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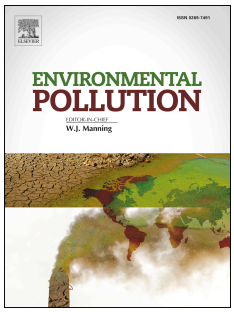

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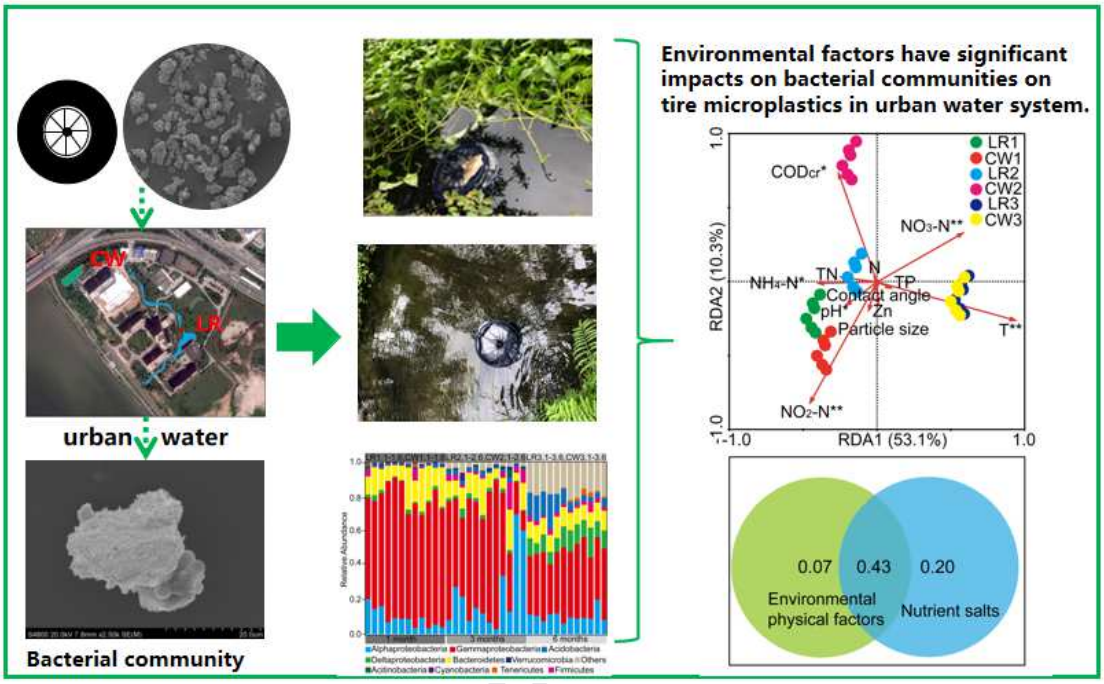

Bacterial community colonization on tire microplastics in typical urban water environments and associated impacting factors

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Abstract: Only limited information is available on bacterial communities' dynamics on tire microplastics in urban water environments. This study exploited 16S rDNA high-throughput sequencing to characterize bacterial communities on tire microplastics, using three different tire brands and tire sizes, in two typical urban water environments, including an influent pond of constructed wetland (CW) and its subsequent effluent into a landscape river (LR) during three different periods, namely, 1 month, 3 and 6 months. Results showed that the abundance of bacterial colonization on tire microplastics will increase over time. Proteobacteria, Bacteroidetes were the dominant bacteria at a phylum level, although they exhibited dynamic changes. At a genus level, the identifiable bacteria found in tire microplastics was generally the common bacteria in wastewater discharge, such as *Aquabacterium* and *Denitratisoma*. Additionally, alpha

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diversity showed no significant differences in bacterial communities at the same locations. While beta diversity showed that the bacterial communities on the tire microplastics in the two locations was different. BugBase revealed that tire microplastics could support pathogenic bacteria in urban water environments. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) indicated that the abundance of microorganisms associated with metabolism and degradation increased with time. Moreover, the ambient environmental factors were the main influencing factors of bacterial communities on tire microplastics. Herein, the contribution rate of nutrient salts (NO_2-N, NO_3-N, NH_4-N, CODcr) was approximately 63%, and that of environmental physical factors of T and pH was 50%. While physicochemical factors, including particle size, contact angle, element content only had a slight impact. Accordingly, tire microplastics, as an emerging environmental pollutant, can act as carries for bacterial colonization and propagation, particularly harmful microorganisms. Therefore, the obtained findings can provide new insight into potential risks of harmful microorganisms that colonize tire microplastics in urban water environments.

Keywords: microplastics; tire; bacterial community; colonization; biofilm

Declaration: We declare no conflict of interest.

Capsule: Our findings can pave the way for understanding bacterial dynamics on tire microplastics in urban water environments.

1. Introduction

The increasing attention being paid to microplastic pollution is the result of the increasing use

of plastic-based products. It is estimated that 83 billion tons of plastic were produced between 1950 and 2017 (Brooks, 2018). The intense consumption and rapid disposal of plastic led to a visible accumulation of plastic fragments (Cozar et al., 2014). In 2004, Thompson et al. (2004) first called the plastic fragments observed under microscope as microplastics. With in-depth research and discussion, microplastics are typically defined as plastic fibers, solid particles or films with an upper limit of 5 mm (Thompson, et al., 2004; GESAMP, 2016).

Although microplastic pollution around the world has been widely concerned, there are relatively few studies on microplastics in freshwater environments, which is reported that less than 4% of the researches on microplastics are related to freshwater (Lambert and Wagner, 2018). The first study about microplastics in freshwater environment was published until 2005 (Moore et al., 2005). In recent years, an increasing number of studies have found that microplastics are ubiquitous in freshwater environment, especially in estuaries and inland waters located in populated urban areas (Fu et al., 2019), and also along the shores of sparsely populated mountain lakes (Free et al., 2014). The average abundance of microplastics in freshwater system varies greatly from few to several million per cubic meter (Li et al., 2018). The wastewater treatment is one of the main sources of microplastics in freshwater (Li et al., 2018), which has also attracted wide attention (Wong et al., 2020).

Being a relatively new material introduced into water environments, microplastics can easily become a microbial carrier due to their small particle size, rough surface and longer half-life (Reisser et al., 2014; Oberbeckmann et al., 2015; Virsek et al., 2017; Gong, et al., 2019). Moreover, their hydrophobic surface can rapidly stimulate the formation of biofilm and hence become carriers for the colonization and transportation of harmful microorganisms (Zettler et al., 2013).

