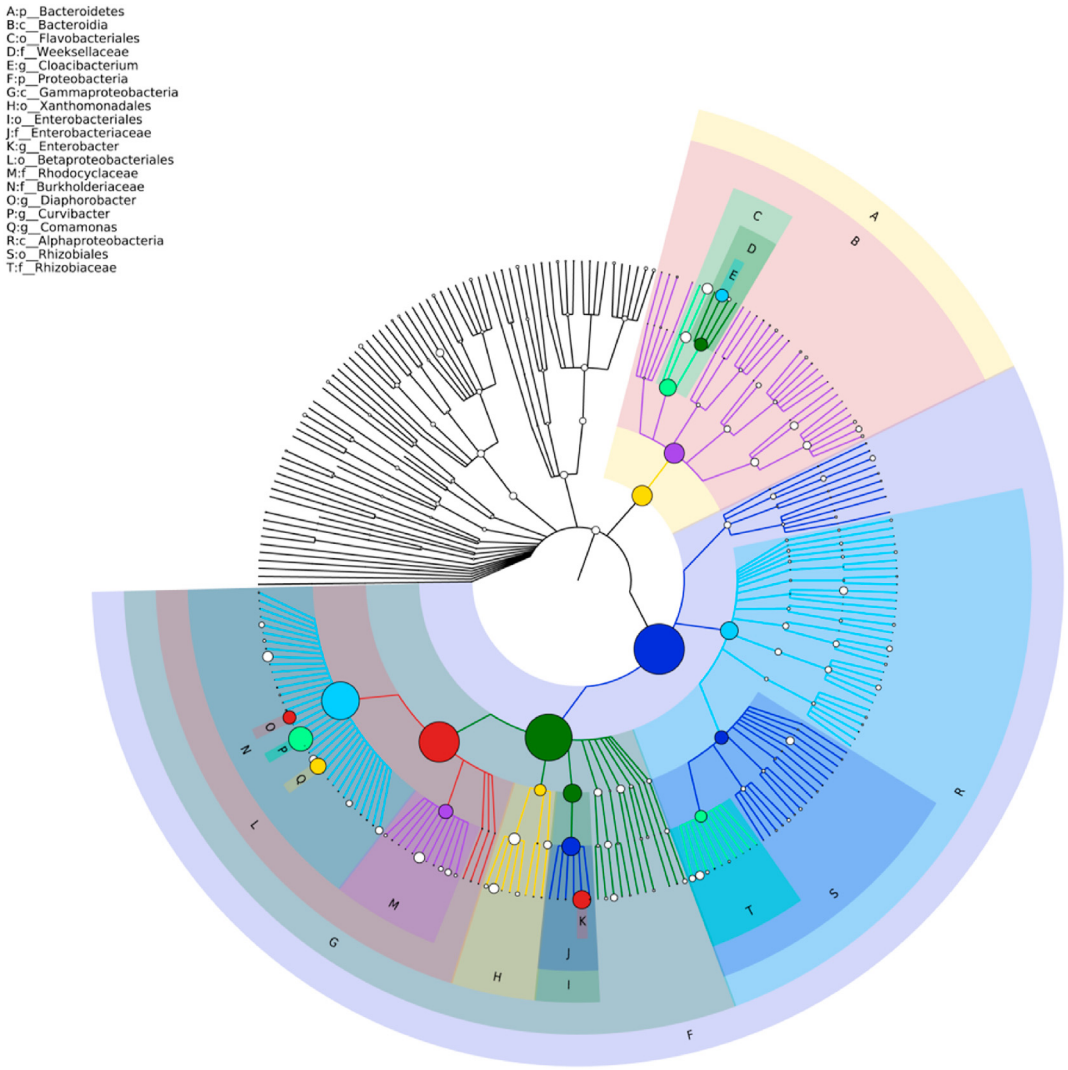

Fig. 6. LDA effect size taxonomic cladogram comparing bacterial communities in R1 reactors with different carriers.

