Contrasting diversity of epibiotic bacteria and surrounding bacterioplankton of a common submerged macrophyte, Potamogeton crispus, in freshwater lakes

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Received 20 June 2014; revised 31 July 2014; accepted 11 August 2014. Final version published online 19 September 2014.

DOI: 10.1111/1574-6941.12414

Editor: Riks Laanbroek

Keywords bacterioplankton; diversity pattern; epibiotic bacteria; submerged macrophyte.

Abstract

Epibiotic bacteria on surfaces of submerged macrophytes play important roles in the ecological processes of shallow lakes. However, their community ecology and dynamics are far from understood in comparison with those of bacterioplankton. Here, we conducted a comparative study of the species diversity and composition of epibiotic bacterial and the surrounding bacterioplankton communities of a common submerged macrophyte, Potamogeton crispus, in 12 lakes at a regional scale in China. We found that in different freshwater lakes, epibiotic bacteria possessed higher taxonomic richness than bacterioplankton did. There existed a marked divergence in the community structure between epibiotic bacteria and bacterioplankton. Alphaproteobacteria was the most dominant group for epibiotic bacteria, whereas Actinobacteria dominated bacterioplankton. Although variations in both bacterioplankton and epibiotic bacterial community compositions in different lakes were better explained by environmental than spatial factors, both environment and space had more intensified effects on epibiotic bacteria. This implied more complex and diverse ‘microhabitats’ for epibiotic bacteria on surfaces of submerged macrophytes, which may lead to higher variations of epibiotic bacteria than bacterioplankton. Our study suggested that epibiotic bacteria exhibited higher diversity and distinct community composition than the surrounding bacterioplankton. More attention should be focused on the productive and diverse microbial habitats on submerged macrophytes.

Introduction

In lacustrine ecosystems, plants such as submerged macrophytes usually play important roles in influencing the dynamics and processes of the entire ecosystem (Brix, 1997). Similar to terrestrial plants, a large amount of bacteria (epibiotic bacteria) lives on the surfaces of submerged macrophytes (Pollard, 2010). Many previous studies that described these epibiotic bacteria, sometimes referred to by very vague terms such as ‘epiphytes’, addressed their roles in the secondary production of lakes (Theil-Nielsen & Sondergaard, 1999), antibiotic activities (Bushmann & Ailstock, 2006), biogeochemical cycling (Coci et al., 2010) or pollutant-removal potential (Patrick & Loutit, 1977; Körner & Vermaat, 1998; Caslake et al., 2006). Despite the important ecological roles of epibiotic bacteria in aquatic ecosystems, the community ecology of these bacteria is far from well understood.

Epibiotic bacteria may have complex interactions with their host plants as well as the surrounding water column. Macrophytes can leach different types of dissolved organics (Bastidas Navarro et al., 2009) and inorganics (Anesio et al., 1999), which can be nutritional or detrimental to epibiotic bacteria (Hempel et al., 2008), onto their leaf surfaces. The different physical or biochemical characteristics on leaves could result in host-specific communities among different plant species (Grump & Koch, 2008; Hempel et al., 2008; Lachnit et al., 2011; He et al., 2012). Epibiotic bacteria could also be influenced directly by the surrounding water body. The surrounding water
bacterioplankton can serve as a major seeding source for epibiotic bacteria of submerged macrophytes (Rimes & Goulder, 1985). Also, epibiotic bacteria may emigrate from the macrophyte leaves and diffuse into the surrounding bacterioplankton community (Rimes & Goulder, 1986). However, almost no studies have systematically compared the community diversity and composition of the two kinds of bacteria in freshwater ecosystems.

According to the meta-community theory, local communities are linked to each other through the dispersal of multiple potentially interacting species. Total community variation on a regional scale (reflecting the beta diversity) can be explained by niche-based environmental filtering, neutral theory-based spatial variations (i.e. dispersal limits) or their combined effects (Holyoak et al., 2005). Although bacterioplankton variations in lakes have largely been explained by spatial factors (Lindström et al., 2006), others have argued that these variations are primarily regulated by local environmental factors (Van der Gucht et al., 2007) or a combination of both types of factors (Langenheder & Ragnarsson, 2007; Jones et al., 2012). With regard to epibiotic bacteria of submerged macrophytes, it is conceivable that submerged macrophytes may possess core communities that are specific to host plants, attributable to the regulatory effects exerted by host plants, to counter the influences of spatial or environmental factors. One contrasting study examining Methylobacterium on two species of terrestrial plants indicated that sites were more important than plant species in terms of their effect on the epibiotic bacterial community (Knief et al., 2010). However, almost no evidence has been found for possible variations of epibiotic bacteria in aquatic habitats, especially comparing the beta diversity of them with surrounding water bacterioplankton on a regional scale.