Previously, the rapid formation of bacterial biofilms was observed on microplastic surfaces within 1–2 weeks in an aquatic environment (Tender et al., 2017). Particularly, *Aeromonas salmonicida*, a pathogenic fish bacterium, was found in bacterial communities on the surface of microplastics in the North Adriatic, indicating that microplastics have become an important means in transmitting bacteria during fish feeding activities (Virsek et al., 2017). Additionally, microplastic surfaces provide a protective niche that supports a variety of different microorganisms, referred to as a "plastisphere" (Zettler et al., 2013). This biotope can be used as an important carrier for the persistence and transmission of pathogens, fecal indicator organisms and harmful algal blooms within aquatic environments (Keswani et al., 2016). The impacts of microplastics as microbial carrier is similar in seawater and freshwater environment (Li et al., 2018). Bacterial biofilm experiments conducted on microplastics and natural matrices in Xuanwu Lake (Nanjing, China) have found that the bacterial richness of microplastic matrix surfaces is much higher than that of natural matrices. Particularly, microplastic biogeochemical processes can potentially have a comparably additional and significant impact (Miao et al., 2018), which can be associated with carbon (C) and nitrogen (N) cycling processes of biofilm on microplastics. Therefore, understanding bacterial dynamics on microplastics in water environments is of critical importance.

Tire microplastics are considered an important microplastic, the second largest contributor of microplastics to aquatic environments on a global scale (Verschoor, 2015; Boucher et al., 2017). Automotive tires are known to be mainly composed of approximately 60% styrene-1.3 butadiene rubber (SBR), natural rubber as well as numerous additives, all of which derive from synthetic rubber (Verschoor et al., 2014). Thus, tire microplastics mainly derive from tires used on roads even though tires are made to be highly wear-resistant. It is estimated that the quantity of tire

88 microplastics produced in the European Union (EU) is 1 327 000 tons/year, while the
89 corresponding tonnage in the United States of America is 1 120 000 tons/year and that of Germany
90 alone is 133 000 tons/year (Wagner et al., 2018). In Australia, the total amount of tire wear was
91 calculated to be 20,000 tons/year (Kole et al., 2017). The per capita emissions of tire microplastics
92 range from 0.23 to 4.7 kg/year, with a global average of 0.81 kg/year (Kole et al., 2017). Under
93 forces of wind or gravity, some of these tire microplastic particles fly into the air and some
94 directly fall onto road surfaces and the surrounding soil, subsequently flowing into sewers or
95 surface water along with rainwater where they finally enter river and ocean systems as well as
96 other water environments (Ziajahromi et al., 2020). It has been demonstrated that approximately
97 18% of tire microplastics in the Seine, France, is imported into freshwater environments and 2% is
98 imported into the estuary of the river (Unice et al., 2019). What's more, the tire microplastics
99 (approximately 17.1%) were one of the most abundant microplastic particle types observed in the
100 tributaries of the Charleston Harbor estuary, South Carolina, the United States of America (Leads
101 and Weinstein, 2019). And approximately 15-38% of the tire microplastics were supposed to exist
102 in the sediment of the wetlands on Queensland's Gold coast (Ziajahromi et al., 2020). As a result,
103 these tire microplastics can become the potential carriers of microbial colonization in water
104 environments, which is risky to human health given that it is transmitted globally through the food
105 chain or through the movement of ocean currents (Yang, 2015; Bouwmeester, 2015; Seltenrich,
106 2015). Moreover, urban water environments are one of the main discharge hotspots of tire
107 microplastics, while also being freshwater environments that have a close association with human
108 beings during urbanization processes (Hu et al., 2018). Therefore, tire microplastics as bacterial
109 carriers and their associative influencing factors in urban water environments warrant far greater

attention.

In this study, we hypothesized that as an important source of microplastics, tire microplastics may become the carriers of bacteria in urban water environment, and the composition and structure of bacterial communities will change with time, and then the functional diversity of bacterial communities will change, which may have an unpredictable impact on urban water environment ecology. Concurrently, we also hypothesized that different water factors and different physicochemical properties of tire microplastics may affect the bacterial communities, which will make the risk of tire microplastics discharged to different water environment more difficult to predict. Accordingly, the objectives of this study were (1) to identify bacterial communities on tire microplastics over time in two typical urban water systems; (2) to evaluate the functional diversity of bacterial communities that colonize tire microplastics; and (3) to determine influencing factors of bacterial communities on tire microplastics, including their ambient water quality in different site and different periods and their specific physicochemical properties. Accordingly, we selected three popular tire brands, namely, Bridgestone, Goodyear and Michelin, as well as mixed tire microplastics of different sizes to act as our model tire microplastic. Following this, we added our series of prepared samples taken from the selected microplastics into two typical urban water systems, namely, an influent pond of constructed wetland (CW) and its subsequent effluent into a landscape river (LR) during three consecutive time periods, that is, 1 month, 3 and 6 months. This study exploited 16S rDNA high-throughput sequencing and associative statistical methods to characterize bacterial communities on tire microplastics and its corresponding influencing factors. The obtained findings from this first study of bacterial community colonization on tire microplastics can provide new insight into the potential ecological risks of microplastics in urban

environmental water systems.

2. Materials and Methods

2.1. Tire microplastics preparation

We obtained tire powders from the three aforementioned tire brands using a metal saw to scalp the surficial 2 cm (approximately) of each tire tread and using a tire grinding machine. At the same time, we purchased mixed tire powders from the ShiJiazhuang Yuxin Building materials company, China. Both tire powders were individually cleaned two separate times for 10 min using an ultrasonic cleaner at 68 Hz. After 3 days of freeze drying, tire powders were pulverized at 60, 515 g (26 000 rpm) for 6 min to prepare the different tire microplastics along with the different tire brands, namely, BRIDGESTONE (BS), GOODYEAR (GY) and MICHELIN (MC), and the mixed samples (Table S1). After cooling, tire microplastic samples were individually sieved through 75, 100 and 150 μm to obtain different particle sizes of the three different brands and mixed samples (Table S1), which were stored in a desiccator for further analysis.

2.2. Physicochemical properties of tire microplastics

The average particle size of tire microplastics was measured within an ethanol absolute (AR) solvent using a submicron/micron (laser) particle size/particle number analyzer (MS2000, Malvern, UK). The contact angle was measured by using an optical video contact tester (KRUSS-DSA100). The specific surface area of tire microplastics was measured using a fully automatic surface area, microporous pore and chemical adsorption instrument (ASAP 2020M+C, USA). The surface morphology of tire microplastics was observed by the field emission scanning electron microscope (S-4800, Hitachi, Japan). The relevant content of C, N and sulfur (S) was

154 measured using an elemental analyzer (vario MAX, Elementar Analysensysteme GmbH,
155 Germany). Prepared samples were further digested using the acid digestion method to measure
156 heavy metal content using inductively coupled plasma mass spectrometry (ICP-MS, Agilent
157 7500cx, CA, USA).

