Microbial carbon metabolic functions of biofilms on plastic debris influenced by the substrate types and environmental factors

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\begin{abstract}
As an artificial type of microbial carrier, plastic debris has been widely detected in freshwater habitats, and the potential impacts of the plastisphere (biofilms colonized on plastics) in aquatic ecosystems have drawn increasing attention. Distinct community compositions and structures of biofilms in plastic and natural substrates have been recorded in freshwater environments. However, the microbial metabolic functioning of the plastisphere was underestimated, especially in freshwater environments. In this study, the effects of substrate types on the carbon metabolic functions of biofilms were studied by \textit{in situ} cultivation of biofilms on plastics (polyvinyl chloride, PVC and polyethylene, PE) and natural substrate (cobblestone) for 44 days in two rivers (the Niushoushan River and the Qinhuai River) and two lakes (Donghu Lake and Xuanwu Lake). Biofilms on plastics showed higher biomasses than those on natural substrates in all ecosystems. Variations in the micro-structure and compactness of biofilms developed under different substrates were observed from scanning electron microscope and confocal laser scanning microscope image analyses. The carbon metabolic activities of the biofilms evaluated by BIOLOG EcoPlate were different between plastics (PVC and PE) and natural substrate (cobblestone) in the four freshwater ecosystems. In the Niushoushan River, PE-associated biofilms had different capacity in using carbon sources from cobblestone-associated biofilms as illustrated by the Shannon-Wiener diversity index and Shannon evenness index. Additionally, the metabolic functional diversity profiles of biofilms on PVC were significantly different from those on cobblestone in the other three aquatic ecosystems. Moreover, results from variation partitioning analysis suggested that the impact of environmental factors (contribution: 21%) on microbial carbon metabolic functions was much greater than that of substrate types (contribution: 6%). These findings illustrated distinct microbial functions of biofilms inhabited on plastics, and environmental factors play a decisive role in the differentiation and specificity of carbon metabolism of the plastisphere. This study offers new insights that plastics serving as artificial microbial niches have the ability to affect the microbial-mediated carbon cycling process in aquatic ecosystems.
\end{abstract}

\section{Introduction}

The accumulation of plastics in aquatic ecosystems has become a global environmental issue, and plastic production will reach 2 billion tons by 2050 if no action is undertaken to slow down the tendency of production and utilization (Liu et al., 2020). In recent decades, plastic debris has been frequently detected in freshwater ecosystems, such as rivers and lakes (Mani et al., 2016), attracting worldwide attention. Approximately 1.15–2.41 million tons of plastics, mainly resulting from improper disposal of plastic waste, have been released into the ocean annually from surface run-off (Blettler and Wantzen 2019; Wang et al., 2020). Due to the wide existence of plastics in freshwater habitats, researchers have studied the fate and transport models of plastic debris in freshwater catchment areas as well as their interaction with organisms, transfer along the aquatic food chain, and capacity to carry organisms and pollutants (Horton et al., 2017; Richard et al., 2019).

Additionally, the physicochemical properties of plastics can affect their behaviours, i.e., interactions with organisms in freshwater ecosystems (Lobelle and Cunliffe 2011). Most of plastics are less dense than water and thus may drift in water for a period of time. With the various physical, chemical and biological effects of plastics in water, they eventually enter the sediments (De Tender et al., 2015). During
floating, plastics can easily assemble organisms capable of biofilm cultivation, such as algae, bacteria and pathogens, thus providing a special niche for microbial colonization called “plastisphere” (Zettler et al., 2013). Biofilms on plastics exhibited dissimilar in microbial community composition and structure, nutrient transport and pollutant accumulation from the ambient water (Frère et al., 2018; Wang et al., 2020). Surprisingly, a recent study has estimated that 1,000–15,000 tons of global microbial biomass comes from the biofilms on plastic debris (Richard et al., 2019). Therefore, the potential impacts of the plastisphere in freshwater ecosystems should be addressed more.

Biofilms are ubiquitous on the surface of natural substrates (i.e., rocks and plant residues) and consists of multiple species (bacteria, fungi, and algae) in aquatic habitats (Wu et al., 2019). Our previous study has illustrated that the alpha diversity of microbial communities colonized on microplastics (polyethylene, PE and polypropylene, PP) cultured in Xuanwu Lake, China was lower than those on natural substrates (cobblestone and wood) (Miao et al., 2019b). Another study has demonstrated that bacterial communities developed on polystyrene (PS) fragments showed different bacterial combinations compared with PE and PP samples from Brest Bay (Frère et al., 2018). The differences of microbial communities might lead to the functional specificity of biofilms. However, the microbial functioning of biofilms in the plastisphere was overlooked because biofilms play a momentous role in regulating chemical exchange and controlling the biogeochemical cycle in aquatic environments (Battin et al., 2016). Biofilm is recognized as fundamental site of dissolved organic carbon degradation, in which heterotrophic microbes play a significant role in the mineralization of organic carbon in freshwater ecosystems (Battin et al., 2016). A very recent study reported that the carbon metabolic functional diversities of microplastics-associated communities were consistently different from those in the surrounding lake waters (Arias-Andres et al., 2018). Hence, the introduction of the plastisphere might change the carbon metabolic function of biofilms and alter carbon cycling in freshwater ecosystems.