Here, we conducted a comparative study of the diversity patterns and composition of epibiotic bacteria and the surrounding bacterioplankton of a common submerged macrophyte, Potamogeton crispus, in relation to environmental factors and geographical distance in lakes on a regional scale. Potamogeton crispus was selected as a model plant because of its wide distribution and rigorous growth during the season of our sampling period. High-throughput sequencing technology was employed to acquire sufficient numbers of sequences for estimation and comparison of alpha or beta diversity. We aimed to reveal (1) differences in the diversity patterns (both alpha and beta diversity) and community composition of epibiotic bacteria and bacterioplankton surrounding a common submerged macrophyte in lake ecosystems and (2) the relative importance of spatial and environmental factors for the two kinds of bacterial communities.

### Materials and methods

#### Field sampling and collection of bacterial biomass

We selected and sampled 12 lakes along the reaches of the Yangtze River in China in April 2012 (Fig. 1), when *P. crispus* was the only dominant submerged macrophyte in these lakes. The names and geographical coordinates of these lakes are given in Supporting Information, Table S1. All these lakes are within an area of 10–100 km² with similar hydrological regimes and affected by the Yangtze River through natural or artificial trickles and water channels.

During sampling, representative plant samples were collected by hand with gloves or with a stainless steel hook. Samples from three to five plant replicates were stored in aseptic plastic bags, preserved at about 4 °C and transferred to the laboratory within 2–4 h. In addition to plant sampling, 1 L of water surrounding the sampled *P. crispus* population was also sampled for physicochemical and bacterioplankton community analyses. The water samples were kept in an ice box and taken to lab within 2–4 h for chemical analyses. To detach epibiotic bacteria, an ultrasonic plus surfactant method (Thomaz & Esteves, 1997; Yang et al., 2001; Hempel et al., 2008) was used. Approximately 2 g of fresh canopy leaf samples was cut away from the *P. crispus* plants and placed into 50-mL sterile polypropylene tubes containing 40 mL of epibiont cleaning buffer (2 mM phosphate buffer solution, 0.01% v/v Tween 80) with sterile scissors and forceps. The tubes were subjected to a 5-min ultrasonic bath, then vortexed for 30 s and again placed in an ultrasonic bath for another 5 min. The ultrasonic treatment did not visibly damage plant leaves. The epibiotic bacteria suspensions in the tube contained nearly 10⁷ mL⁻¹ DAPI (4’,6-diamidino-2-phenylindole)-stained individuals, and a second washing of the remaining leaves resulted in < 10⁶ mL⁻¹ cell counts (data not published). The epibiotic bacteria were collected by filtration through 0.2-μm Isopore membrane filters (Millipore Ireland Ltd., Ireland). Bacterioplankton biomass was collected by filtering 200-mL water samples through 0.2-μm Isopore membrane filters (Millipore Ireland Ltd.). All filters were then stored at −20 °C before further processing.

#### Determination of environmental parameters

Water temperature, oxidation reduction potential (ORP), dissolved oxygen (DO), and conductivity were determined *in situ* with a YSI 6600 water quality monitoring sonde (YSI, Yellow Springs, OH) while sampling. Water clarity (Secchi depth) was determined with a Secchi disk. Total nitrogen (TN), total phosphorous, nitrate nitrogen (NO₃⁻),
nitrite nitrogen (NO$_3^-$) and orthophosphate (PO$_4^{3-}$) were measured by continuous colorimetric flow analysis (Skalar SAN PLUS system; Skalar Analytical BV, Breda, the Netherlands). Dissolved organic carbon (DOC) was monitored with a TOC analyzer (ET-1020A; Euro Tech). Chlorophyll a (Chl-a) was extracted with a 90% acetone solution for 24–36 h and then evaluated by colorimetry. The data of these parameters are shown in Table S1.