158 **2.3. Placement of tire microplastics into two typical urban water systems**

159 Approximately 0.2 g of the different series of prepared tire microplastics were weighed out
160 and placed into hollow quartz sand glass tubes of approximately 8 cm length and 2 cm diameter.
161 Both sides of the tubes were covered with an 8 cm diameter and 5 μm aperture glass fiber filter
162 membrane, which were fixed using the rubber bands, and then the outer wall of the glass tubes
163 ware wrapped with the sealing films with a length of about 5 cm. This approach sealed tire
164 microplastics in place while allowing microbes and water to pass through freely. All tubed
165 samples were then packed into net bags to place into the LR and the CW treatment locations for 1
166 (December 6th, 2018 to January 6th, 2019), 3 (December 6th, 2018 to March 6th, 2019) and 6
167 months (December 6th, 2018 to June 6th, 2019), respectively. The LR and CW treatments were
168 situated within the campus of the Institute of Urban Environment, Chinese Academy of Sciences
169 (Fig. S1). Specifically, CW influent derived from discharge from a wastewater treatment plant
170 within the campus. Therefore, as typical urban water systems, the selected sampling sites of CW
171 and LR could to some extent represent the situations of urban water environments, which could
172 favor to demonstrate clearly the ecological differences of bacterial communities and their
173 potentially negative effects resulted from tire microplastics in urban water systems. For accuracy,
174 bacterial samples in the two different study locations were labelled, namely, LR1 (1 month), LR2
175 (3 months) and LR3 (6 months) for LR and CW1 (1 month), CW2 (3 months) and CW3 (6 months)

for CW. As shown in Table S1, a sample number from 1 through 6 was further assigned to BS, GY, MC and the three different sized tire microplastic mixtures (Table 1), respectively.

2.4. Pretreatment, DNA extraction, amplification, product recovery and 16S rDNA high-throughput sequencing of samples

After 1, 3 and 6 months, respectively, the aforementioned tubes were retrieved and then tube surfaces were rinsed with sterilized water. Afterward, tubes were disassembled to access the tire microplastics for DNA extraction. The retrieved tire microplastics samples were poured into a 500 mL beaker covered with a 30 μm sterilized mesh gauze to filter out the water, and residue that had adhered to the inside of the glass tubes was rinsed using sterilized water. The mesh gauzes were then wrapped until only the tire microplastics samples left, and then they were placed into disposable Ziplock bags and stored at -80 °C for further DNA extraction.

Genomic DNA from all samples was extracted using the FastDNA SPIN Kit for Soil (Qbiogene, MP Biomedicals, Irvine, CA, USA). And the selected amplification regions included 16S V3–V4. Polymerase chain reaction (PCR) was performed using the Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs), a specific primer with barcodes and high-efficiency high-fidelity enzymes to ensure amplification efficiency and accuracy. Products were recovered using a gel recovery kit (GeneJET, Thermo Scientific), while the library was constructed using the 48 reaction Ion Plus Fragment Library Kit (Thermo Scientific). After qubit quantification and library testing, the Ion S5TMXL System (Thermo Scientific) was used for sequencing.

2.5. Data analysis

2.5.1. Data processing

198 Cutadapt (version 1.9.1; http://cutadapt.readthedocs.io/en/stable/) (Ward et al., 2017) was
199 used to partially cut reads of low quality, and barcode and primer sequences were truncated to
200 obtain raw data. Read sequences (https://github.com/torognes/vsearch/) (Martin, 2011) were
201 compared to the species annotation database to detect chimeric sequences which we subsequently
202 removed to obtain the final valid data (i.e., clean reads) (Rognes, 2016).

203 Clean reads from all samples were clustered using UPARSE software (UPARSE version
204 7.0.1001; http://www.drive5.com/uparse/) (Haas et al., 2011) at a 97% consistency default
205 (Identity) cluster sequence into operational taxonomic units (OTU). The mothur method and the
206 SSU rRNA database (Wang et al., 2007) from SILVA (release 132) (http://www.arb-silva.de/)
207 (Edgar et al., 2013) were used for species annotation analysis (with a set threshold value between
208 0.8–1), and taxonomic information was obtained at each taxonomic level: kingdom, phylum, class,
209 order, family, genus and species, which were used to calculate the community composition of each
210 sample.

211 **2.5.2 Diversity and bacterial functional prediction**

212 Samples were subjected to alpha diversity (α-diversity) analysis using QIIME software
213 (version 1.9.1) (Caporaso et al., 2010), including observed species, the Chao1 index, the Shannon
214 index and the Simpson. Petal chart based on OTUs of bacterial communities was analyzed by
215 “venn” package in R software (version 3. 6. 0). Beta diversity (β-diversity) analysis was
216 performed by Principal Co-ordinates Analysis (PCoA), which based on weighted UniFrac distance
217 matrix in terms of bacterial communities on tire microplastics, was generated using the “vegan”
218 package in R software (version 3. 6. 0) to visualize sample differences (Magali et al., 2013).
219 Linear discriminant analysis effect size (LEfSe) analysis was used to analyze species abundance

data between groups (i.e. LR and CW) applying the rank-sum test method and to detect different species between different groups (i.e. LR and CW). Linear discriminant analysis (LDA) was then used to reduce the dimension and evaluate its effect on species richness, using the LDA score to draw the histogram of the LDA value distribution of the different species, wherein the default setting was 4. The t-test and the Wilcox signed-rank test were used to analyze differences between the diversity indices.

BugBase uses an OTU table (with reference clustering and reference sequence: the Greengenes 97% OTU dataset) as the input file. First, the predicted 16S copy number is used to normalize the OTU table. The preprocessed database and the BugBase tool are then used to automatically select thresholds to predict bacterial phenotypes. Based on the tree of OTU and the gene information of OTU in Greengene database, PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to predict the metabolic function of bacterial communities. Finally, a mantel test was used to explore the relationship between the taxonomic and functional structures of bacterial communities on tire microplastics with 9999 permutations by “ade4” package in R software (version 3. 6. 0).

2.5.3 Correlation analysis between bacterial communities and physicochemical properties and environmental factors

Furthermore, we collected 100 mL of water samples from the two sites (i.e., LR and CW) after 1, 3 and 6 months (i.e., January 6th, 2019, March 6th, 2019, June 6th ,2019, respectively). The water temperature (T) is detected by the thermometer in the water immediately. The pH value of water was measured using a pH meter. Ammonia nitrogen (NH_4-N), nitrate nitrogen (NO_X-N) and phosphate (PO_4-P) concentrations were determined using flow injection analysis (Lachat QC8500,

USA). Total phosphorus (TP) was determined by spectrophotometry. Anions were determined using an ion mass spectrometer (ICS-3000, USA). Total nitrogen (TN) was determined using a TOC/TNVC pH analyzer (Shimadzu, Kyoto, Japan). The content of the chemical oxygen demand (COD) was determined using the potassium dichromate method. As it pertains to statistical analysis, different durations (i.e., 1, 3 and 6 months) were evaluated using a t-test at a significant level of $P < 0.05$. Furthermore, relationships between bacterial communities on tire microplastics and their specific physiochemical properties and environmental factors were analyzed by redundancy analysis (RDA) using Canoco version 5.0 (Dang et al., 2010). Variation partitioning analysis (VPA) for determination of the contributions of different environmental factors to the variations of bacterial communities were conducted in R with the package "vegan".

