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Structures and Anaerobic Metabolic Activity of Attached Microbial Communities During Biofilm Formation in Aquaculture Systems

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ABSTRACT

In this study, the structural characteristics of the attached microbial community during biofilm formation and the metabolic requirements under anaerobic conditions were evaluated. In particular, 16S rDNA sequencing technology was used to study the structural changes of the attached microbial community during biofilm formation (day 0, 10, 20, and 30) in an aquaculture system, and Biolog microplate technology was used to study metabolic characteristics under anaerobic conditions during biofilm formation. The AWCD (average well colour development), an indicator of carbon metabolism, of attached microorganisms during biofilm formation under anaerobic conditions differed significantly among time points ($P < 0.05$), and the carbon source utilization capacity was enhanced. Under anaerobic conditions during biofilm formation, the utilization of amines in six major carbon sources was the highest, followed by polymers, carbohydrates, amino acids, and carboxylic acids, and the utilization of phenols was the lowest. Under anaerobic conditions, the utilization rate by the attached microorganisms during biofilm formation was highest for Tween 40, followed by Tween 80, D-xylose, 4-hydroxybenzoic acid, α-D-glucose-1-phosphate, L-phenylalanine, and phenylethylamine. The 16s rDNA sequencing results showed that the dominant phyla of attached microorganisms during biofilm formation were mainly Proteobacteria, Bacteroidetes, Firmicutes, and Cyanobacteria. On the 10th and 20th days of biofilm formation, attached microorganisms were enriched for *Rhodobacter* of α-Proteobacteria and *Janthinobacterium* of β-Proteobacteria, which play important roles in biofilm denitrification. However, on the 30th day, enriched bacteria included the Burkholderiales of β-Proteobacteria, the Xanthomonadaceae and *Thermomonas* of γ- Proteobacteria, which function in cyanide decomposition.

INTRODUCTION

Aquaculture organisms generally use only 20-30% of the nitrogen source in their feed, and the remainder enters the water body as residual matter and faeces (Avnimelech et al. 2006, Piedrahita et al. 2003). The resulting increase in non-ionic ammonia and nitrite nitrogen in the water will inhibit the growth of aquaculture organisms and even lead to death (Xia et al. 2010, Reddy-Lopata et al. 2006). This is a major issue in high-density intensive farming.

Denitrification by biofilms is a core step in ecological restoration. As an economical, effective, and eco-friendly biological denitrification technology (Huang et al. 2017), biofilms contain both anaerobic and aerobic zones for simultaneous aerobic nitrification and anaerobic denitrification. This will enrich both nitrifying and denitrifying bacteria and accelerate the oxidation of ammonia nitrogen and nitrite nitrogen into nitrate nitrogen, thereby improving the removal efficiency (Flemming et al. 2010). Biofilms consist mainly of microorganisms and their extracellular polymers. The spatial distribution of microorganisms in a biofilm is distinct, and denitrification is directly related to the structure of the attached microbial communities (Rittmann et al. 2018, Zhang et al. 2010). In the early stage of biofilm formation, the surface is dominated by an aerobic layer, where aerobic bacteria are dominant. Eventually, facultative anaerobic and anaerobic layers form (Nogueira et al. 2002, Kang et al. 2005). As biofilm formation progresses, denitrifying bacteria dominate (Qian et al. 2011).

The metabolic characteristics of denitrifying bacteria in the facultative anaerobic and anaerobic layers can provide a basis for the screening of carbon sources (electron donors) required for denitrification by biofilms. Biolog technology for studying carbon source metabolic characteristics is a well established method for screening biofilm carbon sources (denitrifying electron donors). Liu et al. (2015)

studied the carbon metabolism characteristics of microbial communities in different water treatment units of recirculating aquaculture systems for eels and found that microbial communities show preferences for certain carbon sources on ECO plates, which functioned as electron donors that increased the rate of denitrification. Zhu et al. (2017) and Li et al. (2014) used Biolog technology to study the carbon source metabolism of biofilm microbial communities in aquaculture systems and achieved the improved metabolic activity.