**Bacterial DNA extraction**

Bacterial DNA was extracted with a method modified from Biteau et al. (2012). Briefly, a 1/2 filter was placed into a 2-mL microcentrifuge tube and cut into small pieces. A 650-µL lysis buffer (50 mM Tris-HCl, 40 mM EDTA, 1% w/v Cetyl trimethyl ammonium bromide, 2% w/v Polyvinylpyrrolidone (MW 40 000), 0.7 M NaCl, 0.4 M LiCl, 0.5% v/v Igepal CA-630; pH 8.0), and 34.2 µL 20% SDS (final concentration: 0.5%), 6.91 µL 1 M dithiothreitol (DTT; final concentration: 10 mM) were added to the tube, mixed thoroughly by vortexing for 30 s and incubated in 65 °C water for 30 min. Then, 1 vol of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) was added to the tube and mixed gently by inversion for 3–5 min. The tube was centrifuged at 16 000 g for 5 min. The supernatant was transferred to a new 2-mL tube, and 1 vol of chloroform : isoamyl alcohol (24 : 1) was added to the tube and mixed. The tube was centrifuged at 16 000 g for 5 min. The supernatant was again transferred to a new 2-mL tube, 2 vol cold ethanol (100%) was added and the tube was kept at −20 °C for 30 min. The tube was centrifuged at 17 000 g for 10 min. The supernatant was discarded, and 500 µL of 70% cold ethanol was added. The tube was centrifuged at 10 000 g for 1 min. The supernatant was discarded, and the DNA was dried in a freeze-drier. Finally, the DNA was dissolved in 50–100 µL sterile water and preserved at −20 °C. DNA quantity was determined with a Nanodrop 1000 (ThermoScientific, Wilmington, DE).

**Sequencing bacterial 16S rRNA genes**

DNA samples from three replicates were pooled and then sent to the sequencing center at MajorBio Company in
Shanghai. The V1–V3 region of bacterial 16S rRNA genes was amplified with the primer 533R (5′-TTACCGCGGTCTGCTCGG-3′), which had the Roche 454 ‘A’ pyrosequencing adapter and a unique 10-bp barcode sequence, and the primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′), which had the Roche 454 ‘B’ sequencing adapter at the 3′-end of each primer. Three replicates for each sample were amplified in a 20-μL reaction under the following conditions: 24 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s with a final extension at 72 °C for 5 min. PCR products were checked by performing gel electrophoresis with pooled replicates and further purified with the AxyPrepDNA gel purification kit (Axygen Biotechnology Hangzhou Ltd., Hangzhou, China). The purified PCR products were combined at equimolar ratios and then run on a Roche 454 FLX pyrosequencing machine.

**Sequence processing and estimation of diversity**

The pre-processing of 16S rRNA gene amplicon sequencing data was conducted using the software MOTHUR 1.30 (http://www.mothur.org/wiki/Download_mothur). Tags and primer sequences were removed. All sequences with lengths of <200 nucleotides and quality scores of <25 were discarded. Sequences were aligned based on the SILVA-compatible alignment database. After performing the de-noising or simplifying procedures (screen, filter, precluster, unique) suggested by the MOTHUR manual (http://www.mothur.org/wiki/Schloss_SOP), chimeras were identified and removed with the Uchime algorithm implemented in MOTHUR. Community taxonomy information was obtained from the RDP database (Wang et al., 2007) and those sequences not belonging to the bacterial kingdom were discarded. A distance matrix was generated, and operational taxonomic units (OTUs) were clustered out by the furthest neighbor algorithm at a 97% similarity level. A phylogenetic tree of representative sequences of all OTUs was built with FASTTREE2 (Price et al., 2010). For alpha or beta diversity estimates and comparisons between different groups, samples were rarefied into the minimum number of sequences from the sample with the poorest sequencing effort. The alpha diversity of bacterial community was estimated based on both OTU0.03 table (97% similarity) and phylogenetic tree. OTU richness was calculated by the command ‘summary.single’ in MOTHUR. Phylogenetic diversity was calculated as Faith’s phylogenetic diversity (Faith, 1992) by the package ‘picante’ (Kembel et al., 2010) in R 3.0.1 (R Core Team, 2013). The significances of the alpha diversity comparisons between epibiotic and bacterioplankton were tested by paired Student t-test. The beta diversity was reflected in variation (OTU- or phylogeny-based dissimilarity of bacterial communities at different sampling sites; Rodrigues et al., 2013).