3. Results and Discussion

3.1. Physiochemical properties of tire microplastics

The average size of tire microplastics among the three brands differed, namely, 120 µm for BS, 136 µm for GY and 102 µm for MC (Table 1). At the same time, the average size of the mixed tire microplastics also differed, namely, 132 µm for MIX-1, 94 µm for MIX-2 and 71 µm for MIX-3. Furthermore, the specific surface area of tire microplastics was small (Table 1), which is comparable to other microplastics, including polyethylene (PE) and polypropylene (PP) (e.g., Pang, 2018). Additionally, the contact angle of tire microplastics among the different brands or the mixed tire microplastics of different particle sizes exhibited no significant differences ($\theta > 120°$) ($P > 0.05$; Table 1), which implied that tire microplastics had higher hydrophobicity.

Table 2 provides the elemental constituents of several tire microplastics. The table shows that

carbon (C), nitrogen (N), sulphur (S), copper (Cu), zinc (Zn), arsenic (As) and lead (Pb) content was similar among the three different tire brands and the mixed tire microplastics of three different particle sizes. Among these, C content was highest, and this was because carbon black is the main raw used material in tire production (Kim and Lee, 2018). Moreover, the vulcanization process was the main reason for high S content, while, being an additive, Zn content in tire microplastics was also high (Degaffe and Turner, 2011). Additionally, as potential additives, many heavy metals can be found in tire microplastics, such as Cu, As, Pb, etc.

The morphology of tire microplastics obtained under laboratory conditions in this study was similar to that collected from roads, all having typical slender shapes (Kreider et al., 2010). Furthermore, we observed extensive microbial colonization in both the LR and CW treatments (Fig. 1). We inferred that microbial secretions that adhere to the surface of tire microplastics can alter their surficial morphology by increasing their roughness (Zettler et al., 2013).

3.2. Bacterial community composition and structure

Results from 16S rDNA high-throughput sequencing showed that bacterial richness after 1 month (976 OTUs) was significantly lower than after 3 months (2685 OTUs) and 6 months (3941 OTUs) ($P < 0.01$; Fig. S2). Bacterial communities on the three-stage tire microplastics shared a number of OTUs: 62, and about 30% of OTUs were shared in different locations in each stage, though the unique OTUs increased with time. Therefore, bacterial communities on the tire microplastics in different locations also appeared to have a “core” of taxa that characterized them as mentioned in the study of Zettler et al. (2013).

At a phylum level (Fig. 2), the dominant bacteria on tire microplastics after 1 month was identical to that after 3 months, namely, Proteobacteria, Bacteroidetes. These results were

consistent with the dominant bacteria on polyethylene (PE) and polypropylene (PP) in a freshwater system reported by Miao et al. (2018). Moreover, these dominant bacteria are typically present during the primary stage of biofilm formation as well as being typical bacteria found in freshwater environments (Newton et al., 2011; Hoellein et al., 2014). While after 6 months, the biofilm entered a mature population state, which was characterized by a greater richness of bacteria. Acidobacteria had become one of the dominant bacteria, which was consistent with long-term structure and diversity of a biofilm formed in a model drinking water system (Martiny et al., 2003). As reported, surfaces exposed to water can adsorb extensive amounts of organic nutrients within a few hours, and this so-called “conditioning” film can immediately attract microbial colonizers that utilize these adsorbed nutrients (Oberbeckmann et al., 2015). Hence, the formation of biofilm on microplastic surfaces in water occurs rapidly, generally within 24 h (Oberbeckmann et al., 2015). Consequently, typical primary colonizers are invariably *Gammaproteobacteria* and *Alphaproteobacteria* (initial phase 0–24 h) (Oberbeckmann et al., 2015). Over time (24–72 h), the abundance of members of the phylum Bacteroidetes increased in collective bacterial communities in water (Dang et al., 2000). As biofilm matured (after approximately 2 weeks), one study found that the relative abundance of *Alphaproteobacteria* decreased, whereas that of Bacteroidetes increased (Elifantz et al., 2013). In a 3-week culture study conducted by Miao et al. (2018), Proteobacteria remained the dominant phylum in all collected biofilm samples, followed by Bacteroidetes. Our results showed that the relative abundance of Proteobacteria and Bacteroidetes decreased from 1 month to 6 months, during which time Acidobacteria increased. Collectively, the richness of bacteria on biofilms that formed by these three periods increased. Moreover, Proteobacteria was the dominant bacteria in biofilm

irrespective of time, mainly *Gammaproteobacteria* and *Alphaproteobacteria*, followed by Bacteroidetes, although all of them exhibited dynamic changes. Longer bacterial culture studies on tire microplastics in the natural world are necessary since data associated with dominant bacteria under such conditions remain limited.

At a genus level, the relative abundance of identifiable bacteria on tire microplastics increased from 1 month to 3 months, while decreased at 6 months ($P < 0.05$; Fig. S3). After 1 month, the relative abundance of *Aquabacterium* and *Devosia* was highest in the LR treatment, while the relative abundance of *Sterolibacterium*, *Azospira*, *Cloacibacterium* and *Aquabacterium* was highest in the CW treatment (Fig. S3a). In contrast, the relative abundance of *Aquabacterium* in the CW treatment tended to increase after 3 months while *Bradyrhizobium* remained relatively high (Fig. S3b). After 6 months, the most abundance in all samples was *Denitratisoma*, next is *Geothrix* in LR treatment and *Thiobacillus* in CW treatment (Fig. S3c). Among these genera, *Aquabacterium* was found to be the most widespread species in drinking water and the dominant species on polyethylene (PP) biofilm in drinking water (Kalmbach et al., 1999). *Aquabacterium* was also identified as a dominant member in soil contaminated by hydrocarbons, which could assimilate C from benzene (C_6H_6) (Jechalke et al., 2013). Additionally, *Bradyrhizobium* is a bacterial genus capable of degrading methoxychlor (Satsuma et al., 2013). The genus *Devosia* can be found in soil, glaciers, dump sites, nitrifying inoculum, marine sediment and even on the surface of medical leech (Nor et al., 2017). *Cloacibacterium* belongs to the family *Flavobacteriaceae*, ubiquitous to aquatic habitats, for which they are generally thought to play a role in the breakdown of complex organic matter (Allen et al., 2006; Gay et al., 2016). At the same time, *Cloacibacterium* are also commonly found in activated sludge and other components