Moreover, biofilm adapts to the spatial and temporal variations in environmental conditions by regulating its community composition and inter-species and intra-species interactions (Pinto et al., 2019; Wang et al., 2020). Meanwhile, the microbial function of biofilms may change accordingly to external environmental pressure. A research study has demonstrated that different water environmental conditions could affect the microbial community structures of biofilms on plastic and natural substrates (Oberbeckmann et al., 2018). More importantly, another research has documented that nutrients, i.e., total nitrogen (TN) and total phosphorus (TP) and salinity could influence the average rate of biofilm colonization on plastics from Haihe Estuary (Li et al., 2019). However, there is still a large knowledge gap with respect to microbial functioning of the plastisphere, especially in various freshwater ecosystems. Accordingly, autotrophic and heterotrophic microorganisms in biofilms both drive carbon cycling in aquatic ecosystems (Battin et al., 2016). Therefore, it is necessary to compare the carbon metabolism of biofilms on plastic and natural substrates in different freshwater ecosystems, thus revealing the potential impact on carbon cycle.

Here, we hypothesized that the community functional characteristics of biofilms attached to plastics and natural substrates might have significant differences and that various environmental factors might also influence the functional differentiation of biofilms. To test this hypothesis, two plastic substrates, i.e., polyvinyl chloride (PVC) and PE, and one natural substrate, i.e., cobblestone, were used for in situ cultivation of biofilms in two rivers and two lakes in Nanjing, China. The measurement of biomass is used to reflect the basic characteristics of biofilms. Moreover, the micromorphology of biofilms was analysed via confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM). Additionally, the BIOLOG EcoPlate was applied to analyse and determine the carbon metabolic functions of biofilms, which favoured the comparison between the plastic and natural substrates in different freshwater ecosystems. Furthermore, the functional diversity of carbon metabolism was compared and analysed to further explore the potential ecological impact of biofilms attached to different substrates for carbon cycle in different freshwater ecosystems.

2. Materials and methods

2.1. Plastic and natural substrates

Plastic flooring composed of PVC and a plastic sealing bag composed of PE were sliced (length of 10 cm; width of 10 cm) and used as plastic substrates. These plastics could represent relatively new anthropogenic substrates for microbial colonization in aquatic ecosystems (Ogonowski et al., 2018) because PVC and PE are common in aquatic environments (Imhof et al., 2013; Koelmans et al., 2019; Muñá Shafiq 2019; Zhao et al., 2014). The size of plastics apparently has no effect on the microbial community structures (Fahrenfeld 2019; Frère et al., 2018); thus, we chose plastic substrates with relatively large surface areas. Cobblestone (C, diameter 3.0 ± 0.5 cm) is widely distributed in freshwater and was thus selected as a natural substrate.

The total surface area, specific surface area, contact angle and surface roughness were used to qualitatively describe the characteristics of substrates. Contact angle, measured with the optical video contact tester (JY-82B Kruss DSA), was used to characterize the hydrophobicity of substrates. The surface roughness was determined by laser microscope (KEYENCE, VK-X150). The detailed information on substrates’ characteristics are available in Table S1.

2.2. Biofilm incubation

Cylindrical stainless steel wire mesh (diameter 25 cm, depth 15 cm, mesh size < 100 μm) were made to culture biofilms. The setup of the mesh size was used to ensure the free exchanges of water and microbes in the devices. The PE and PVC squares were tied vertically to the steel wire using fishing line (Chen et al., 2019) to ensure that the plastic surface was in full contact with the waters. Meanwhile, cobblestones were placed at the bottom of the devices. The devices were fitted with 30 particles of cobblestones (natural substrate) and 4 pieces of plastic substrates (PVC and PE) (Table S1), ensuring that the total surface area of substrates was at the same level. Similar treatment methods were used to compare the biofilms on plastic and natural substrates in previous studies (Ogonowski et al., 2018; Miao et al., 2019b).