**Statistical analyses**

The significances of variation differences between epibiotic bacteria and bacterioplankton were tested by permutation-based t-test owing to the independence of dissimilarity values of pairwise samples. The permutation-based t-test was done with the package ‘Deducer’ (Fellows, 2012) in R. Non-metric multidimensional scaling (NMDS) was used to visualize bacterial community difference. The significance tests for patterns observed by NMDS were performed with analysis of similarity (ANOSIM). NMDS and ANOSIM were conducted with the ‘vegan’ package (Oksanen et al., 2013) in R. The indicator analysis was utilized to identify OTUs (with the top 10 statistics) that were specific to bacterioplankton or epibiotic bacteria, which was performed with the ‘indic interchange’ package (De Caceres & Legendre, 2009) in R. The ‘niche breadth’ value proposed in Logares et al. (2012) for each OTU was calculated to identify the cosmopolitan (with highest niche breadth value) occurring for both epibiotic bacteria and bacterioplankton.

A permutation-based method, multiple regressions on distance matrices (MRM), was used to partition the variations of bacterial communities into pure environment, pure space, space/environment mixed, and unexplained components as proposed by Lichstein (2007). However, we used the ‘bioenv’ function of ‘vegan’ package in R to select a subset of z-transformed (scaled to zero-mean and unit-variance) environmental parameters best correlated with the community distances (Trumbo et al., 2013). This procedure not only selected those more important parameters for community variations, but also alleviated the collinearity among parameters, for example, the variance inflation factor (VIF) values (calculated by ‘vegan’ in R) of each selected parameters were all < 5. According to Borcard et al. (2011) this is acceptable for variance partition analysis. Another permutation based-Mantel (partial Mantel, simple Mantel) test was used to test the significance of the correlations between bacterial communities and geographical distance or selected environmental parameters. The Mantel test and MRM were performed with the ‘ecodist’ package (Goslee & Urban, 2007) in R with a permutation number of 9999.

**Nucleotide sequence accession numbers**

Sequences obtained in this study were deposited in GenBank under accession nos SAMN02439562 to SAMN02439585, in the biosample no. SUB402173.
Results

Description of overall sequences

Across all epibiotic bacterial and bacterioplankton samples in 12 lakes, we obtained 69,334 quality sequences, with an average read length of 483 bp. The sequence reads spanned a portion of the bacterial Escherichia coli 16S rRNA gene corresponding to positions 8 to approximately 533. The total OTU richness was 3856 at a 97% similarity level for all the rarefied samples (to the minimum number of sequences, 2046). Three OTUs consisted of more than 1000 sequences (the maximum was 1369). Additionally, the number of OTUs containing 10, 10 and 2 sequences and singletons was 42, 313, 1028 and 1454 for epibiotic bacteria and 43, 276, 628 and 869 for bacterioplankton, respectively. The Good’s coverage value at a 97% similarity level was 91.6% on average (SD 3.7%).

Of all sequences of rarefied samples, 67,794 (97.8%) could be classified at the phylum level and 25 phyla (subphyla) were identified using the RDP Classifier (listed in Table S2). Only nine phyla (subphyla) constituted over 0.5% of all communities (Table S2): Betaproteobacteria (29.6 ± 10.5%; mean ± SD), Alphaproteobacteria (23.0 ± 15.8%), Actinobacteria (22.4 ± 15.8%), Bacteroidetes (11.8 ± 7.9%), Gammaproteobacteria (4.7 ± 3.9%), Firmicutes (0.94 ± 1.65%), Armatimonadetes (0.91 ± 2.48%), Planctomycetes (0.71 ± 0.68%) and TM7 (0.61 ± 1.61%).