Anaerobic denitrification mainly occurs in the facultative anaerobic and anaerobic layers. Therefore, it is important to investigate the metabolic activity of carbon sources in biofilm microorganisms under anaerobic conditions to identify appropriate carbon sources for denitrification. In this study, an ecological base was used for the growth of microorganisms in an aquaculture system, and Biolog microplate technology was employed to study the characteristics of carbon metabolism in microbial communities under anaerobic conditions during biofilm formation. High-throughput sequencing was used to examine the structures of microbial communities during biofilm formation. We aimed to explore the differences in carbon metabolism under anaerobic conditions and structural changes of attached microbial communities during biofilm formation. The results improve our understanding of the process of anaerobic denitrification in biofilms and provide a theoretical basis for studies of biofilm denitrification mechanisms and for the improvement of the biofilm denitrification capacity.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted at the test site of the Pearl River Fisheries Research Institute of the Chinese Academy of Fishery Sciences. A 2 × 2 m cement pond filled to a depth of 1.0 m with water was used. The cement pond was exposed to sunlight and disinfected before use. Forty grass carps were placed in the pond, weighing 75 ± 0.5 g. The daily feeding amount was 2% of their total weight which gradually increased to 3%. The ecological base was AquaMat, which was 1 × 1 m in size and 0.2 cm thick. The specific surface area was 200 m^2/m^2, and its relative density was 240 g/m^2. One end of the long side of the AquaMat was kept intact, and the other end was cut into strips of 0.05 m × 0.8 m, which were ideal dimensions for the flow of water and fish movement.

At the beginning of the experiment, one AquaMat was placed in the aquaculture water. Samples were collected from the upper, middle and lower parts of the AquaMat on day 0, 10, 20, and 30. The samples were placed in a sterilized 50 mL centrifuge tube, kept at 4°C, and brought to the laboratory for subsequent processing.

Water Quality Testing

The pH, temperature, dissolved oxygen, electrical conductivity, and total dissolved solids were tested on-site using a portable multi-parameter water quality meter (YSI Professional Plus, Yellow Springs, OH, USA). Total nitrogen (TN) was determined by the alkaline potassium persulphate digestion-UV spectrophotometric method (GB 11894-1989).

Analysis of Anaerobic Metabolism of Biofilm Microorganisms Using Biolog-ECO Microplates

Five grams of the samples were added to 150 mL of 0.9% sterile saline solution. Pure nitrogen gas was injected into the solution to ensure an anaerobic environment, and the mixture was shaken for 2 h. The fully mixed microbial suspension was sealed and placed in an anaerobic incubator and aliquots of equal size were obtained. The sample solution was poured into a sterile pipette basin in the anaerobic incubator and then pipetted into a pre-heated 25°C Biolog-ECO microplate, where 150 mL of the sample was added to each well. The loaded Biolog-ECO microplate was covered and cultured at a constant temperature of 25°C. After 48 h, the microplate was removed from the anaerobic incubator and absorbance at 590 nm was determined. The average optical density at 48 h was used for subsequent data processing.

The average well colour development (AWCD) was used as an indicator of carbon metabolism of the microbial communities. The AWCD was calculated from the measured absorbance of Biolog-ECO with the formula AWCD = (Ci – R)/n. Here, Ci is the absorbance of the carbon source wells, R is the absorbance of the control wells, and n represents the total number of carbon sources (i.e., 31). The change in AWCD over time was plotted.

Diversity indexes were obtained, including the Pielou's index, Simpson's index (i.e., the dominance index), Shannon-Wiener index, and the McIntosh's index. The Shannon-Wiener index reflects the abundance and evenness of microorganisms, the Simpson's index indicates the dominance of the most abundant species in the microbial community, and the McIntosh's index is based on the Euclidian distance of the community in multidimensional space (Magurran et al. 1988).

The utilization rates of carbon sources by biofilm microbial communities under anaerobic conditions were calculated. The physiological and metabolic characteristics of the microorganisms in water using carbon substrates in the 96 wells were analysed. The Biolog-ECO microplate (ECO Microplate; Matrix Technologies Corporation, Maumee,

OH, USA) contained 3 sets of 31 different carbon sources (including 6 amino acids, 12 saccharides, 5 carboxylic acids, 4 polymers, 2 amines, and 2 phenols), and there were 3 replicates for each microplate. Based on the colour change of the wells, the rate of carbon utilization under anaerobic conditions was determined. The maximum value for the average AWCD of the six types of carbon sources at the four sampling time points was set to 100%, from which the relative utilization rates of the six types of carbon sources at different time points were calculated.