The experimental devices were used for in situ cultivation of biofilm at four stations in Nanjing, East China (Fig. 1): Donghu Lake in Hohai University (D, 31°54′59.0″N118°46′55.3″E), Xuanwu Lake (X, 32°04′19.7″N118°47′9.9″E), the Niushoushan River (N, 31°55′14.4″N118°48′15.7″E) and the Qinhuai River (Q, 32°03′36.2″N118°44′38.1″E). There were three cylindrical culture devices in each station. The devices were placed 0.5 m below the surface water with fish line to receive the same light intensity (Arias-Andres et al., 2018) and ensure that all devices were kept within the water in case of water level fluctuation. The devices were subjected to regular cleaning of dirt on the surface to prevent blockages of the tank aperture and the material circulation (Arias-Andres et al., 2018). The three different samples at the four stations were correspondingly marked as DC, DPE, and DPVC; XC, XPE, and XPVC; NC, NPE, and NPVC; and QC, QPE, and QPVC. Background water samples in the incubation sites were collected in triplicate every twenty days, and the morphology of biofilms on the substrate surface was observed simultaneously. 44 days (from August 31 to October 13, 2019) of in situ cultivation was used to obtain mature biofilms on each substrate (Wu et al., 2014). The experimental devices in each cultivation station were transported to the lab for further analysis.
C is the ab- 

and represents the maximum carbon metabolism rate, 

\[ A_{\text{max}} = 0.06 \]

for the wells is 

\[ A_{\text{max}} = A_{\text{T}} - A_{\text{R}} \]

A, nitrite nitrogen and re- 

\[ A_{\text{T}} \]

, ammonium nitrogen, 

\[ A_{\text{R}} \]

is the maximum value of the 

K is the absorbance value of each well at 590 nm, 

, and represents the utili-

\[ K = \sum_{i=1}^{n} (C_i - R)/n \] (1)

where \( C_i \) is the absorbance value of each well at 590 nm, \( R \) is the absorbance value of the blank control well, and \( n \) is a representative of the amount of wells. Additionally, a value of \( < 0 (C_i - R) \), 06 for the wells is counted as zero.

The three metabolic functional diversity indices mentioned above are the Shannon–Wiener diversity index (H’), Simpson diversity index (D) and Shannon evenness index (E), and the formulas are available in Table S4 (Miao et al., 2019a). Data for calculation were selected when the AWCD changed stably (incubation time: 114 h).

The dynamic curve of AWCD changes over time can be fitted by the logistic growth equation (Eq. (2)).

\[ \text{AWCD} = \frac{K}{1 + e^{-c(t-t_0)}} \] (2)

where \( K \) is the maximum value of the AWCD and represents the utilization capacity of carbon sources, \( p \) the maximum value of the slope of AWCD and represents the maximum carbon metabolism rate, \( t_0 \) an independent variable called incubation time, and \( t \) represents the time when AWCD = K/2 and reflects the adaptation time of microbes to the carbon source substrates (Salomo et al., 2009).

2.5. Biofilms metabolic function

Some corresponding indicators, such as the average well-colour development (AWCD) and three metabolic functional diversity indices, were measured to determine the microbes’ ability to utilize various carbon sources and characterize their metabolic proficiency. The AWCD was calculated as Eq. (1).

2.6. Physicochemical analysis and biofilm biomass measurement

During the 44-day cultivation, the water samples were collected twice (Sept 11, 2019 and Oct 13, 2019). Triplicates of 500 mL of water at four stations from the corresponding cultivation depths were transported to the laboratory and analysed for TN, nitrate nitrogen (NO\(_3\)–N), nitrite nitrogen (NO\(_2\)–N), ammonium nitrogen, (NH\(_4^+\)–N),
TP, pH, chemical oxygen demand (COD\textsubscript{meq}), suspended solids (SS), dissolved oxygen (DO), oxidation-reduction potential (ORP) and turbidity (NTU). The average values of two test results were used to represent the environmental conditions in the culture period.

Biofilm biomass was determined gravimetrically as dry weight (DW) and ash free dry weight (AFDW). Biofilms on certain area on the substrates were scraped off and placed in crucible. Samples were dried in an oven at 105°C for 24 h to determine DW. Samples were ignited in muffle furnace for at 450°C for 5 h to determine AFDW.

2.7. Morphology of biofilms on substrates

The morphology of biofilm samples was observed using a SEM (Hitachi S-4800, Japan) (Wu et al., 2019). In addition, the spatial distribution of algae and polysaccharides in biofilms were visualized by CLSM with fluorescence staining. Biofilm samples were washed with pure water and fixed with 2.5% glutaraldehyde for 2 h. Next, calcofluor white was used to visualize α- and β-D-glucopyranose polysaccharides for 1 h. Excessive stain was then washed with pure water. Ultimately, the tinted samples were examined with a CLSM (Zeiss LSM 800, Germany). Self-fluorescence of chlorophyll was excited by a 633 nm laser (Zhao et al., 2018), and α- and β-D-glucopyranose polysaccharides were observed at 400/435 nm (excitation/emission wavelengths) (Wang et al., 2018). Blue and green colours in the CLSM image (3D) represented polysaccharides and algae, respectively.