Diversity comparisons between epibiotic bacteria and bacterioplankton

The alpha diversities of epibiotic bacteria and bacterioplankton were compared based on both OTU and phylogenetic indexes. We found that the epibiotic bacterial communities overall were significantly higher in alpha diversity than the bacterioplankton communities (Fig. 2a and b). This is true for both the average OTU richness (two-tailed paired Student t-test: t = 2.92, P = 0.01, d.f. = 11) and phylogenetic diversity (two-tailed paired Student t-test: t = 3.79, P < 0.01, d.f. = 11).

In addition, epibiotic bacteria exhibited higher alpha diversity than bacterioplankton for each specific phylum except Actinobacteria (Fig. 3a and b). For OTU richness, there were significantly more bacteria from groups Alphaproteobacteria, Gammaproteobacteria, the total of the rare phyla (with a proportion < 0.5%) and unclassified bacteria in epibiotic bacterial communities than in bacterioplankton (in all cases: two-tailed paired Student t-test, t = 2.20, P < 0.05). For phylogenetic diversity, there were significantly more bacteria affiliated to Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Firmicutes, Armatimonadetes, TM7 and unclassified bacteria in epibiotic bacterial communities than in bacterioplankton (in all cases: two-tailed paired Student t-test, t = 2.20, P < 0.05). Bacteria from Actinobacteria were found to exhibit lower alpha diversity in epibiotic bacterial communities than in bacterioplankton communities, the difference being significant for OTU richness (t = 2.20, P < 0.05) but not for phylogenetic diversity (t = 2.20, P > 0.05).

The average variation in pairwise lakes for epibiotic bacteria was 0.83 ± 0.09 (OTU-based) or 0.82 ± 0.03 (phylogeny-based), which was higher than that for bacterioplankton (taxonomic, 0.76 ± 0.08; phylogenetic, 0.78 ± 0.04; Fig. 4a and b). A permutation-based t-test confirmed that the variation among different lakes was significantly higher for epibiotic bacteria than for bacterioplankton (two-tailed permutation based t-test, Welsh t = 4.47, P < 0.001, for OTU-based; Welsh t = 5.46, P < 0.001, for phylogeny-based). The higher beta diversity of epibiotic bacteria was also reflected by the relatively lower OTU overlap ratios (the percent of shared OTUs in total OTUs of two groups) of epibiotic bacteria.
the phylum Actinobacteria, which decreased from an average of 35.8 ± 8.7% (mean ± SD) percent in bacterioplankton communities to 9.1 ± 7.3% in epibiotic bacterial communities, followed by slight decreases for Betaproteobacteria, Armatimonadetes and TM7. The largest proportional increase in epibiotic bacteria was the phylum Alphaproteobacteria, which increased from 12.0 ± 7.0% in bacterioplankton to 34.1 ± 14.5% in epibiotic bacteria, followed by the phyla Gammaproteobacteria, Firmicutes and Planctomycetes (Fig. 5).

Among the identified taxonomy groups of all bacterial communities, 88.0% phyla, 71.7% classes, 72.0% orders, 62.9% families and 52.2% genera were shared by epibiotic bacteria and bacterioplankton. The number of groups specific to epibiotic bacteria was higher than that for bacterioplankton at all hierarchy levels (Table 1). However, in the analyses of the total 3856 OTUs of overall bacterial communities, most OTUs were only detected in either epibiotic bacterial (52.9% of total OTUs) or bacterioplankton (26.4% of total OTUs) communities, and the percent of shared OTUs between epibiotic bacteria and bacterioplankton was only 20.7% (Table 1). In each lake, the overlap ratio of epibiotic bacteria and bacterioplankton was 10.4 ± 3.8% (mean ± SD). In the analyses of OTUs in each specific phylum group, the numbers of OTUs specific to epibiotic bacteria were all higher compared with bacterioplankton (from 1.4- to 3.3-fold), except for Actinobacteria (0.7-fold). The overlap ratio between epibiotic bacteria and bacterioplankton was from 4.8% to 29.5% (21.6 ± 8.1%, mean ± SD; Table 1).