Analysis of Microbial Community Structures

Four grams of AquaMat was added to a conical flask filled with 150 mL of sterile deionized water and mixed for 2 h in a shaker at 25°C and 180 r/min. The mixture was then vacuum-filtered with a 0.2 μm microbial membrane filter, and the membrane filter was cut into pieces with sterile scissors and stored in a 50 mL sterile centrifuge tube. For microorganisms in the aquaculture water body, a 150 mL water sample was obtained using a measuring cylinder and vacuum-filtered with a 0.2 μm microbial membrane filter. The subsequent steps were the same as those described above. Microbial DNA was extracted using the OMEGA Water DNA Kit (Omega, Norwalk, CT, USA).

The concentration and purity of DNA were evaluated by 1% agarose gel electrophoresis. The DNA was diluted to 1 ng/μL with sterile deionized water, and the V4 hypervariable region of the 16S rDNA gene was amplified using 515F and 806R primers with sample-specific barcodes (Ni et al. 2017). PCR was performed using a 30 μL reaction volume, containing 15 μL of a high-efficiency high-fidelity enzyme mixture (Phusion® High-Fidelity PCR Master Mix; New England Biolabs, Ipswich, MA, USA). The PCR products were mixed in equal proportions, and the mixture was purified using a GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, MA, USA). The sequencing library was constructed with the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs) and adapter sequences were added. Finally, the library was sequenced using the Illumina MiSeq platform, and 250-bp paired-end reads were generated. Miseq sequencing was carried out by Beijing Nohe Corporation (Beijing, China).

The paired-end reads of the original DNA fragments were combined using Flash, and the combined labels were assigned to each sample based on the sample-specific barcodes. A sequence analysis was performed using UPARSE (Edgar et al. 2013), and sequences with ≥97% similarity were assigned to the same operational taxonomic unit (OTU). Representative sequences for each OTU were selected and annotated with appropriate classification information using the RDP classifier (Wang et al. 2007). Perl scripts were used to analyse α-diversity and β-diversity.

Data Analysis

A principal component analysis (PCA) and heat map analysis were performed using the vegan package in R. Other statistical analyses were implemented in SPSS18.0, and the experimental data were presented as arithmetic means ± standard deviation. One-way analysis of variance (ANOVA) with Duncan's multiple range test was used to compare means among groups and the significance level was set to $P < 0.05$.

RESULTS

Basic Water Quality in the Pond

During the experiment, the water temperature of the grass carp pond was kept at about 30°C. The dissolved oxygen was kept at above 6.0 mg/L. The pH was maintained at about 7.4. The electrical conductivity was kept between 0.31 ± 0.03 and 0.40 ± 0.01 μS/cm. The TN was maintained at 4.89 ± 1.31 to 6.97 ± 1.48 mg/L. The water quality was generally stable (Table 1).

Analysis of the AWCD and Functional Diversity of Biofilm Microbial Communities under Anaerobic Conditions

The AWCD, Shannon index, Simpson's index, Pielou's index, and McIntosh's index were calculated from the optical density (C-R) of the ECO microplates at 48 h (Table 2). The AWCD values for the attached microorganisms during biofilm formation under anaerobic conditions differed significantly among time points ($P < 0.05$). The AWCD value for the attached microorganisms during biofilm formation

Table 1: Characteristics of aquaculture water.

	Temperature (°C)	Dissolved oxygen (mg/L)	pH	SPC (μS/cm)	TN (mg/L)
0d	30.7±0.75	5.78±1.16	7.22±0.02	0.35±0.10	4.89±1.31
10d	29.8±0.95	6.2±0.46	7.64±0.24	0.40±0.01	5.74±2.07
20d	31.8±0.26	5.94±0.35	7.32±0.26	0.31±0.03	6.97±1.48
30d	31.3±0.4	6.07±0.08	7.48±0.34	0.36±0.03	5.27±0.18

Table 2: AWCD and diversity indexes for biofilm microbial communities under anaerobic conditions.