2.8. Data and statistical analyses

Principal component analysis (PCA), which is a multivariate statistical analysis method for selecting fewer important variables via linear transformation of multiple variables using the R packages FactoMineR and factoextra, was used to compare the differences of carbon metabolism in the different microbial communities. Furthermore, a non-metric multidimensional scaling (NMDS) analysis, which is a more comprehensive and multi-dimensional method for analysing the effect of sample types on microbial community and diversity, was performed and visualized using R packages vegan and ggplot2 (Shepard, 1962; Wickham, 2009). Moreover, distance-based redundancy analysis (dbRDA) was applied to study the complex relationship between microbial function and environment using Canoco 4.5. And collinearity factors and factors with small contribution were eliminated in the analysis process (Legendre and Legendre, 2012). The classification of aquatic ecosystems was based on hierarchical clustering, and the environmental values were standardized by Euclidian distance before hierarchical clustering (Oberbeckmann et al., 2018). Statistical comparisons between different samples were performed based on a permutational multivariate analyses of variance (PERMANOVA) (Zhang et al., 2015). Additionally, variation partitioning analysis (VPA) was performed to analyze the contributions of different abiotic factors to microbial community and functional variation, using in R with the package “vegan”. Mantel test was used to determine the correlation between individual of environmental factors or substrate properties and the carbon metabolic functions of biofilms.

All biochemical analyses of the biofilm samples on plastics (PVC and PE) and natural substrate (cobblesone) were performed in triplicate, and the values are presented as the mean ± standard deviation (Frère et al., 2018). The three diversity indices, i.e., Shannon-Wiener diversity index, Simpson diversity index and Shannon evenness index, of the plastic and natural substrates in different freshwater ecosystems were compared by one-way analysis of variance (ANOVA) to evaluate the effects of different substrates and freshwater ecosystems on microbial functions. A P-value of 0.05 was regarded as significant and calculated by an ANOVA for all analyses, and Origin was used to create all pictures.

3. Results

3.1. Biofilm biomass and characteristics of different culture environments

Two rivers and two lakes were selected as in situ culture sites of biofilms, and their physical and chemical characteristics are shown in Table S5. The WW, DW and AFDW per unit area were measured to describe the basic characteristics of biofilms (Table S6). The WW and DW of biofilms on plastic substrates (PE and PVC) were greater than those on natural substrate (cobblesone) in all incubation stations (Table S6), indicating that microbes more easily colonized the plastic substrates than natural substrates. Furthermore, compared with the three other freshwater ecosystems, biofilms on plastics in Donghu Lake contained the least amount of organic matter as illustrated by the AFDW/DW values (Table S6), which might be related to the concentrations of nutrients in water (Table S5).

3.2. Apparent morphology and community structure of biofilms on substrates

In this study, SEM images were used to investigate the microscopic structure of biofilms colonized on plastics (Hou et al., 2019b; Wang et al., 2020). As shown in Fig. 2, the biofilms in all samples were dominated by algal groups. The microscopic structure of biofilms in Donghu Lake (Fig. 2 a and b) exhibited heterogeneous with complex reticular structures with more filamentous algae compared with those in other sampling sites. Relative dense morphology of biofilms in Xuanwu Lake (Fig. 2 c and d) and the Qinhuai River (Fig. 2 g and h) were observed with more sphere form microbes on the plastic substrates (PE and PVC). These results indicated that the external nutrient conditions and fluid flow might have a certain impact on the morphology and structure of the biofilms, such as deformation, breakup and detachment (Mattei et al., 2018).

Observations by CLSM indicated that biofilms colonized on different substrates were morphologically heterogeneous. In Donghu Lake, algae (in green) were the dominant species and large quantities of EPS (in blue) were weaved into a network of material transport channels. Biofilms on PVC and cobblesone were thicker and denser than those on PE (Fig. S1 a, b, and c). However, in the other three in situ cultivation stations, the content of algae (in green) on biofilms was generally lower indicated by the CLSM images, revealing that different freshwater ecosystems might affect the composition and structure of biofilms (Fig. S1). In Xuanwu Lake (Fig. S1 d, e, and f) and the Qinhuai River (Fig. S1 j, k, and l), the content and abundance of algae (in green) in natural substrates were lower than those in plastic substrates. Additionally, the EPS (in blue) on PVC was generally greater than that on the other substrates in all aquatic ecosystems, demonstrating that the compactness and structure of biofilms varied when developed on different substrates (Zhao et al., 2018) (Fig. S1).