**Linking environmental and spatial factors to community structure**

Variance of bacterial community compositions in different geographical sites could be partitioned into four components: pure environmental, pure geographic, mixed effect, and unexplained. As shown in Table 2, for both kinds of bacteria, the selected environmental factors had greater explanatory power than pure geographic distance. For epibiotic bacteria, pure environment explained 9.9% (OTU-based) or 13.3% (phylogeny-based) more of the whole variance compared with pure geographic distance. For bacterioplankton, pure environmental factors explained 9.9% (OTU-based) or 18.2% (phylogeny-based) more of the whole variance compared with pure geographic distance. For bacterioplankton, pure environmental factors explained 9.9% (OTU-based) or 13.3% (phylogeny-based) more of the whole variance compared with pure geographic distance. For bacterioplankton, pure environmental factors explained 9.9% (OTU-based) or 13.3% (phylogeny-based) more of the whole variance compared with pure geographic distance. For bacterioplankton, pure environmental factors explained 9.9% (OTU-based) or 13.3% (phylogeny-based) more of the whole variance compared with pure geographic distance.
Discussion

Higher alpha and beta diversity of epibiotic bacteria than bacterioplankton

Very few studies have directly comparing the diversity of epibiotic bacteria and surrounding water bacterioplankton. In a 16S rRNA gene clone library sequencing-based study of the marine macro algae species *Ulva australis* at one site (Burke *et al.*, 2011), the OTU number was 135 ± 26 (mean ± SD; for 882 ± 164 sequences) for epibiotic bacteria and 133 ± 28 (for 1088 ± 28 sequences) for seawater bacterioplankton. In this study, the pyrosequencing approach enabled us to acquire more sequences to uncover more rare taxa. The OTU number of *P. crispus* was 436.6 ± 136.8 for epibiotic bacteria and 323.0 ± 44.0 for bacterioplankton in the 12 freshwater lakes. Our results indicated higher alpha diversity of epibiotic bacteria than bacterioplankton. Unlike many terrestrial plants, there is no desiccation and less UV stress (Lindow & Brandl, 2003) on aquatic plant leaves, which may create ideal habitats for bacterial survival. Their interface lifestyle between a body of water and plant tissues endows epibiotic bacteria with competitive advantages to acquire nutrients from both sources. Despite the largely unrevealed sophisticated relationships between epibiotic bacteria and their host plants, it is evident that the surface of submerged macrophytes is a hotspot for productivity and element cycling. There may be plenty of carbon sources for epibiotic bacteria to utilize on the productive macrophyte surface. For example, epibiotic algae and submerged macrophytes themselves together were estimated to account for more than 80% of primary productivity in Lawrence Lake, whereas the phytoplankton in bulk water only constituted 13%, and much of the leachates or DOC on macrophyte surfaces could be efficiently utilized by epibiotic microorganisms (Wetzel & Søndergaard, 1998). According to Wright’s species-energy theory (Wright, 1983), the higher productivity on the submerged macrophyte surface could supply a greater variety of resource types, and would support more bacteria species. Compared with the bulk water, leaves of submerged macrophytes might serve as ‘concentrators’ of bacterial community by supplying more nutrients and micro-niches for the immigrating bacteria cells. In addition, the
endophytic bacteria might also make a special contribution to the epibiotic bacteria community after emigrating outwards to the leaf surface (Beattie & Lindow, 1995), which could add partly to the increase of epibiotic bacterial community richness and a disparate community composition from the bulk bacterioplankton. This could also help explain why attached biofilms in stones in glacial-fed streams have a lower alpha diversity than stream water communities—these stone biofilms have much lower productivity, fewer carbon sources and undergo strong environmental sorting, whereas stream water communities may be recruited from a larger species pool in the river sediments through mass effect (Wilhelm et al., 2013).

Although not a definite, it is often observed that higher alpha diversity is coupled with higher beta diversity (Kunte et al., 1999; Fontaneto & Ricci, 2006). In some cases, the greater number of species in local habitats could mean a higher probability of differing community assemblies in different sites of a region, subjected to current or historical environmental heterogeneity, spatial dispersal limits and random perturbations (Leibold et al., 2004). Our study also captured the coupling of alpha and beta diversity in the comparison of epibiotic bacteria and bacterioplankton. The higher beta diversity implied that the surface characteristics of the same host plant _P. crispus_ or its interactions with epibiotic bacteria were not stable or monotonous. In a previous study, Osmond et al. (1981) discovered that the photosynthetic processes for the same submerged macrophytes could be very different in different sites.