	AWCD	Shannon-Weiner index	Simpson index	Pielou index（E)	McIntosh index
day 0	0.37±0.03[a]	2.53±0.21[a]	0.99±0.01[a]	1.04±0.08[b]	3.53±0.21[a]
day 10	0.72±0.10[b]	2.86±0.10[b]	0.98±0.002[b]	0.69±0.02[a]	5.75±0.51[b]
day 20	0.79±0.07[b]	3.00±0.31[b]	0.98±0.003[b]	1.01±0.10[b]	6.04±0.48[b]
day 30	0.77±0.10[b]	2.91±0.09[b]	0.98±0.01[a]	0.99±0.03[b]	5.87±0.56[b]

Note: Different superscript letters in the same column show significant differences between treatments ($P < 0.05$), n = 6

under anaerobic conditions at day 0 was significantly lower than those at day 10, day 20 and day 30 ($P < 0.05$), but the AWCD values at day 10, day 20 and day 30 were not significantly different from each other ($P > 0.05$). During biofilm formation, the capacity of biofilm microorganisms to utilize carbon sources under anaerobic conditions improved significantly. The Shannon index and the McIntosh's index at day 0 were significantly lower than those at day 10, 20 and 30 ($P < 0.05$).

The Simpson's indices at day 10 and day 20 were significantly higher than those at day 0 and day 30 ($P < 0.05$); the Pielou's index was at lowest on day 20.

Analysis of the Efficiency of Carbon Source Utilization by Biofilm Microorganisms under Anaerobic Conditions

Utilization rates were highest for amines under anaerobic conditions, followed by polymers, carbohydrates, amino acids, and carboxylic acids, and the utilization rate of phenols was the lowest (Fig. 1). The capacity to utilize amines under anaerobic conditions was significantly different among sampling times ($P < 0.05$). Amine utilization was lower at day 0 than at other sampling times. The amine utilization rate was highest at day 10, followed by day 20 and day 30. The utilization rate of polymers increased over time and reached a maximum of 58.18% on day 30. The carboxylic acid utilization rates were significantly different among time points ($P < 0.05$), with a continuous increase until day 20 (reaching a maximum of 49.37%), followed by a decrease on day 30. The carbohydrate utilization rates were 48.05%, 49.24% and 48.57% on day 10, day 20 and day 30, respectively, and the lowest rate was observed on day 0. The phenol and amino acid utilization rates were similar; the utilization rates for the two carbon sources were lowest on day 0 and decreased on day 20. Across sampling times, the quantity of microorganisms was lowest on day 0, as was their carbon source utilization rate. Over time, biofilm microorganisms gained a greater capacity to utilize the six major carbon sources under anaerobic conditions, but the carbohydrate utilization rate did not change substantially after day 10. In general, biofilm microorganisms showed metabolic preferences for amines, polymers, carbohydrates, amino acids, and carboxylic acids under anaerobic conditions, and their metabolic capacity for the six carbon sources improved over time.

Under anaerobic conditions, there were significant differences in the ability of the microorganisms to use 31 differ-

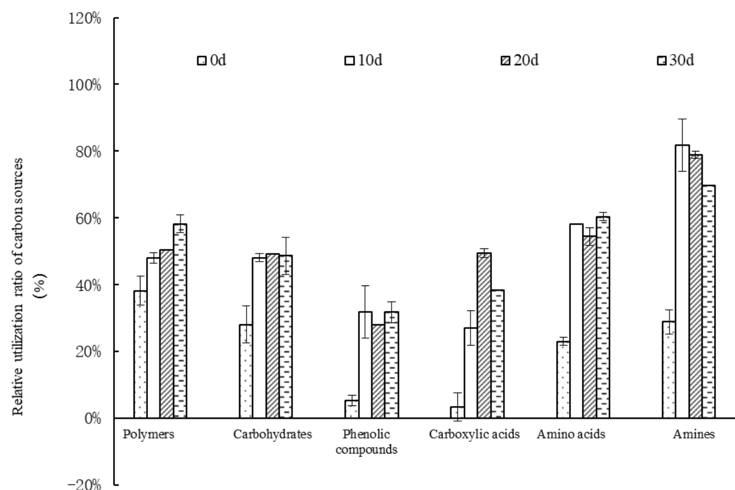

Fig.1: Relative utilization rates for six carbon sources at different sampling points under anaerobic conditions.

ent carbon sources under anaerobic conditions (Fig. 2). Only half of the 31 carbon sources were utilized by the biofilm microorganisms under anaerobic conditions, and the relative AWCD values for each carbon source were different. The carbon sources with high utilization rates exhibited greater demand by the microorganisms under anaerobic conditions, while low utilization rates indicated reduced or no demand for those carbon sources under anaerobic conditions. In this study, we set the baseline carbon source utilization rate as a total utilization rate of 4% on each microplate.