3.3. Metabolic pattern of the biofilm microbial community and the kinetic profile

The utilization of carbon sources was positively related to the metabolic capacity of corresponding microbes, which could be quantified by the AWCD values. Fig. 3 shows that the AWCD values changed over time and that the growth pattern was similar to an S-shaped curve. This regular pattern was highly consistent in all twelve samples, and Eq. (2) fit all the dynamic curves of the AWCD (Fig. 3). The results revealed that the AWCD of all samples generally had a stagnation period in the initial 20 h. However, over time, the growth rate of the AWCD that represented the average rate of microbial carbon metabolism, gradually increased and reached the maximum value (p) in 30–50 h (Table 1). The AWCD was in a relatively stable stage when the culture time reached approximately 100 h (Fig. 3), demonstrating that the carbon utilization capacity of cultivable microbial community had reached the
Fig. 2. The SEM images of 8 biofilm samples on plastic (PE and PVC) incubated in four freshwater ecosystems, including Donghu Lake (D), Xuanwu Lake (X), the Niushoushan River (N) and the Qinhua River (Q). The 8 biofilm samples including DPE (a), DPVC (b), XPE (c), XPVC (d), NPE (e), NPVC (f), QPE (g), QPVC (h).
In Donghu Lake, the biofilms cultured on natural substrates showed a higher ability to utilize carbon sources than those on plastic substrates (PVC and PE), with biofilms on PVC revealing the lowest carbon metabolism capability, thus indicating that the substrate types had a significant effect on carbon metabolism (Fig. 3a). However, biofilms on plastic reflected a stronger ability to utilize carbon sources in the Niushoushan River, indicating that environmental factors also played an important role in carbon metabolism (Fig. 3c). In Xuanwu Lake and the Qinhuai River, biofilms on PVC entered the stable stage ahead of time and they showed lower carbon utilization capacity than the others, illustrating that biofilms on PVC had a strong adaptability to carbon source substrates but a weak utilization ability (Fig. 3c, d; and Table 1). The biofilms cultured in the Niushoushan and the Qinhuai River mostly showed a higher carbon metabolism rate (p) and shorter adaptation time (s) than those cultured in Donghu and Xuanwu Lake (Table 1), suggesting that the different metabolic patterns of biofilms might be ascribed to the comprehensive effects of different environmental factors.

Based on the biochemical characteristics and structural composition, 31 different carbon sources were divided into five categories, namely, carbohydrates, polymers, carboxylic acids, amino acids and amines/amides (Table S2). Overall, microbes had the lowest metabolic consumption of carbohydrates and showed a preference for the other four carbon sources (Fig. 4). In addition, there was no significant difference in the utilization of polymers in four stations regardless of substrate types. For carbohydrates, polymers, carboxylic acids, and amino acids, there was no significant difference in the carbon consumption among the three substrates in Donghu Lake. Nevertheless, the utilization of amines/amides by the microbial community on PVC was lower as shown in Fig. 4a compared with that on the other substrates (ANOVA, P-value < 0.05), and the same phenomenon was observed in the Qinhuai River (Fig. 4d). In Xuanwu Lake, biofilms on PVC had significant differences with biofilms on PE in the utilization of carboxylic acids, which was consistent with the results in the Niushoushan River, and biofilms on PVC in the utilization of amino acids (ANOVA, P-value < 0.05) (Fig. 4 b and c). Furthermore, the usage of carbohydrates by biofilms on the natural substrates was apparently lower than that on plastic substrates.

### Table 1

The kinetic curve parameters of 12 biofilm samples in four ecosystems.

| Samples  | K     | p     | s (h) | r²   |
|----------|-------|-------|-------|------|
| DPVC     | 1.572 | 0.077 | 44.2  | 0.986|
| DPE      | 1.798 | 0.076 | 45.3  | 0.989|
| DC       | 1.890 | 0.072 | 46.8  | 0.994|
| XPVC     | 1.644 | 0.101 | 39.1  | 0.993|
| XE       | 1.923 | 0.074 | 42.8  | 0.995|
| XC       | 1.927 | 0.079 | 46.9  | 0.990|
| NPVC     | 1.747 | 0.097 | 40.4  | 0.983|
| NPE      | 1.911 | 0.097 | 39.4  | 0.991|
| NC       | 1.710 | 0.071 | 41.2  | 0.992|
| QPVC     | 1.813 | 0.090 | 31.3  | 0.997|
| QPE      | 2.069 | 0.103 | 39.5  | 0.994|
| QC       | 2.040 | 0.092 | 39.6  | 0.989|

K (the maximum value of AWCD), p (the maximum carbon metabolism rate), s (the time when AWCD = K/2), r² (correlation coefficient).

In Donghu Lake, the biofilms cultured on natural substrates showed a higher ability to utilize carbon sources than those on plastic substrates (PVC and PE), with biofilms on PVC revealing the lowest carbon metabolism capability, thus indicating that the substrate types had a significant effect on carbon metabolism (Fig. 3a). However, biofilms on plastic reflected a stronger ability to utilize carbon sources in the Niushoushan River, indicating that environmental factors also played an important role in carbon metabolism (Fig. 3c). In Xuanwu Lake and the Qinhuai River, biofilms on PVC entered the stable stage ahead of time and they showed lower carbon utilization capacity than the others, illustrating that biofilms on PVC had a strong adaptability to carbon source substrates but a weak utilization ability (Fig. 3c, d; and Table 1). The biofilms cultured in the Niushoushan and the Qinhuai River mostly showed a higher carbon metabolism rate (p) and shorter adaptation time (s) than those cultured in Donghu and Xuanwu Lake (Table 1), suggesting that the different metabolic patterns of biofilms might be ascribed to the comprehensive effects of different environmental factors.