More intensified effects of both environment and space for epibiotic bacteria

The link of bacterial community variations with the environmental parameters and geographical distance could help explain bacterial beta diversity as well (Tuomisto & Ruokolainen, 2006). In our results, both kinds of bacteria were explained better by environmental parameters than by geographical distance. All the lakes in this study were within the reaches of the Yangtze River. The dispersal of the bacterioplankton, although not examined directly, could be easy and unconstrained among these lakes, resulting in the non-significant pure effect of geographical distance on bacterioplankton variation. Overwhelmingly, epibiotic bacteria were linked more with both environmental and spatial explanatory factors than were bacterioplankton. Besides the conductivity and NO$_3^-$ for both types of bacteria, TN and NH$_4^+$ were exclusively correlated with epibiotic bacteria. The surrounding water variables can affect epibiotic bacteria by other possible means, having a direct effect or an indirect effect on the biochemical characteristics of host plants. For instance, increasing NH$_4^+$ levels can lead to a reduced soluble sugar and increased soluble amino acid content of _P. crispus_.
leaves (Cao et al., 2004), thus potentially affecting the biochemical properties of leaf surfaces. This study only captured a very small part of the environmental heterogeneity in different sites; for example, the temperature did not differ greatly between the lakes, and we also failed to include inorganic carbon parameters, low molecular organics, etc., and the physical, biochemical characteristics on leaves per se. In spite of this, the insufficient numbers of environmental factors and their mixed effects with geographical distance exhibited more power to explain epibiotic bacterial community variations. This implied that more environmental and geographical heterogeneities were encountered by epibiotic bacteria, which thus contained higher beta diversity. The more intensified effects of both environment and space for epibiotic bacteria also indicates the potential of submerged macrophytes to provide a stable and diverse environment for epibiotic bacterial community variations, which are capable of degrading polycyclic aromatic hydrocarbons in oil pollutants (Hiraishi et al., 2002; Leys et al., 2004; Wang et al., 2012). The Duganella (Betaproteobacteria), which is dominant and is only detected in epibiotic bacteria (Table S3), contains yellow pigments and is also found in leaf surface of terrestrial plants (Kämpfer et al., 2012); these bacteria were reported to have potential in bioremediations of heavy metals (Tanner et al., 1998; Jackson et al., 2009). Some studies also reported bacteria from this group as endophytic bacteria of plants, and stressed their possible roles in phytoremediation processes (Moore et al., 2006).

**Differences in community composition of epibiotic bacteria and bacterioplankton**

The differentiation of the epibiotic bacterial community from bacterioplankton was evident at different phylogenetic resolutions. These results agreed with the marine U. australis study, in which the OTU overlap ratio of the two kinds of bacteria was even lower (1.88%; Burke et al., 2011). The higher OTU overlap ratio (10.4 ± 3.8%) in each lake in this study might result partly from the higher sequencing intensity. The phylum Alphaproteobacteria, which dominated in epibiotic bacteria, was also found to be dominant in epibiotic bacteria of other aquatic plants (Crump & Koch, 2008; Hempel et al., 2008; Tujula et al., 2010; Burke et al., 2011). The most specific epibiotic bacterial OTUs were affiliated with Rhodobacteraceae (Alphaproteobacteria; Table S3); bacteria from this group were key members of the initial biofilms forming in one study (Elifantz et al., 2013). They possessed many surface colonization traits based on genomic studies (Slightom & Buchan, 2009). Another specific epibiotic bacterial OTU was affiliated with Porphyrobacter (Alphaproteobacteria; Table S3). Bacteria from this taxon usually contain chlorophyll and frequently have been found to be capable of degrading polycyclic aromatic hydrocarbons in oil pollutants (Hiraishi et al., 2002; Leys et al., 2004; Wang et al., 2012). The Duganella (Betaproteobacteria), which is dominant and is only detected in epibiotic bacteria (Table S3), contains yellow pigments and is also found in leaf surface of terrestrial plants (Kämpfer et al., 2012); these bacteria were reported to have potential in bioremediations of heavy metals (Tanner et al., 1998; Jackson et al., 2009). Some studies also reported bacteria from this group as endophytic bacteria of plants, and stressed their possible roles in phytoremediation processes (Moore et al., 2006). Actinobacteria was predominant in water bacterioplankton and was much less frequently detected in epibiotic bacteria. Several specific and dominant OTUs in the bacterioplankton communities were affiliated with Actinomycetales (Actinobacteria) and Limnophilantans, Polynucleobacter (both Betaproteobacteria; Table S3). These taxa are typical and dominant bacterioplankton taxa in freshwater bodies (Brusn et al., 2003; Ježberová et al., 2010; Kasalický et al., 2013). They may be specialists for water columns and lack the capacity to live in certain interfaces such as the leaf surfaces of submerged macrophytes. We also observed some cosmopolitans living in both habitats, such as Hy-