The number of high-utilization (>4%) carbon sources under anaerobic conditions was lowest on day 0, with only three carbon sources, while on day 10, day 20 and day 30, there were 11 high-utilization carbon sources (Fig. 2). During biofilm formation, the change in high-utilization (>4%) carbon sources under anaerobic conditions was similar to the change in AWCD activity. Both were lowest on day 0, with significantly higher values on day 10, day 20 and day 30 than those on day 0 ($P < 0.05$). With biofilm formation and the improvement in the carbon utilization capacity under anaerobic conditions, the utilization rates of α-D-glucose-1-phosphate, β-methyl-D-glucoside, Tween 40, D-galacturonic acid, 4-hydroxybenzoic acid, L-phenylalanine, and phenethylamine were significantly higher compared with those on day 0. The carbon sources that consistently showed high utilization rates were Tween 80 and D-xylose.

Analysis of Attached Microbial Communities during Biofilm Formation

In a cluster analysis, applying a standard threshold of 97% similarity, 24566 bacterial sequences were generated and 10154 OTUs were identified. During biofilm formation, the relative abundances of biofilm microorganisms differed significantly over time (Fig. 3). On day 0, the dominant phyla in the aquaculture water were Proteobacteria (29.1%), Bacteroidetes (20%), Planctomycetes (14.8%), Cyanobacteria (9%), Verrucomicrobia (9%) and Actinobacteria (8.6%). On day 0, the dominant phyla in biofilms were Proteobacteria (51.1%) and Bacteroides (26.9%). On day 10, the structures of the biofilm microbial communities in biofilms changed, and Firmicutes (32%) became a dominant phylum. On day 20, the dominant phyla in the

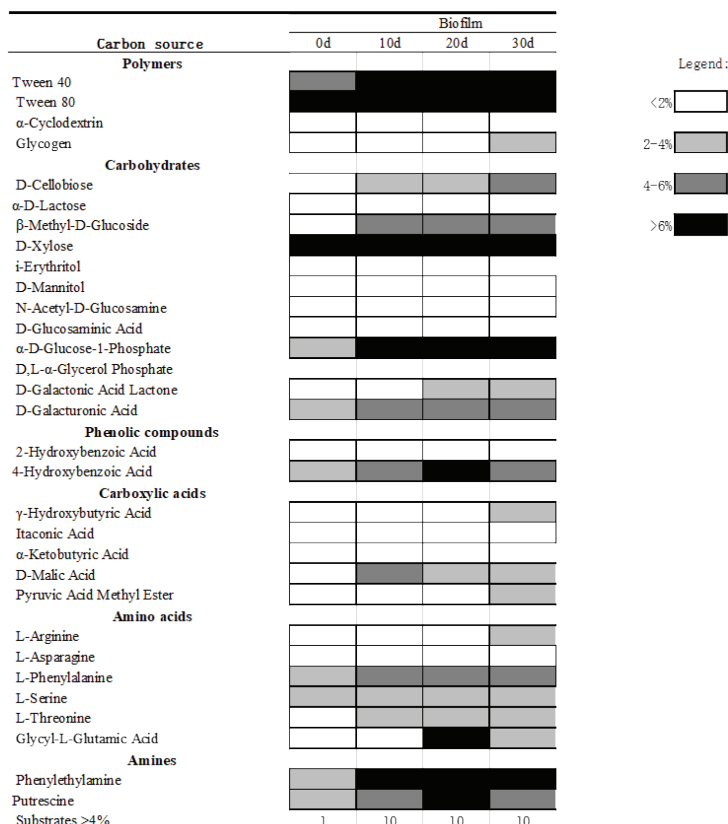

Fig. 2: Pattern of utilization (based on AWCD values) for 31 carbon substrates by adherent microbes in biofilms under anaerobic conditions.

attached microorganisms also included Planctomycetes (5.9%), while Proteobacteria (45.7%), Firmicutes (32.8%), and Bacteroides (11%) were still among the dominant phyla. On day 30, the dominant phyla in biofilms included Proteobacteria (51.1%), Bacteroidetes (21.4%), Planctomycetes (8.6%) and Cyanobacteria (6.5%).