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that on plastic substrates (PVC and PE) (ANOVA, P-value < 0.05). In addition, there was no consistent regular pattern to explain these phenomena, which might be the result of the comprehensive effect of different substrates and different freshwater ecosystems.

3.4. Microbial metabolic functional diversity

The diversity of metabolic functions of the microbial communities could be evaluated by metabolic functional diversity indices, which consisted of the Shannon-Wiener diversity index, Simpson diversity index and Shannon evenness index (Table 2). In Donghu Lake, the biofilm samples from the cobblestone exhibited the highest values of the three indices, and obvious differences were observed compared with those on the plastic substrates (PVC and PE) (ANOVA, P-value < 0.05) as illustrated by the Shannon–Wiener diversity index and Simpson diversity index. In Xuanwu Lake, the higher indices for cobblestone suggested that the biofilm samples from natural substrates were more diverse than those on PVC (ANOVA, P-value < 0.05).

The above results indicated that the biofilms on the natural substrates cultured in the lake ecosystems had higher metabolic levels and diversity. In the Niushoushan River, the highest Shannon–Wiener diversity index and Shannon evenness index were observed on C, while the lowest were observed on PE. Nevertheless, the diversity of the microbial community on the plastic substrates (PVC and PE) in the Qinhuai River was more abundant than those on the cobblestone. In addition, there was no significant difference among the three substrates.

Table 2

| Samples | AWCD (incubation time: 114 h) | Shannon-Wiener diversity index (H') | Simpson diversity index (D) | Shannon evenness index (E) |
|---------|-------------------------------|-------------------------------------|----------------------------|--------------------------|
| DPVC    | 1.504 ± 0.164a                | 3.328 ± 0.008b                      | 0.962 ± 0.000b             | 0.978 ± 0.002a           |
| DPE     | 1.802 ± 0.133a                | 3.337 ± 0.005b                      | 0.963 ± 0.000b             | 0.981 ± 0.001a           |
| DC      | 1.873 ± 0.071a                | 3.391 ± 0.019a                      | 0.965 ± 0.001a             | 0.988 ± 0.005a           |
| XPVC    | 1672 ± 0.054a                 | 3.332 ± 0.012b                      | 0.963 ± 0.000b             | 0.970 ± 0.004b           |
| XPE     | 1.917 ± 0.171a                | 3.396 ± 0.010a                      | 0.966 ± 0.000a             | 0.989 ± 0.003a           |
| XC      | 1.926 ± 0.044a                | 3.378 ± 0.005a                      | 0.965 ± 0.000a             | 0.984 ± 0.002a           |
| NPVC    | 1.798 ± 0.035b                | 3.387 ± 0.013ab                     | 0.965 ± 0.001a             | 0.986 ± 0.004ab          |
| NPE     | 1.933 ± 0.039a                | 3.374 ± 0.006b                      | 0.965 ± 0.000a             | 0.983 ± 0.002b           |
| NC      | 1.712 ± 0.037b                | 3.406 ± 0.008a                      | 0.966 ± 0.000a             | 0.992 ± 0.002a           |
| QPVVC   | 1.785 ± 0.041a                | 3.407 ± 0.011a                      | 0.966 ± 0.000a             | 0.992 ± 0.003a           |
| QPE     | 2.086 ± 0.034a                | 3.400 ± 0.003ab                     | 0.966 ± 0.000a             | 0.990 ± 0.001ab          |
| QC      | 2.070 ± 0.186a                | 3.359 ± 0.023b                      | 0.964 ± 0.001a             | 0.978 ± 0.007b           |

AWCD (The average well-color development; incubation time: 114 h), H' (Shannon-Wiener diversity index), D (Simpson diversity index), E (Shannon evenness index). Value in the table are the mean ± SD, n = 3. Using one-way ANOVA followed by Tukey's posthoc tests respectively, and different letters after values represent the significant difference at P-value < 0.05 (comparison of biofilm samples from the same station).
in the river ecosystems in terms of the Simpson diversity index, demonstrating that the substrate type did not play a significant role in the utilization of the most common carbon substrates in the BIOLOG EcoPlate.

NMDS was performed based on the metabolic pattern of carbon utilization (Fig. S2). As shown in Fig. S2, three different substrates could be divided into three groups (PVC, PE and C), although the lack of significant differences in community function between single substrates (Table S7) might be due to the lack of parallel samples (n = 3) (Miao et al., 2019b; Ogonowski et al., 2018). In the Qinhuai River, biofilms on C showed significant differences with those on PE and PVC (PERMANOVA, P-value < 0.05) (Fig. S2 d; and Table S7). However, there was no obvious difference between the natural and plastic substrates in the other three freshwater ecosystems (Fig. S2 a, b and c; and Table S7), which is consistent with the PCA (Fig. S3). The results of the PERMANOVA comparisons are listed in Table S7.