of wastewater treatment plants, which contribute directly to phosphate removal in activated sludge (Allen et al., 2006; Gay et al., 2016). Therefore, certain observable bacterium could be beneficial to the degradation/removal of pollutants in water, such as the plasticizers sebacate, azelate and adipates, which are used to varying degrees in microplastic production as well as several common pollutants, such as N and P (Kalmbach et al., 2000; Gay et al., 2016; Liu et al., 2018; Kleinteich et al., 2018). *Denitratisoma*, belongs to *Rhodocyclaceae* family, involved in ammonium-oxidizing and denitrification in wastewater treatment, the abundance greatly influenced by the water quality, which presented strong positive correlations with the influent effluent concentration of COD and ammonium nitrogen (Xu et al., 2017). In the groundwater remediation process, *Geothrix* is one of the important bacterial group, which is capable of acetate and ethanol degradation, mainly by Fe (III) reduction, as well as by denitrification (Cardenas et al., 2008). Collectively, these abovementioned bacteria can be also found in wastewater discharge. This confirms that the observable bacteria can to some extent represent the conditions of urban water environments, since the sampling sites investigated in this study are one of typical urban waters where are the discharge sites of wastewater treatment plants. Furthermore, these observable bacteria can rapidly accumulate on tire microplastics and then migrate in the urban water environments, acting as a potential environmental pollutant and putting aquatic organisms and human at risk (Smith et al., 2018).

3.3. Diversity analysis

This study used OTU-based α-diversity to analyze observable bacterial communities, applying the Observed species index, Chao1, Shannon and Simpson indices (Fig. S4). The observed species index and Chao 1 index showed that the average of species diversity on tire

351 microplastics tended to be higher in the LR treatment compared to the CW treatment after 1 and 3
352 months, but after 6 months, the average of species diversity in LR treatment is considerably lower
353 than CW treatment ($P < 0.05$). The Shannon index and Simpson index revealed that the index
354 values of the LR treatment were relatively smaller than those of the CW treatment after 1 month
355 and 6 months, indicating that species diversity and bacterial community richness of the LR
356 treatment were also smaller, but the opposite effect was seen after 3 months. However, in general,
357 the *P* value of the four α-diversity indices was greater than 0.05; thus, there were no significant
358 differences between the different locations (i.e., LR and CW).

359 This study used PCoA based on weighted UniFrac distance matrix in terms of bacterial
360 communities on tire microplastics (OTU level) for β-diversity analysis. PCoA showed that
361 samples collected in the same area were more aggregated, and their corresponding bacterial
362 composition was more similar (Fig. 3). After 1 month and 6 months, samples were obviously
363 clustered into two groups (LR and CW), the bacterial communities on the tire microplastics in LR
364 were significantly different with those in CW. After 3 months, the difference between LR bacterial
365 communities and CW decreased, but the difference was still significant. These two groups were
366 significantly different as conformed by the Adonis ($P<0.01$) and Anosim analyses ($P<0.01$)
367 (TableS2). Moreover, samples from the same periods were also clearly distinguished (Fig. S5). At
368 the same time, differences in bacterial communities on tire microplastics between the two different
369 urban sites (LR and CW) tended to decrease firstly and then increase over time (Fig. S6).
370 Specifically, we found 13 different types of biomarker in the LR and the CW after 1 month,
371 namely, *Alphaproteobacteria*, *Rhizobiaceae*, *Sterolibacterium*, *Burkholderiaceae*,
372 *Sediminibacterium*, *Cloacibacterium*, etc. (Fig. S6a); While we found 11 different types of

biomarker in the LR and the CW after 3 months, namely, *Denitratisoma, Chitinophagaceae, gamma_proteobacterium, Sediminibacterium, Aquabacterium, Oceanospirillales*, etc. (Fig. S6b). And after 6 months, 18 different types of biomarker were found in LR and CW treatment, including *Thiobacillus, Denitratisoma, Hydrogenophilaceae, Burkholderiaceae, Holophagaceae, Holophagales*, etc. (Fig. S6c). This decrease in tendency related to differences in bacterial communities can be attributed to the similarity of bacterial communities in primary stage under similar urban water environments, and differences may to a certain extent have become less evident as the number of colonized bacterial species increased (Hu et al., 2018). However, with the increase of time, biofilm entered a mature population state, differences in bacterial communities become apparent. Additionally, the temperature after six months (June) is much higher than that in the first two periods (January and March) (Table S3). Therefore, it can also be inferred that the temperature may affect the community composition on the tire microplastics in different locations.

3.4. Potential Functional Consequences

The abundance of potential pathogenic bacteria was determined using the BugBase tool for functional prediction, which confirmed that tire microplastics in urban water environments can act as carriers of pathogenic bacteria. In this study (Fig. S7), in general, the relative abundance of potential pathogens on tire microplastics had tended to increase with time in the urban water treatments. Specifically, during the first two periods, both the relative abundance of pathogenic bacteria in LR treatment were comparable to those in CW treatment, but after 6 months, the former was significantly lower than the latter ($P<0.05$; Fig. S7). Additionally, there is no obvious trend of potential pathogenic relative abundance in different brands of tire microplastics, while the

relative abundance in the same treatment seems to be related to the particle size (Fig. 4). As the relative abundance of the tire microplastics with large particle size were much bigger, we inferred that pathogenic bacteria appear to favor the larger size. In these three periods, the pathogenic bacterial relative abundance in different treatment of tire microplastic sizes were relatively high. Accordingly, our study found that tire microplastics provided colonization carriers for potential pathogens, although their abundance varies dynamically over time, and the relative abundance differed in different tire brands and tire microplastic sizes.

Bacterial community-based biofilm formation on tire microplastics can serve as a protective mechanism given that microorganisms grown in these matrix-enclosed aggregates are more resistant to antibiotics and host defenses (Hall-Stoodley et al., 2004). Therefore, the carrier role that tire microplastics play is conducive to long-distance pathogenic bacteria transport in water environments, subsequently increasing its potential ecological risks. There is considerable evidence that shows that aquatic organizms intake microplastics (Avivo et al., 2015; Farrell and Nelson, 2013), which allows for the ingestion of colonized microbes, particularly pathogenic bacteria. A previous study has shown that the pathogenic fish bacterium *Aeromonas Salmonicida* was found on microplastics in the North Adriatic (Virsek et al., 2017). Both LR and CW are typical and important urban aquatic habitats. At the same time, tire microplastics are transported within urban water systems and subsequently ingested by aquatic organisms. This will not only put aquatic organisms at risk, but may also threaten human health through the food chain (Kole et al., 2017). Accordingly, tire microplastics that enter urban water environments are a unique bacterial habitat that could potentially act as a new carrier to transport bacteria downstream (McCormick et al., 2014).