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**Table 2. MRM and Mantel analyses of bacterial communities distance with the ln geographical distance and Euclidian distance of standardized environmental parameters among sampling lakes**

<table>
<thead>
<tr>
<th>Parameters†</th>
<th>TN, NH₄⁺, NO₂⁻</th>
<th>Cond., NO₃⁻</th>
<th>Cond., NH₄⁺, NO₂⁻</th>
<th>Cond., NO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRM (R²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure G.</td>
<td>0.039</td>
<td>0.021</td>
<td>0.012</td>
<td>0.007</td>
</tr>
<tr>
<td>Pure E.</td>
<td>0.148</td>
<td>0.110</td>
<td>0.194</td>
<td>0.140</td>
</tr>
<tr>
<td>Mix effect</td>
<td>0.358</td>
<td>0.145</td>
<td>0.374</td>
<td>0.123</td>
</tr>
<tr>
<td>Residuals</td>
<td>0.455</td>
<td>0.724</td>
<td>0.421</td>
<td>0.730</td>
</tr>
<tr>
<td>Partial Mantel (r)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure G.</td>
<td>0.281*</td>
<td>0.168</td>
<td>0.165</td>
<td>0.095</td>
</tr>
<tr>
<td>Pure E.</td>
<td>0.495***</td>
<td>0.363*</td>
<td>0.562***</td>
<td>0.401*</td>
</tr>
<tr>
<td>Mantel (r)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G.</td>
<td>0.630***</td>
<td>0.408***</td>
<td>0.621***</td>
<td>0.361***</td>
</tr>
<tr>
<td>E.</td>
<td>0.711***</td>
<td>0.505***</td>
<td>0.753***</td>
<td>0.513***</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001.

Cond., conductivity; E, environmental parameters; EPB, epibiotic bacteria; G., geographical distance; PTB, bacterioplankton.

The parameters were selected by the ‘bioenv’ function in the ‘VEGAN’ package in R.
drogenophaga (Betaproteobacteria), Rhizobiales (Alphaproteobacteria), Brevundimonas (Alphaproteobacteria), Flavobacterium (Flavobacteriales; Table S3). They may be versatile in adapting to different kinds of habitats, and represent the inter-communication of epibiotic bacteria and bacterioplankton communities.

We observed some taxonomic groups dominant and specific to epibiotic bacteria that were known for biofilm formation and pollutant removal, which implied the possible divergence of functional traits between epibiotic bacteria and bacterioplankton. However, to obtain detailed knowledge about epibiotic bacterial functions on submerged macrophytes and the relationship with bacterioplankton, further investigations are needed.

Conclusions

Our results indicated that epibiotic bacteria of submerged macrophytes are more diverse than bacterioplankton in terms of both alpha and beta diversity. Although variations in both bacterioplankton and epibiotic bacterial community compositions in different lakes were best explained by environmental factors, there were more intensified effects of both environment and space for epibiotic bacteria. In future studies, the exact physical, biochemical characteristics on the surfaces of more different submerged macrophytes, and the functional traits of bacterial community should be considered.

Acknowledgements

We thank Jiwen Zheng, Huabing Li and Xue Wang for their kind help in field sampling work. Thanks are also given to Guilin Liu for helping map sampling sites. This work was supported by the National Science Foundation of China (31225004, U1202231) and the Chinese Academy of Sciences (KZZD–EW–TZ–08).

References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Summary of the site characteristics and water environmental parameters of the investigated lakes.

**Table S2.** Relative abundance (%) of different phyla in the bacterial communities of the investigated lakes.

**Table S3.** Taxonomic groups of the top 10 OTUs specified to epiphytic bacteria (EPB), bacterioplankton (PTB) and top 10 OTUs with highest niche breadth.