### 3.5. Roles of substrate types and environment factors in microbial carbon metabolism

In this study, db-RDA was applied to study the complex relationship between microbial carbon metabolism and environment (physico-chemical properties of substrates and environmental factors in the sampling points). As shown in Fig. 5a, the distribution of biofilm carbon metabolism was affected by both substrate types and environmental factors. Specifically, the distribution of biofilm carbon metabolism cultured in the two rivers overlapped, indicating that their carbon metabolic functions of biofilms were similar. This might be due to the similarity of environmental factors between the two rivers, which was consistent with the clustering results (Fig. S4). Surprisingly, PVC
sample points of these two rivers gathered in the second quadrant and the distribution distance of PE sample points was relatively close, which demonstrated that different substrate properties also affected the carbon metabolic functions of biofilms. The biofilm carbon metabolism of Donghu and Xuanwu Lake were relatively independent in the two groups, demonstrating that there were significant differences in carbon metabolic functions of biofilms cultured in the two lake ecosystems (P-value = 0.001) (Table S8).

Furthermore, after screening out the factors of collinearity and small contribution on the biofilm carbon metabolism, five influence factors (TP, TN, SS, roughness and contact angle) were preserved. As shown in Fig. 5a, roughness and contact angle were the main driving forces of functional differentiation of carbon metabolism. These two factors had great influences on the carbon metabolic functions of the biofilms on PVC. In addition, the primary driving environmental factors of carbon metabolic functions of biofilms were TP, TN and SS, which had great influences on the biofilms cultured in Xuanwu Lake.

As illustrated in Table S9, mantel test was used to analyse the effect of single influencing factor on carbon metabolic functions of biofilms. Most of the environmental factors except pH and NH$_4^+$~N had significant effects on the carbon metabolism of biofilms ($r > 0.1$, significance level $< 0.05$) (Table S9). Additionally, roughness and contact angle also showed a high correlation ($r > 0.1$, significance level $< 0.05$) (Table S9). However, specific surface area and density had little even no correlation with carbon metabolism (Table S9). In addition, to better analyse the roles of substrate types and environmental factors in biofilm carbon metabolism, VPA analysis was performed to determine the contribution of substrate types and environmental factors (as classified in Table S9). As showed in Fig. 5b, the impact of environmental factors (contribution: 21%) was much greater than that of substrate types (contribution: 6%).

4. Discussion

We comprehensively compared the functional diversity of biofilms on plastic and natural substrates in river and lake ecosystems based on the BIOLOG EcoPlate. We demonstrated that different types of the plastic substrates could affect the diversity of the carbon metabolic functions of biofilms to some extent. More importantly, the environmental factors had more significant effects on carbon metabolic than the different types of the plastic substrates. These results indicated that plastic, a relatively new anthropogenic substrate (Ogonowski et al., 2018), would change the mechanism of biofilm colonization so that it could better adapt to the local water environments, which might have a certain impact on the carbon cycle of freshwater ecosystems.

4.1. Effects of substrate types (plastic and natural) on the microbial functions of biofilms

According to Fahrenfeld (2019), the microbial assemblages significantly differed between PS and PE particles, which might be related to the different surface characteristics of the plastic debris. A similar study demonstrated that PS exhibited distinct bacterial community structures compared with both PE and PP samples collected in the Bay of Brest (Frère et al., 2018). Additionally, recent research found that the bacterial communities developing on PVC and other plastics (PE and PP) clustered in two groups (Pinto et al., 2019). In our study, the biofilms cultured on PVC had a lower comprehensive ability to utilize carbon sources than biofilms on PE and cobblestone except in the Niushoushan River as expressed by the stability value of the AWCD. Therefore, plastic substrates could impact the community structures of biofilms, and then affect the metabolic functions of the biofilms. In the present study, under the same environmental conditions, different substrate types had different preferences for the five types carbon sources, thus indicating the differentiation of carbon metabolic functions of biofilms. However, there was no significant difference in the utilization of polymers in the four aquatic ecosystems regardless of substrate types. This result was consistent with previous studies (Lyons and Dobbs 2012) and illustrated that water-associated and aggregate-associated communities tend to metabolize polymers, which were the most representative carbon sources. Additionally, polymers have been reported as the most actively metabolized group in native ice samples (Grzesiak et al., 2015). These results suggested that the communities of aquatic organisms commonly utilized polymers, while functional discrepancies were observed among the utilization of other types of carbon substrates.