A PCA showed that PC1 (53.65%) and PC2 (15.62%) explained up to 69.27% of the total variance. The scores for these two principal components were plotted to visually evaluate the changes in microbial community structure during biofilm formation (Fig. 4). On day 0, the microorganisms in the water body differed substantially from the biofilm microorganisms at each subsequent point; the microbial community structures were quite different. Points for day 10 and day 20 were highly clustered, and the biofilm microbial community structures were similar. The points at these two sampling times were distant from those at day 0 and day 30, and the biofilm microbial community structures were significantly different.

During biofilm formation, the microbial community structure formed by microbial succession (Fig. 5). At the beginning of the experiment, the microbial community

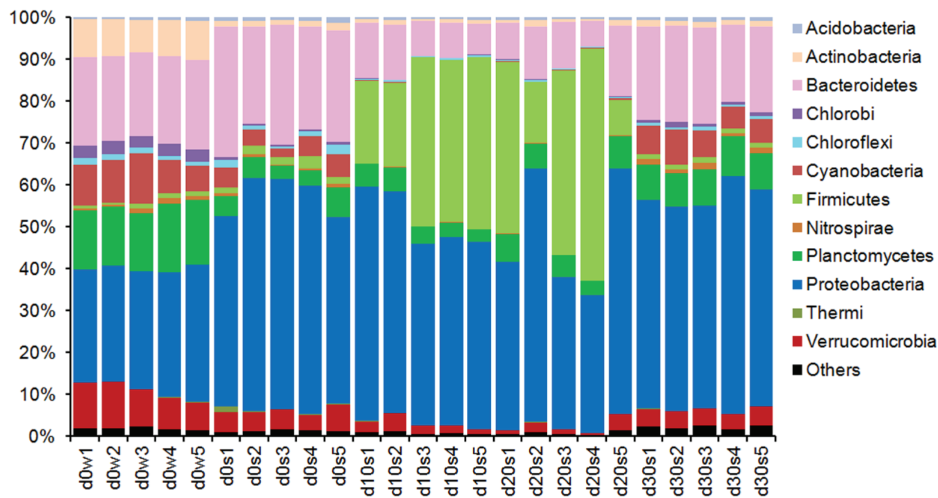

Fig. 3: Composition of dominant phyla during biofilm formation. d, sampling time; w, water body; s, biofilm.

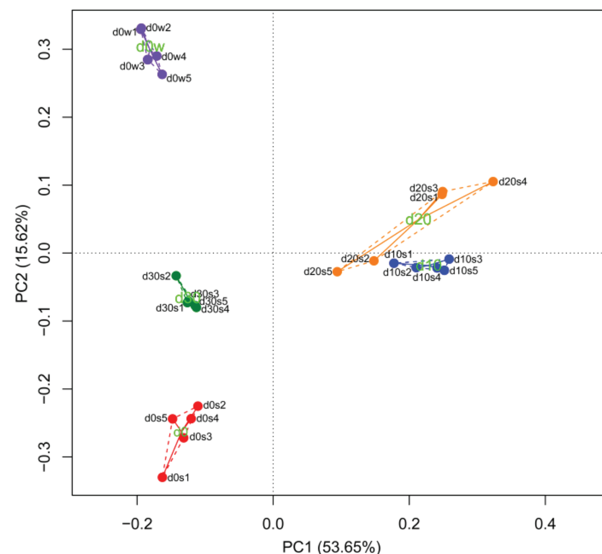

Fig. 4: Principal component analysis of community structure during biofilm formation. d, sampling time; w, water body; s, biofilm.

structure in the water body was highly similar to that of the biofilm at day 0. The clustering patterns of biofilm microorganisms on day 10 and day 20 indicated that they had similar community structures; the biofilm microorganisms on day 0 and 30 also clustered together. On day 0, Pirellulaceae, Cryomorphaceae, Stramenopiles, and Cerasicoccaceae did not attach to the biofilm substrate on day 0, and the biofilm microorganisms were dominated by Saprospiraceae and *Rubrivivax*. Over time, the abundances of *Rhodobacter*, Planococcaceae, *Flectobacillus*, *Bacillus* and *Aeromonas* increased significantly; the abundances of Saprospiraceae and *Rubrivivax* were lower on day 0 than on day 10. On day 20, there were significant increases in *Janthinobacterium* and *Alicyclobacillus* in the biofilm compared with abundances on day 10. On day 30, *Paucibacter* and *Thermomonas* showed high abundances in biofilms.