Furthermore, PICRUSt program was used to predict and analyze the functional genes. Based on the prediction results of KEGG database (Kyoto Encyclopedia of genes and genes), six kinds of functional analysis of biological metabolic pathways were obtained at the first level: metabolism, genetic information processing, environmental information processing, cellular processes, organic systems and human diseases. Among them, metabolism, genetic information processing and environmental information processing were the main components, accounting for approximately 49.19%, 14.92%, 14.71%, respectively, which was consistent with the results of Jing et al. (2019). Heatmap of top 20 functional gene prediction (hierarchy level 2) (Fig. S8) indicated that several predicted pathways were significantly enriched ($P < 0.05$) after 3 and 6 months' bacterial communities compared with those after 1 month, especially those genes associated with genetic information processing (e.g., transcription, folding, sorting and degradation). Genes associated with metabolism (e.g., amino acid, lipid, carbohydrate metabolism and biosynthesis of other secondary metabolites) also enriched. In a word, the content of functional microorganisms on the tire microplastics changed with time, the abundance of microorganisms associated with metabolism and degradation increased, and the functional genes of bacterial communities on the tire microplastics of LR and CW were also different. Additionally, PCoA indicated that functional structure of samples in the different area were obvious distinguished, as well as in the different periods (Fig. S9). Although a Mantel test demonstrated that the functional structure was highly correlated with the taxonomic structure ($r = 0.629$, $P< 0.001$).

3.5. Relationship analysis

Results from RDA showed that physiochemical properties of tire microplastics and surrounding environmental factors that affect bacterial community colonization on tire

439 microplastics impacted the two typical urban water systems during three consecutive time periods
440 investigated (Fig. 5). RDA results could explain 53.1% and 10.3% in X and Y axes, respectively. It
441 can be found from Fig. 5 that bacterial growth on tire microplastics exhibited a similar tendency at
442 the same place in each time period. However, certain water quality parameters intensified impacts
443 on bacterial communities, including T, NO_2-N, NH_4-N, NO_3-N, pH, COD_{cr}, TN, TP, the influence
444 of the first six factors were significant ($P < 0.05$). In the first period, the main influencing factors
445 for microorganisms on tire microplastics in the LR and CW treatments were available NO_2-N,
446 NH_4-N and pH. After 3 months, CODcr influenced bacterial communities, while after 6 months, T
447 and NO_3-N were the main influencing factors in LR and CW treatments. The results of VPA
448 showed that a total of 70% variance of bacterial communities could be explained by selected
449 variables (Fig.5). Among them, nutrient salts including NO_2-N, NO_3-N, NH_4-N, CODcr were the
450 most important factors, with a contribution rate of 63%, followed by environmental physical
451 factors (T and pH), with a contribution rate of 50%. There was a common correlation between the
452 two groups of factors, and the shared contribution rate was 43%. Even though the impact of
453 physiochemical properties of tire microplastics was insignificant ($P>0.05$), it cannot be ignored.
454 Owing to manufacturing processes among tire brands differ slightly, the constituents that comprise
455 their products also differ (Degaffe and Turner, 2011). Among these constituents, Zn content had a
456 slightly larger impact on bacterial communities for different brands of tire microplastics, while N
457 content had almost no effect. Skjolding et al. (2016) found that factors such as carrier particle size
458 and shape influence microbial communities. Moreover, the contact angle of tire microplastics has
459 a very slight effect, which may result from the reduction of the connection to the surface by
460 hydrophobic interactions due to the biofilm coating that formed on the surface of tire microplastics

in the primary stage (Harrison et al., 2018; Moraes et al., 2019). In our study, biofilm tended to be at mature stage, and bacterial communities on biofilms were mainly affected by environmental factors, while the impact of almost all physiochemical properties was slighter. Additionally, it should be noted that microplastics are more buoyant and durable than natural matrices, while the half-life of the former is longer than the latter (Zettler et al., 2013). These relevant tire microplastic properties provide a new microbial niche for microbial colonization and long-distance transportation (Keswani et al., 2016).

4. Conclusions

As we supposed, the obtained results showed that tire microplastics, as an emerging environmental pollutant, act as carries for bacterial colonization and propagation, particularly harmful microorganisms. As this new pollutant increasingly enters urban water systems, the abundance of bacterial colonization on tire microplastics will increase over time, especially for pathogenic bacteria, and the abundance of bacterial function associated with metabolism and degradation also increased with time, which could put aquatic organisms and potentially even human health at risk. Except for the main impact of environmental water factors, the smaller surface area, particle size, hydrophobic surface and longer half-life of tire microplastics can provide better colonization and propagation vectors for microbes, consequently increasing their ecological risks. Furthermore, bacterial communities residing on tire microplastics will undoubtedly be impacted by many other influencing factors given that urban water environments are inherently diverse. Practically, this study is limited by several factors associated with insufficient data, such as size dependency, the number of study sites and the duration of the study period. Accordingly, more relevant studies are therefore necessary to better understand the

formation of bacterial communities on tire microplastics as well as their associated changes with respect to physiochemical properties.

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Figure captions

Fig. 1. Scanning electron microscope (SEM) image of tire microplastics prior to the experiment (a) and following placement into the influent pond of constructed wetland (CW) for 3 months (b).

Fig. 2. Relative abundance of the bacterial communities at a phylum level (Proteobacteria is identified as the class level) on tire microplastics in the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months. The bacterial phyla out of the top 10 are included as Others.

Fig. 3. Principal Co-ordinates Analysis (PCoA) based on weighted UniFrac distance matrix in terms of bacterial communities on tire microplastics (OTU level) in the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months.

Fig. 4. Cylindrical map illustrating the potential pathogenic abundance of bacterial communities on tire microplastics in the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months, 1, 2, 3, 4, 5, 6 represent sample number

Fig. 5. Redundancy analysis (RDA) plot illustrating the relationship between environmental factors and bacterial communities on tire microplastics, and variation partitioning analysis (VPA) differentiating effects of environmental physical factors (T and pH) and nutrient salts (NO_2-N, NO_3-N, NH_4-N, CODcr) in the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months.

Fig. S1 The locations of the influent pond of constructed wetland (CW) and the landscape river (LR) and within the campus of the Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China.

Fig. S2 Petal chart based on OTUs of bacterial communities on tire microplastics for the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months. 37.2%, 29.8%, 32.9% represented the shared proportion of OTUs in LR1 and CW1, LR2 and CW2, LR3 and CW3, respectively.

Fig. S3 Relative abundance of bacterial communities at a genus level on tire microplastics for the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month (a), after 3 months (b) and 6 months (c). The bacterial phyla out of the top 10 are included as Others.

Fig. S4 Comparison between α-diversity indices of bacterial communities on tire microplastics for the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and after 6 months.

Fig. S5 Principal Co-ordinates Analysis (PCoA) based on weighted UniFrac distance matrix in terms of bacterial communities on tire microplastics (OTU level) in the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months.

Fig. S6 Linear discriminant analysis (LDA) value distribution histogram for different species of bacterial communities on tire microplastics after 1 month (a), after 3 months (b) and 6 months (c) for the landscape river (LR) and the influent pond of constructed wetland (CW).