Our previous study demonstrated that the alpha diversity of natural substrate-associated communities was higher than that on plastic (PE and PP) substrates. In particular, distinct community structures and functions were exhibited between two substrate types (Miao et al., 2019b). Similarly, we found that the biofilm samples from the natural substrate (cobblestone) expressed the highest values that were significantly different from those on the plastic substrates (PVC and PE) in Donghu Lake as illustrated by the Shannon-Wiener diversity index and Simpson diversity index (Table 2). In fact, the physicochemical properties of substrates, including surface roughness, hydrophobicity and specific surface area can affect microbial colonization and formation (Frère et al., 2018; Liu et al., 2020; Ogonowski et al., 2018). In the present study, contact angle showed a high correlation with carbon metabolism ($r = 0.1031$, significance level $= 0.028$) (Table S9). In addition, the growth and development of biofilms might be affected by the roughness and texture of the substrate surface, because roughness and irregularly shaped surfaces could provide more biofilms attachment points (Fahrenfeld 2019). In our study, the surface roughness had a significant effect on the differentiation of carbon metabolic functions ($r = 0.2685$, significance level $= 0.001$) (Table S9). These results together with our findings indicated that the substrate types (plastic and natural substrates) could affect the function diversity of biofilms in a specific aquatic environment, thereby further impacting the material cycle in freshwater ecosystems (Arias-Andres et al., 2018; Gryta et al., 2014).

4.2. Effects of environmental factors on the microbial functions of biofilms

Microbes reacted rapidly to the environmental changes via changes in the community structure and microbial functions (Gryta et al., 2014). In addition, the formation of biofilms on substrates by microbial aggregation was the result of environmental selection and adaptation, which was observed in hasher environments with stronger species sorting (Ogonowski et al., 2018). According to Kettnner et al., (2017), the ambient water conditions, i.e., local environmental parameters and source communities, shaped fungal aggregation patterns on wood, PE and PS, indicating the possible habitat and location specificity of fungi. In the present study, the functional differences in biofilms in the different ecosystems were consistent with the clustering results of environmental conditions. Accordingly, different environmental factors might affect the specificity of biofilm communities and functions. No significant differences were observed in the carbon metabolism between natural and plastic substrates in freshwater ecosystems except in the Qinhuai River. Additionally, significant differences in the physiological properties between natural aggregates in water and microbial communities attached to microplastics were observed in three lakes with different nutritional states (Arias-Andres et al., 2018). Thus, plastic, as a new-type carrier of microbial communities, does influence the assemblage of microbes, although the ambient environment might have a dominant effect on the biofilm communities on different substrates.

Additionally, our results indicated that biofilms cultured in river ecosystems mostly had a higher carbon metabolism rate and shorter adaptation time than those cultured in lake ecosystems, suggesting that different metabolic patterns of biofilms might due to the comprehensive effects of different environmental factors. Simultaneously, the biomass
of biofilms cultivated in mountain areas was lower than that of biofilms collected in agricultural and urban areas because of the different nutrient inputs (Liao et al., 2018). In this study, biofilms on plastic in East Lake had the lowest organic matters compared with the three other freshwater ecosystems as illustrated by the values of the AFDW/DW, which might be related to the concentration of nutrients in water. Consequently, the formation, growth, and maturity of biofilms were the result of multiple environmental factors, thus affecting the microbial community structure and function diversity. A recent study illustrated that nutrients (TN and TP) and salinity were the main environmental factors affecting average growth rate of biofilm communities cultivated in the Haihe River Basin (Li et al., 2019). In the present study, the primary driving environmental factors of carbon metabolic functions of biofilm were TP, TN and SS, which had great influence on the biofilms cultured in Xuanwu Lake (Fig. 5a). More importantly, according to VPA analysis in our study, the impact of environmental factors (Contribution: 21%) was much greater than that of substrate types (Contribution: 6%) (Fig. 5b). Therefore, the significant influence of environmental factors on microbial carbon metabolic functions illustrated that comprehensive water environment assessment was of great significance for further study on functional properties of the plastisphere.

5. Conclusions

Although the community structures of biofilms on plastic and natural substrates have attracted extensive research attention, their functional characteristics must be studied, especially their impacts on the carbon cycle in freshwater ecosystems. In this study, results showed that the carbon metabolism of biofilms colonized on plastics (PVC and PE) in a single freshwater ecosystem were different from those on the natural substrate (cobblestone), indicating that the plastisphere had a significant impact on the carbon cycle in the aquatic environments. In addition, results from VPA suggested the effect of environmental factors on carbon metabolism of biofilms was more significant than that of substrate types. Overall, our findings suggested that plastics serving as artificial microbial niches have the ability to affect the carbon cycling process in aquatic ecosystems. Due to the complex nature of the in situ aquatic environments, additional studies are required to include more environmental factors and the dynamic changes of microbial functions of the plastisphere should be studied to further expand our understanding of plastic-biofilm functional relationship.

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 A. Supplementary data

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