DISCUSSION

The capacity of biofilm microorganisms to utilize carbon sources under anaerobic conditions gradually improved during the process of biofilm formation in the aquaculture system. The AWCD value reflects the overall metabolic capacity on different carbon sources; a faster rate of change indicates higher metabolic activity (Xi et al. 2005). Under anaerobic conditions, the AWCD value for biofilm microorganisms was lowest on day 0 ($P < 0.05$); on day 20 and day 30, the biofilm microorganisms had the highest metabolic capacity on carbon sources under anaerobic conditions. These results are consistent with those of Li et al. (2014) for changes in the carbon source metabolism of microbial communities in grass carp aquaculture ponds. Changes in the Shannon index, Simpson's index, Pielou's index, and McIntosh's index were generally consistent with the results for carbon source metabolic activity. It is possible that during biofilm formation, increases in the quantity and variety of microorganisms directly cause changes in metabolic activity under anaerobic conditions (Liang et al. 2015).

During biofilm formation, the amine utilization rate under anaerobic conditions was highest among the six types of carbon sources, followed by polymers, carbohydrates, amino acids, and carboxylic acids, and the phenol utilization rate was lowest. According to previous studies of efficient carbon sources under aerobic conditions, biofilm microorganisms have the greatest preference for amino acids, followed by carboxylic acids and sugars (Zhu et al. 2017, Wang et al. 2013, Yang et al. 2009), indicating preferences for carbon sources under anaerobic conditions. The difference might be explained by the fact that biofilm microorganisms limit the metabolic activity of aerobic microorganisms under anaerobic conditions, while the metabolic activities of anaerobic and facultative anaerobic microorganisms are less affected, resulting in reduced demand for carboxylic acids and sugars. We speculated that amines were the main carbon sources contributing to anaerobic denitrification mediated by biofilms. In this study, Biolog technology was used to analyse the dynamic characteristics of carbon source metabolism in biofilm microbial communities under anaerobic conditions. We found that carbon sources utilized under anaerobic conditions were significantly different from those utilized under aerobic conditions. The carbon sources with the highest utilization rates included α-D-glucose-1-phosphate, Tween 40, D-xylose, Tween 80, 4-hydroxybenzoic acid, L-phenylalanine, and phenethylamine. Compared

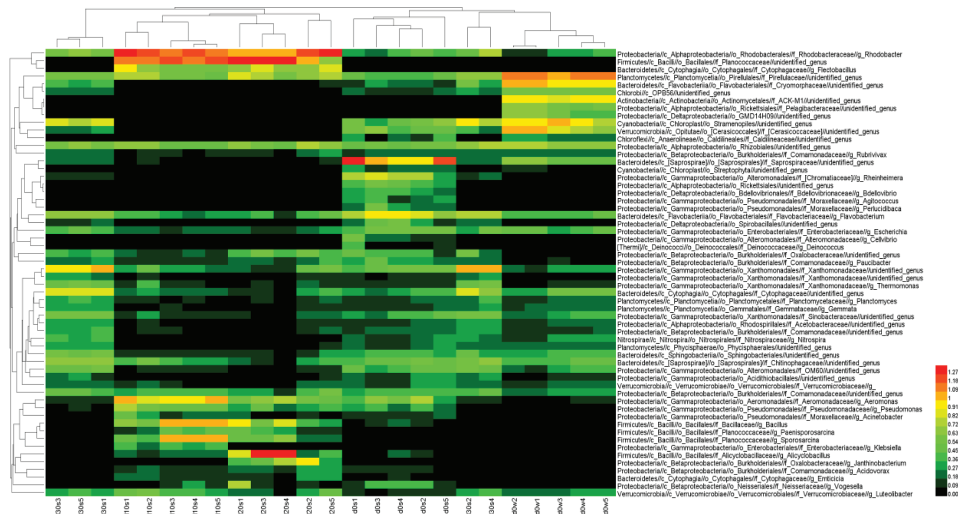

Fig. 5: Heatmap profile of the dominant OTUs of attached microorganisms during biofilm formation d, sampling time; w, water body; s, biofilm.