microorganisms mentioned above improved the TN removal rate of the reactor. According to Shen et al. (2016), a packed-bed bioreactor with 1,4-PBS as a microbial carrier showed a volumetric denitrification rate of 0.60 kg m^{-3} day^{-1}. The dominant bacteria were *Dechloromonas*, *Alicycliphilus*, *Azospira* and *Sinobacteraceae*-uncultured. These microbes belong to *Proteobacteria*, and their dominance agreed with the results of this study. Thus, 1,4-PBS affects the relative abundances of microorganisms belonging to *Proteobacteria* and causes changes in reactor performance. In addition, a high COD/N ratio is beneficial to the denitrification process. However, high denitrification efficiency is also obtained with a low influent COD/N ratio (Su et al., 2019). Compared with R1 (without a carrier), R2 (with a 1,4-PBS carrier) exhibited significantly increased denitrification efficiency. Overall, microbial carriers of biodegradable materials showed outstanding denitrification performance (Liu et al., 2018; Peng et al., 2006). The material 1,4-PBS is a suitable microbe carrier and aerobic denitrification solid carbon source. The microbial denitrification pathway mainly depends on the conversion rates of nitrification and denitrification (Peng et al., 2006). Initially, the biofilm on the 1,4-PBS carrier had not yet formed, and a high DO led to a faster growth rate for NOB than AOB (Yao and Peng, 2017), resulting in less NO_2^--N accumulation. According to Shen et al. (2016), PBS could be utilized as solid carbon source and biofilm carrier for nitrate removal. The result showed that PBS manifested a promising denitrification performance. The volumetric denitrification rate was 0.60 kg m^{-3} day^{-1} at the NO_3^--N loading rate of 0.63 kg m^{-3} day^{-1}, and the average NO_2^--N concentration was below 0.20 mg L^{-1}. The removal of NO_3^--N is mainly through denitrification, which showed that the PBS carrier can promote the denitrification reaction. AOB bacteria are adapted to survive in an environment with a DO concentration of 0.2–1.5 mg L^{-1} (Mohammed et al., 2014), while NOB bacteria are adapted to survive in an environment with a dissolved oxygen concentration of 1.2–1.5 mg L^{-1} (Almstrand et al., 2011). In the system, ammonia oxidation requires a DO concentration of 1.5 mg L^{-1} or even higher, while the DO concentration required for the accumulation of nitrite is 0.3–0.7 mg L^{-1}. Therefore, when the system is operated under low dissolved oxygen conditions for a long time, the accumulation rate of nitrite would be destroyed, and the abundance and activity of NOB bacteria would be inhibited. The DO concentration of 1,4PBS-SBR reactor is between 1.0 and 2.5 mg L^{-1}, and denitrification was the main way of denitrification in 1,4PBS-SBR system. After 45 days of operation, the removal rate of TN in 1,4PBS-SBR reactor was gradually increased. This shows that in the 1,4PBS-SBR reactor was an anaerobic and hypoxia microenvironment on the PBS carrier. This provided a living

environment for aerobic and anaerobic bacteria. As the amount of carbon source released from 1,4-PBS increased, the biofilm on the 1,4-PBS gradually formed. This caused the DO distribution on the surface of the 1,4-PBS carrier to change. Eventually, accumulation of NO_2−N enhanced the SND process. According to Liu et al. (2018), during the limited aeration phase of PBS carrier reactor, the TN removal rate in the anammox process reached 11%, but decreased to 6% during the full aeration phase. The PBS carrier provided a place for the colonization of different microorganisms. so that denitrifying microorganisms with different functions coexist on the PBS carrier. This function can be used for different microbial denitrification processes that require different content of dissolved oxygen.

4. Conclusions

The material 1,4-PBS is a suitable carrier for the growth of aerobic denitrifying bacteria and can enhance the TN removal percentage by 16.3%. Due to the added 1,4-PBS carrier, the EPS content increased from 216.33 g g^{-1} VSS to 416.33 g g^{-1} VSS. The 1,4-PBS carrier gradually stabilized biofilms, increased the abundances of more microbes and diversity, and changed the microbial community structure. Furthermore, the relative abundances of *Curvibacter, Enterobacter, Comamonas, Cupriavidus* and *Azospira* increased. 3D-EEM spectra indicated that protein-like and humic acid-like substances were the main components of SMPs, and their peak intensities increased in the initial 31 days and then decreased in the following 14 days. The study of the PBS carrier affect the microbial community structure and performance of denitrification enhanced bring insight into the role of the aerobic denitrifying bacteria in SBR reactor.

CRediT authorship contribution statement

Jiafeng Ding: Visualization, Revised- Final Draft. **Bin Chen:** Writing - original draft. **Xueping Ye:** Validation, Conceptualization, Methodology, Project administration, Funding acquisition. **Yan Li:** Formal analysis. **Dongren Zhou:** Investigation. **Ying Ding:** Data curation. **Weiqin Zhu:** Supervision. **Hangjun Zhang:** Investigation, Resources, Writing - review & editing.

Declaration of competing interest

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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Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2020.125279.

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