Fig. S7 Cylindrical map illustrating the potential pathogenic abundance of bacterial communities on tire microplastics in the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months. * on the bars represents significant differences while compared to other groups ($P < 0.05$).

Fig. S8 Heatmap of functional gene prediction (hierarchy level 2) of bacterial communities on tire microplastics for the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and after 6 months.

Fig. S9 Principal Co-ordinates Analysis (PCoA) based on weighted UniFrac distance matrix in terms of bacterial communities on tire microplastics (Functional gene prediction) in the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months.

Figures

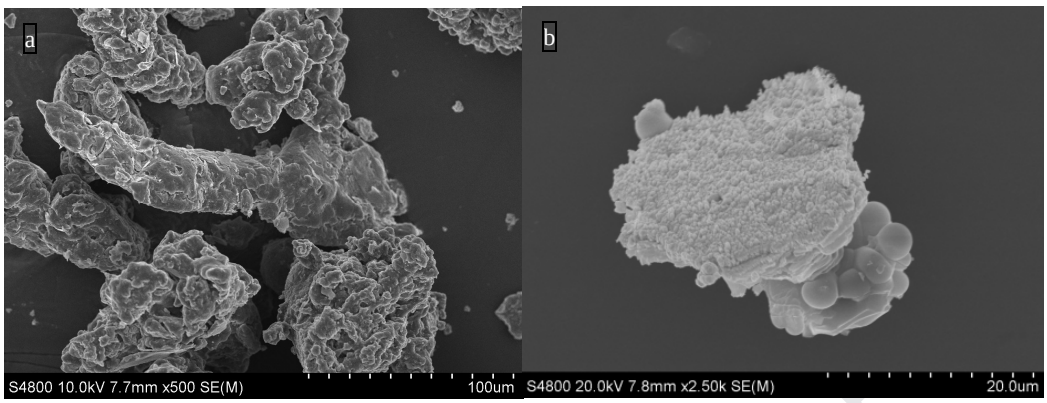

Fig. 1. Scanning electron microscope (SEM) image of tire microplastics prior to the experiment (a) and following placement into the influent pond of constructed wetland (CW) for 3 months (b).

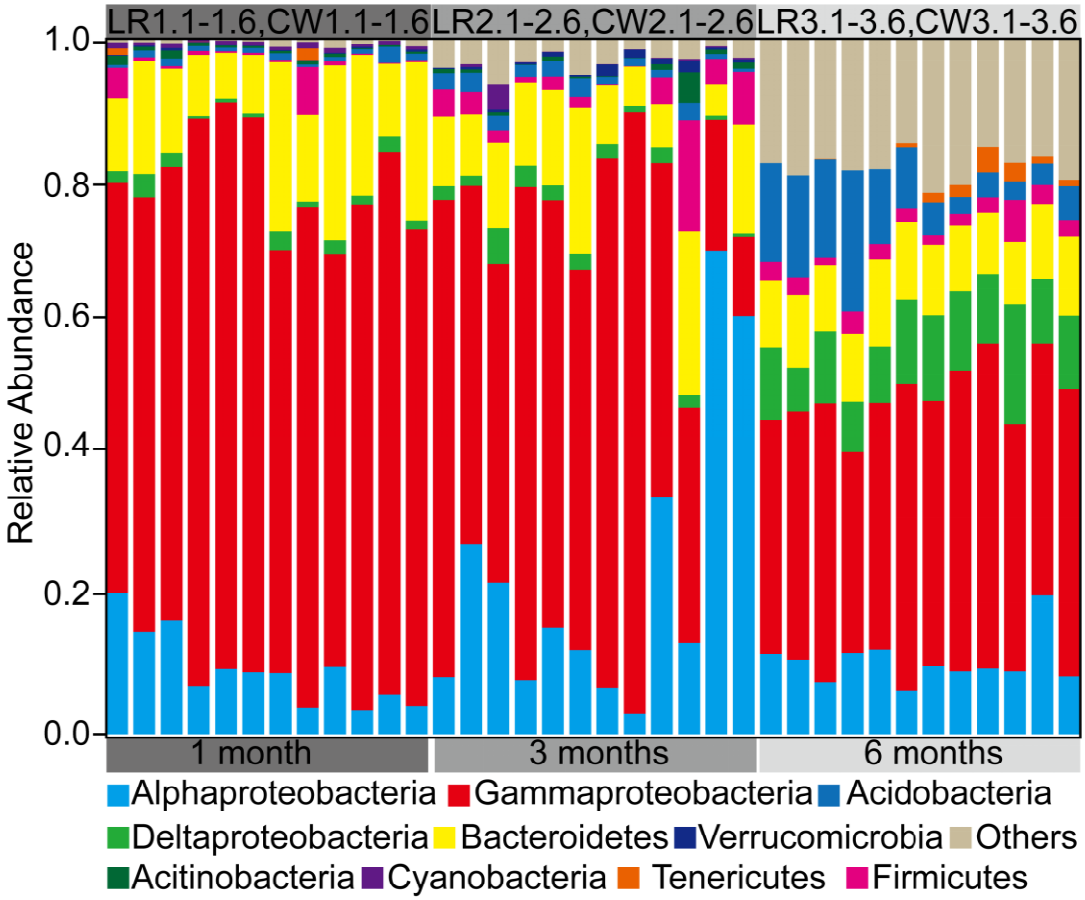

Fig. 2. Relative abundance of the bacterial communities at a phylum level (Proteobacteria is identified as the class level) on tire microplastics in the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months. The bacterial phyla out of the top 10 are included as Others.

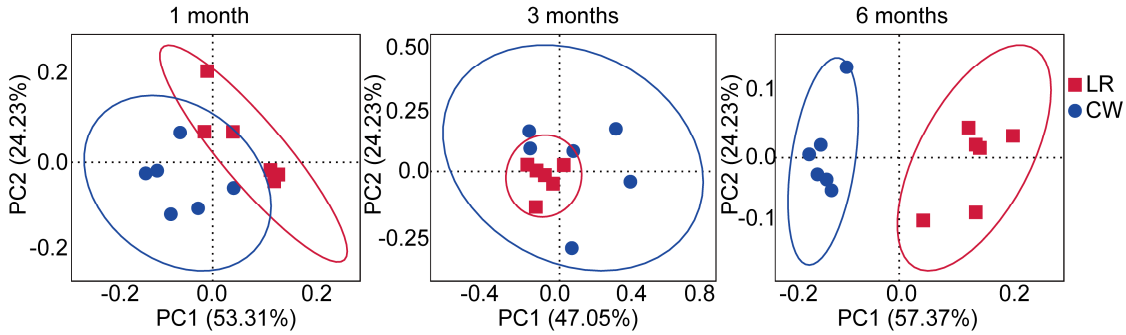

Fig. 3. Principal Co-ordinates Analysis (PCoA) based on weighted UniFrac distance matrix in terms of bacterial communities on tire microplastics (OTU level) in the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months.