with the carbon sources utilized by biofilm microorganisms under aerobic conditions, the types of carbon sources used by denitrifying bacteria under anaerobic conditions were significantly reduced (Liu et al. 2015, Li et al. 2014, Cao et al. 2014), which might be explained by the reduced metabolic activity of the aerobic bacteria under anaerobic conditions and the rather intact carbon source metabolic activity of anaerobic and facultative bacteria.

In this study, we performed a PCA of the microbial community structures in the aquaculture water and during biofilm formation. The microbial community structures during biofilm formation were similar over time, but there was a significant difference between those at the initial stage (day 0) and the final stage (day 30). During biofilm formation, microorganisms became abundant, the thickness of the biofilm increased, and the diversity of the attached microbes increased. After the biofilm stabilized, the dominant phyla began to grow, which limited the colonization of other microbial species and affected the structure of the microbial communities.

The dominant phyla during biofilm formation were Proteobacteria, Firmicutes, Bacteroides, and Cyanobacteria, consistent with previous studies (Li et al. 2017, Yu et al. 2016). Highly abundant bacteria during biofilm formation included Saprospiraceae, *Rubrivivax, Rhodobacter, Janthinobacterium, Paucibacter,* and *Thermomonas*. The dominant bacteria Saprospiraceae at the beginning of biofilm formation (Day 0) might have played an important role in the degradation of carbohydrates and proteins (Fu et al. 2017). Under anaerobic conditions, the photosynthetic bacteria *Rubrivivax* can use ammonia as a hydrogen donor for photosynthesis and hydrogen production (Verméglio et al. 2012). On day 10 and 20, biofilms were enriched for the denitrifying bacteria *Rhodobacter*, belonging to the class α-Proteobacteria. Studies have shown that *Rhodobacter* can perform amination and denitrification using amines as carbon and nitrogen sources (Heylen et al. 2006, Zhang et al. 2005), the denitrifying bacteria enriched on day 20 also included *Janthinobacterium*, belonging to the class β-Proteobacteria, which play important roles in denitrification (Heylen et al. 2007, Fang et al. 2011). On day 30, *Paucibacter*, which belongs to Burkholderiales under the class β-Proteobacteria and the phylum Proteobacteria, was highly abundant in biofilms. On day 30, the dominant bacteria in the biofilms were Xanthomonadaceae and *Thermomonas* in the class γ-Proteobacteria and the phylum Proteobacteria, which can decompose environmental pollutants and compounds that are difficult to degrade (Wang et al. 2015). Proteobacteria is important in the denitrification process, and most denitrifying bacteria belong to the phylum β-proteobacteria (Takahashi et al. 2011, Chu et al. 2013, Ye et al. 2011). In this experiment, most of the highly-abundant bacteria during biofilm formation were denitrifying bacteria, which played an important role in the anaerobic denitrification process mediated by biofilms.

In conclusion, as biofilm formation progressed and as the AWCD values for biofilm microorganisms under anaerobic conditions increased significantly ($P < 0.05$), the utilization capacity of carbon sources gradually improved. During biofilm formation, the utilization of the six types of carbon sources under anaerobic conditions was significantly different from that under aerobic conditions. Under anaerobic conditions, the biofilm microorganisms exhibited the highest utilization of amines, while under aerobic conditions, demand was higher for polymers and amino acids. The utilization of single carbon sources by biofilm microorganisms under anaerobic conditions was also different from that under aerobic conditions. The demand for single carbon sources under anaerobic conditions was significantly lower than that under aerobic conditions. A high-throughput sequencing analysis indicated that the dominant phyla in the biofilm included Proteobacteria, Bacteroides, Firmicutes and Cyanobacteria. Bacteria with high abundances during biofilm formation included *Rhodobacter* of phylum α-Proteobacteria, *Janthinobacterium* and *Burkholderiales* of β-Proteobacteria, and Xanthomonadaceae and *Thermomonas* of γ-Proteobacteria. *Rhodobacter* and *Janthinobacterium* play important roles in denitrification mediated by biofilms.

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