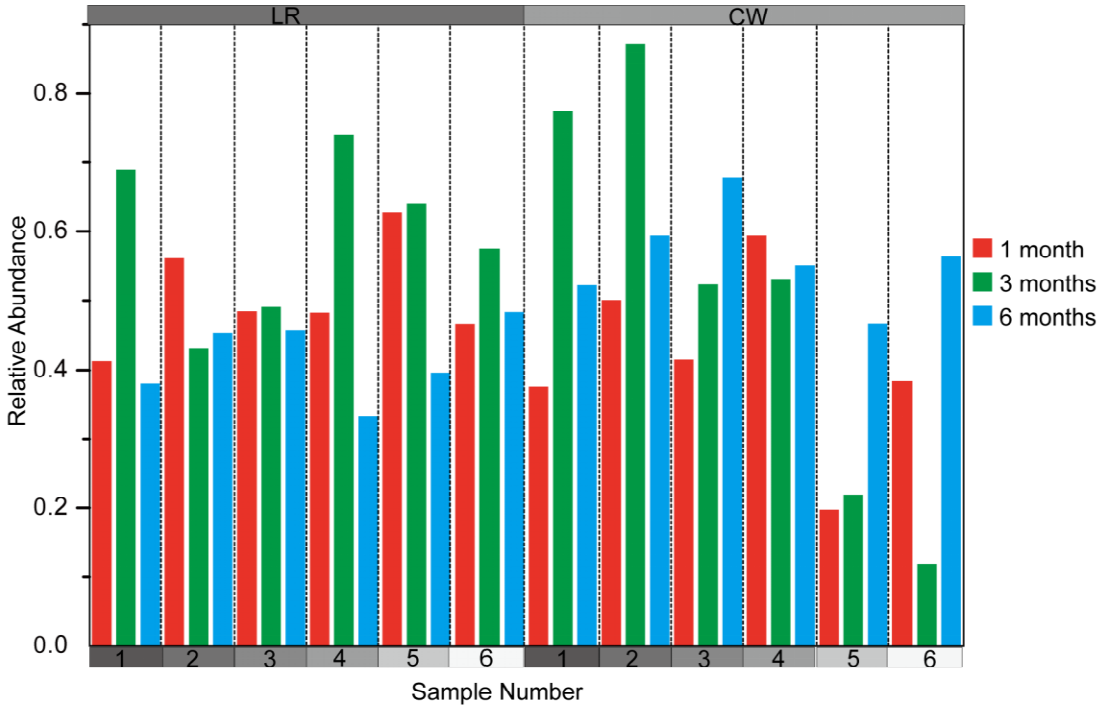

Fig. 4. Cylindrical map illustrating the potential pathogenic abundance of bacterial communities on tire microplastics in the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months, 1, 2, 3, 4, 5, 6 represent sample number.

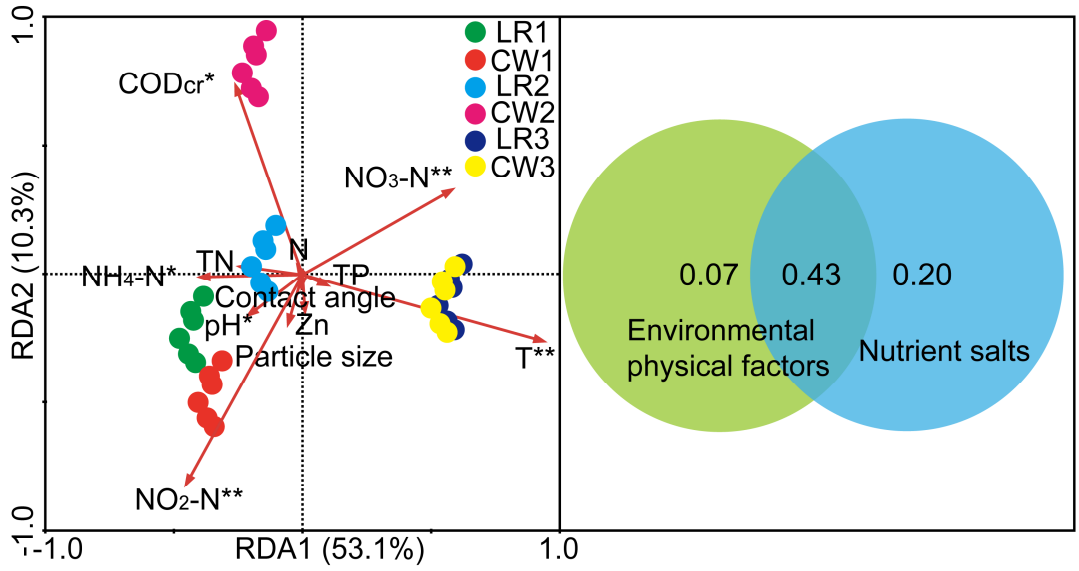

Fig. 5. Redundancy analysis (RDA) plot illustrating the relationship between environmental factors and bacterial communities on tire microplastics, and variation partitioning analysis (VPA) differentiating effects of environmental physical factors (T and pH) and nutrient salts (NO_2-N, NO_3-N, NH_4-N, CODcr) in the landscape river (LR) and the influent pond of constructed wetland (CW) after 1 month, after 3 months and 6 months.

Tables

Table 1 Specific surface area, average particle size and contact angle of three different tire brands and three different particle sizes of tire microplastics.

Sample name	BET surface (m^2/g)	Average particle size (μm)	Contact angle (θ)
BS	0.4657	120±0.5	135.43°
GY	0.1098	136±1.4	132.17°
MC	0.1566	102±0.9	138.17°
MIX-1	0.1463	132±1.7	130.67°
MIX-2	0.0931	94±3.1	128.70°
MIX-3	0.1636	71±2.5	129.73°

Table 2 The content of several elements found in three different brands and three different particle sizes of the mixed tire microplastics.

Sample Name	N (g/kg)	C (g/kg)	S (g/kg)	Zn (g/kg)	Cu (mg/kg)	As (mg/kg)	Pb (mg/kg)
BS	1.70	661.82	12.48	24.93	64.40	19.09	45.89
GY	3.50	676.65	12.06	40.90	34.25	17.02	60.02
MC	2.46	704.38	13.95	84.25	25.49	20.24	18.04
MIX-1	2.57	714.95	10.60	11.00	17.48	17.43	23.38
MIX-2	2.55	684.30	9.88	15.75	39.79	15.34	48.82
MIX-3	2.61	644.86	9.27	15.33	44.37	16.60	47.65

Highlights

- Tire microplastics (TMPs) supported pathogenic bacteria in urban water environment.
- The abundance of bacterial colonization on tire microplastics increased over time.
- Bacterial communities on TMPs varied in different urban water environment.
- Urban water factors have significant impacts on bacterial communities on TMPs.

Author Statement

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: