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Strong impact of anthropogenic contamination on the co-occurrence patterns of a riverine microbial community

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Originality-Significance Statement

Emerging organic contaminants such as pharmaceuticals and personal care products (PPCPs) represent a great threat to the aquatic biome, yet in comparison to toxic effects on macrobiota, the impact of PPCPs on riverine microbiota are very poorly understood. Here we demonstrated that α-diversity of riverine microbial communities can decrease under the stress caused by excessive nutrients and PPCPs. Moreover, PPCPs are shown to play a more important role in shaping the microbial co-occurrence ecological network in the lotic ecosystems studied than regular physico-chemical factors. Our work provides new insights into our understanding of how organic micropollutants affect the assembly of aquatic microbial communities.

Summary

Although the health of rivers is threatened by multiple anthropogenic stressors with increasing frequency, it remains an open question how riverine microbial communities respond to emerging micropollutants. Here, by using 16S rDNA amplicon sequencing of 60 water samples collected during different hydrological seasons, we investigated the spatio-temporal variation and the co-occurrence patterns of microbial communities in the anthropogenically impacted Jiulong River in China. The results indicated that the riverine microbial co-occurrence network had a non-random, modular structure, which was mainly shaped by the taxonomic relatedness of co-occurring species. Fecal indicator bacteria may survive for prolonged periods of time in river water, but they formed an independent module which had fewer interactions with typical freshwater bacteria. Multivariate analysis demonstrated that nutrients and micropollutants (i.e. pharmaceuticals and personal care products, PPCPs) exerted combined effects in shaping α- and β-diversity of riverine microbial communities. Remarkably, we showed that a hitherto unrecognized disruptive effect of PPCPs on the abundance variations of central species and module communities was stronger than the influence of physico-chemical factors, suggesting the key role played

by micropollutants for the microbial co-occurrence relationships in lotic ecosystems. Overall, our findings provide novel insights into community assembly in aquatic environments experiencing anthropogenic stresses.

Key words: Microbial community, spatio-temporal variation, co-occurrence network, nutrient, pharmaceuticals and personal care products, Jiulong River

Introduction

The essential roles of microorganisms in mediating global biogeochemical cycles and maintaining ecosystem services are well known. The recent application of high-throughput sequencing provides an increasingly clear view of the specific and unique microbiomes that characterize different natural or artificial environments, such as freshwater, ocean, soil, sediment, hot springs, animal guts and bioreactors (Tamames et al., 2010). In lake ecosystems, a number of microbial taxa have been termed 'typical freshwater bacteria' (TFB), which is based on phylogenetic analysis of 16S rRNA gene pools from lake bacterioplankton, mainly including α-, β- and γ-Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobcateria and Verrucomicrobia, (Glöckner et al., 2000; Zwart et al., 2002; Newton et al., 2011). Many prior investigations have demonstrated that TFB are widely distributed not only in lake water, but also in the surface waters of rivers and streams (Hu et al., 2014; Savio et al., 2015; Meziti et al., 2016). However, the relative abundance of TFB was found to generally decrease in those parts of the lotic ecosystems that suffer strong disturbances from human activities; Specific allochthonous signals, such as inflows of sewage and the associated human and animal gut bacteria, could also be detected when studying the composition and dynamics of riverine microbial communities (Hu et al., 2014; Meziti et al., 2016). These findings highlight that rivers are among the most vulnerable ecosystems in the context of a growing human population along the riverside and increasing anthropogenic pressure.

Meanwhile, rivers are critical sources of water for drinking, agriculture and industrial production as well as valuable reservoirs of biodiversity (Shiklomanov, 1998), not to mention essential channels for connecting terrestrial and marine ecosystems (Vörösmarty et al., 2010). However, the ecological mechanisms controlling microbial community assembly and interspecies interactions in anthropogenically disturbed rivers have not been resolved with respect to complex abiotic and biotic environmental factors.

So far, evidence has accumulated that both deterministic (species sorting) and stochastic (mass effect) processes govern the assembly of aquatic microbial communities (Savio et al., 2015; Nino-Garcia et al.,

2016). In lotic ecosystems, local physico-chemical factors such as temperature, pH, dissolved oxygen (DO), nitrogen and phosphorus nutrients as well as organic matter usually control species sorting to a considerable extent (Hu et al., 2014; Staley et al., 2015), whereas the hydrological structure of lotic ecosystems (flow direction, fluvial network, water residence time and riparian construction) may have an influence on balancing deterministic and stochastic processes (Widder et al., 2014; Savio et al., 2015; Nino-Garcia et al., 2016). Moreover, chemical micropollutants discharged from riparian human activities, such as pharmaceuticals and personal care products (PPCPs), may act as potential selective forces on riverine microbial communities considering their intensive/widespread use, resistance to wastewater treatment and biodegradation, environmental persistence, and known ecotoxicological effects on aquatic life (Schwarzenbach et al., 2006; Rosi-Marshall and Royer, 2012; Liu and Wong, 2013).

The potential influences of PPCPs on the diversity and function of freshwater microbial communities were also assessed by means of several laboratory-scale microcosm or mesocosm experiments (Yergeau et al., 2012; Lawrence et al., 2015; Sabater et al., 2016). It has been suggested that the pseudo-persistent PPCPs may have contrasting ecological effects on the aquatic community: PPCPs may become carbon and energy sources to certain members of the natural microbial community via biodegradation (subsidy effect), while being toxic to others (stress effect) - even though their environmental concentrations are generally low (ng/L ~ μg/L range) (Luo et al., 2014). Both subsidy and stress effects of PPCPs would, however, be expected to lead to changes in the diversity and composition of freshwater microbial communities and relevant biogeochemical processes (Yergeau et al., 2012).

Apart from abiotic environmental factors, the composition and dynamics of microbial communities are also shaped by ecological factors such as top-down control (i.e. grazing and viral lysis) and microbe-microbe interactions (Chaffron et al., 2010; Faust and Raes, 2012; Lima-Mendez et al., 2015). Microbe-microbe interactions specifically have received little attention until recently (Faust and Raes, 2012). Due to advances in high-throughput sequencing technologies, the increased availability of sequence data across large spatio-temporal scales has provided an opportunity to address this gap in

fundamental knowledge in microbial ecology. Recent studies have used co-occurrence network analysis as a tool to decipher the potential intra- or inter-species interactions in the complex microbial assemblages in oceans (Cram et al., 2015), lakes (Eiler et al., 2012; Kara et al., 2013) and soils (Barberan et al., 2012; Berry and Widder, 2014; Ma et al., 2016) as well as in anthropogenic environments such as activated sludge (Ju et al., 2014), anaerobic digesters (Rui et al., 2015) and the human gut (Faust et al., 2012). These findings revealed that microbial communities generally have non-random co-occurrence patterns and modular structures, which strongly implies the essential role of biotic interactions in governing community assembly and ecosystem function. In addition, it has been suggested that within an ecological network, some central or keystone species play an irreplaceable role in maintaining the structure and function of the whole microbial community (Sazima et al., 2010; Ma et al., 2016). However, little is known about how the combined effects of physico-chemical variables and chemical micropollutants, including PPCPs, affect the population dynamics of co-occurring and central species. Answers to these important questions will improve our understanding of the relative roles of abiotic, biotic and anthropogenic factors in shaping spatio-temporal microbial diversity, composition and species interactions, and will offer insights into the ecological rules that guide microbial community assembly in fluvial ecosystems.

This study aims to obtain an in-depth understanding of riverine microbial interactions and their response to anthropogenic interference (i.e. excessive nutrient loadings and chemical micropollutants). We specifically focus on the following questions: i) Do lotic microbial communities under long-term human interference show non-random co-occurrence patterns? If yes, ii) Do physico-chemical factors and PPCPs interact to shape the structure of co-occurrence networks and the abundance of central populations over spatio-temporal gradients? To this end, we performed a combination of chemical analysis of PPCPs and a 16S rDNA amplicon sequencing-based analysis of microbial communities of Jiulong River, China (Fig. S1), which has been under long-term exposure to pronounced gradients of anthropogenic disturbance (Chen et al., 2013; Hu et al., 2014), thus serving as an ideal system for addressing the

aforementioned microbial ecology questions concerning the lotic ecosystem. We analyzed the data using co-occurrence networks, checkerboard scores (C-score), and multivariate statistics.

Results

Spatio-temporal microbiome dynamics and cosmopolitan community

Water samples were collected from Jiulong River during normal, dry and wet hydrological seasons. Physico-chemical variables and concentrations of PPCPs are listed in Table S1. For river microbiome analysis, in total, 3,733,731 high-quality reads were obtained from 60 samples (42,900 to 155,612 reads per sample). To reduce the biases arising from different sequencing depths across samples, the datasets were randomly sub-sampled at 40,000 reads per sample and then clustered into 109,827 operational taxonomic units (OTUs) (6,782 ± 2,389 per sample) at 97% identity (Table S2). Principal coordinates analysis (PCoA) using Bray-Curtis (Fig. 1a and b) or Jaccard distance (Fig. S2a and b) metrics revealed a clear spatio-temporal pattern of microbial community structure in Jiulong River (Mantel test between Bray-Curtis and Jaccard distances, $\rho = 0.99$, $P < 0.0001$). The first two PCoA components of Bray-Curtis distance explained a higher proportion of variance (32.0%) than those of Jaccard distance (21.7%). The seasonal variation of microbial community composition was more pronounced than geographic variation between the two tributaries (North River (NR) and West River (WR)), as indicated by the Adonis (R^2 = 0.186 vs 0.401, $P < 0.05$) and ANOSIM (R = 0.225 vs 0.071, $P < 0.01$) tests. Furthermore, Venn diagram analysis demonstrated that 15,525 OTUs, which represent 14.1% of the total OTUs and 89.2% of the total reads, were shared among three seasons (Fig. S3), implying that a cosmopolitan, but seasonally dynamic microbiome may be present. In accordance with this result, we found that 460 OTUs (0.4% total OTUs and 63.8% total reads) occurred in more than 80% of all samples and could therefore be considered persistent OTUs according to the definition of Ju and Zhang (2015). In contrast, the intermittent (20-80% occurrence) and transient (≤ 20% occurrence) OTUs harbored 5,845 OTUs (5.3% total OTUs and 22.6% total reads) and 103,522 OTUs (94.3% total OTUs and 13.7% total reads), respectively (Table S2). In the

downstream analysis, we therefore focused on the non-transient OTUs that were present in more than 20% of the samples because they represented the bulk of the biomass of the river microbiome. At the order level, Burkholderiales, Actinomycetales, Clostridiales, Flavobacteriales, Rhizobiales, Rhodocyclales, and Turicibacterales accounted for more than 76.9% of the persistent and intermittent communities (Fig. S4).

General community assembly patterns of the river microbiome

C-score analysis showed that the river microbiome species, regardless of hydrological season, exhibited segregated distributions (Fig. S5) as significantly higher C-scores compared to null models and positive standardized effect size (SES) values were always observed for the real networks ($P < 0.0001$) (Table 1). However, the strength of species segregation varied among seasons. The highest SES value appeared during the normal season (SES = 8.9), while dry and wet seasons had comparable SES values (SES = 7.3 and 7.4, respectively; Table 1), suggesting that the degree of species segregation was higher during the normal season than dry or wet seasons. Notably, the SES values for positively correlated OTUs and negatively correlated OTUs ranged from 10.5 to 15.0, and 15.2 to 30.1, respectively, all of which are significantly higher than values for all OTUs ($P < 0.0001$). The results suggested that both positive and negative biotic interactions, which were reflected by positive and negative correlations between OTU-OTU, play important roles in species segregation in the river microbiome, with a higher contribution from the negative interactions. Similar patterns were also observed for the analysis of C_{var}-score (i.e. SES of C_{var}-score: negative > positive > all OTUs, Table 1), which was proposed to simultaneously check the degrees of both species segregation and aggregation (Fayle et al., 2013). Taken together, we conclude that both negative and positive biotic interactions contribute to the non-random community assembly in the river ecosystem.

The topological and taxonomic properties of the co-occurrence networks

Given the non-random community assembly patterns in the river microbiome, a network interface

was constructed to further explore the topological and taxonomic characteristics of microbial co-occurrence patterns. Based on correlation analysis, 44,680 edges, which depict significant and strong pairwise correlations between species (Spearman's $\rho \geq 0.6$, $P < 0.01$), were captured between 2,194 nodes (i.e. OTUs) (Fig. S6). Among these edges, 2,237 edges represent very strong co-occurrence correlations ($\rho \geq 0.8$, $P < 0.01$) between 648 OTU nodes with very strong correlations (Fig. 2, left panel). The node degree distribution of the co-occurrence network showed a scale-free power-law distribution ($R^2 = 0.95$), implicating the preferential and non-random attachment of new vertices to the more highly connected vertices. This is quite different from the Gaussian distribution ($R^2 = 0.98$) of node degree for a random network of identical size (Erdös-Rényi model) (Fig. S7). Among the three seasons, the sub-network of normal season contained 16,912 edges among 1,036 nodes, which was much higher than the sub-networks of dry (9,078 edges) and wet (6,879 edges) seasons (Table 2), reflecting a higher number of co-occurrence instances in the normal season (edge/node ratio = 20.4). Analogously, the decreasing cluster coefficients (CC) and increasing average path lengths (APL) provided further evidence of a reduction in network complexity from normal to wet seasons (Table 2). Nevertheless, for all seasons we observed that river microbiome networks exhibit values of modularity (MD, 0.423~0.672), CC (0.415~0.511), and APL (3.918~6.107) that were higher than those in their respective Erdös–Rényi random networks (Table 2), suggesting that all river microbiome networks had small-world properties and modular structures.

Moreover, we calculated and compared the incidences of observed (O) and random (R) co-occurrence between all pairwise OTUs to explore the intra- and inter-taxa co-occurrence patterns. The results showed that OTUs within the same phylum, including Firmicutes, Actinobacteria, Planctomycetes and Bacteroidetes, tended to co-occur more often than expected by chance (with O/R ratios > 2.0, Table S3). Similar non-random intra-taxa co-occurrence patterns were also evident at the class (e.g. Clostridia and Actinobacteria), order (e.g. Rhizobiales and Actinomycetales), family (e.g. Clostridiaceae) and genus levels (e.g. *Clostridium* and *Flavobacterium*) (red highlights, Table S3). Apart from the non-random

intra-taxon association patterns, there was also a higher incidence of inter-taxa co-occurrence than expected from random associations. For instance, nodes from different taxa of phylum Firmicutes, (I) Bacilli and Clostridia, (II) Clostridiales and Turicibacterales, (III) Clostridiaceae and Peptostreptococcaceae, (IV) Clostridiaceae and Turicibacteraceae, and (V) *Clostridium* and SMB53 showed higher incidences of inter-taxa co-occurrence than expected by chance (O/R > 8.0, blue highlights, Table S3). On the other hand, certain taxa tended to co-occur less than expected by chance. For example, OTUs of Proteobacteria had much lower observed incidences of co-occurrence with OTUs of Actinobacteria, Planctomycetes and Bacteroidetes than expected from a random network (O/R $\leqslant$ 0.5, green highlights, Table S3).

Taxonomic relatedness determines the modular structure

Modularity analysis demonstrated that the entire network could be divided into 10 major modules, each of which was comprised of a group of OTU nodes that inter-connected more frequently among themselves than with nodes in other modules (Fig. 2). Ternary plot analysis showed that the major modules displayed seasonal patterns (Fig. 3). For instance, OTUs from modules I and II had higher relative abundances in normal and dry than in wet seasons, while modules IV and V tend to occur in dry season. On the other hand, taxonomic relatedness is clearly a key factor in determining the modular structure in the network (see the right panel versus left panel, Fig. 2). The typical examples are the orders Actinomycetales and Clostridiales, with the former dominating modules I (84.9% nodes belonged to Actinomycetales) and V (77.3%) while Clostridiales dominated module II (76.5% of nodes affiliated with Clostridiales, and 90.1% nodes belonged to phylum Firmicutes) (Table S4). Strikingly, however, it is highly probable that module II stands for the impact of polluted freshwater that receives human/animal-oriented fecal pollutants (Table S4 and Fig. S8). This is because i) Family Clostridiaceae, which accounted for 59.3% of module II, may be potential fecal indicator bacteria (FIB), based on analysis of environmental source of microbial taxa (see supplemental text, Table S5 and S8); ii) other

members of the module II, including Lachnospiraceae, Peptostreptococcaceae, Ruminococcaceae (genus *Ruminococcus*), Streptococcaceae (*Streptococcus*) and Turicibacteraceae (*Turicibacter*), are routinely found in human/mammalian intestines and feces (Table S5) (Harwood et al., 2014).

In addition, we found that six of the 10 modules, including modules I (84.9% nodes affiliated with TFB), IV (97.9%), V (81.8%), VIII (100%), IX (88.5%), and X (100%), were primarily occupied by indigenous TFB affiliated with the orders Actinomycetales, Burkholderiales and Flavobacteriales, etc.. Apart from module II, the modules III (35.5% nodes belonged to TFB), VI (40%) and VII (60.5%) also contained relatively low proportions of TFB (Fig. 2).

Central species and their taxonomic distributions

Central species, which play an irreplaceable role in maintaining the structure and function of a microbial community, were identified as having a high degree (> 100) and low betweenness centrality values (< 5000) in co-occurrence networks (Ma et al., 2016). In simple terms, these are those OTUs that have both a central and highly connected position in the network. Based on this criteria, a total of 151 OTUs (the average relative abundances ranged from 0.003% to 2.51%), accounting for only 8.6% of the total reads, were recognized as central species (Fig. 4a). The taxonomic classification illustrated that the majority of central species/OTUs belonged to the orders Burkholderiales (9 OTUs and 6.0% in the central community), Actinomycetales (71 OTUs/47%), Clostridiales (37 OTUs/24.5%), Rhizobiales (4 OTUs/2.6%), Rhodocyclales (1 OTU/0.7%), Turicibacterales (4 OTUs/2.6%), Synechococcales (1 OTU/0.7%) and Gemmatales (2 OTUs/1.3%). 51.7% of the central OTUs were further identified as TFB (78 OTUs, average relative abundances 0.003 to 0.216%). In contrast, 32 of 151 OTUs (21.2%) were affiliated with the family Clostridiaceae (phylum Firmicutes) and may belong to fecal bacteria (the average relative abundances ranging from 0.004-0.534%) (Fig. 4a and Table S4). Comparison of the taxonomic composition of the microbiome and the co-occurrence network (Fig. 2) showed that the relative proportions of bacterial orders were strongly positively correlated ($\rho > 0.9$, $P < 0.001$) between

the whole microbiome and the co-occurrence network. Both the microbiome and co-occurrence network are dominated by the bacterial species of orders Burkholderiales, Actinomycetales and Flavobacteriales (Fig.4b, upper panel). In contrast, Actinomycetales, Clostridiales, Turicibacterales, Synechococcales and Gemmatales were overrepresented in the central community. Conversely, several bacterial orders, including Flavobacteriales, Cytophagales and Saprospirales and Sphingomonadales, were quite abundant in the microbiome and co-occurrence network, but were not- or under-represented among the central species (Fig. 4b). Notably, OTUs of Turicibacterales (genus *Turicibacter*) only represented 2.6% of central species, but they accounted for over 31% of abundance (see the lower panel against upper panel, Figure 4b).

Environmental drivers of riverine microbiome α- and β-diversity

Spearman correlation analyses indicated that water pH, the concentrations of nitrogen species (NH_4-N, NO_2-N, NO_3-N and dissolved inorganic nitrogen (DIN)) and nutrient stoichiometry (the ratio of DIN to soluble reactive phosphorus (SRP)) were negatively correlated with α-diversity indexes, including the observed number of OTUs (OTUs), Chao1 richness (Chao1), phylogenetic diversity (PD), Shannon-Wiener diversity (Shannon), Simpson's reciprocal indexes (R-Simpson), and Pielou's equitability index (Equitability) (ρ between -0.31 to -0.68, $P < 0.05$) (Fig. 5).Among the 26 PPCPs tested, thiabendazole (TBD) and sulfamethazine (SMT) had positive correlations with the richness and diversity of riverine microbial community (ρ between 0.37 to 0.63, $P < 0.05$), whereas methyl paraben (MPB), bisphenol A (BPA), triclosan (TCS) and propyl paraben (PPB) showed the opposite pattern (ρ between -0.30 to -0.60, $P < 0.05$). Moreover, significant negative correlations between community diversity metrics and the sum concentrations of PPCPs were also observed. This correlation was slightly enhanced when TBD and SMT were excluded (Fig. 5).

The step-wise DistLM revealed that the selected physico-chemical variables (i.e. temperature, pH, NO_3-N, total phosphorus (TP), and SRP) together explained 19.8-56.0% of compositional variation of

total microbiome, network (modules) and central communities, while 24.1-58.9% of the observed variation in the datasets could be explained by a combination of PPCP compounds alone ($P < 0.05$) (Table S7), suggesting that PPCPs may play as important role as typical physico-chemical variables in shaping the distribution and co-occurrence patterns of riverine prokaryotic communities. The PPCPs that account for most variation in step-wise DistLM models include a fungicide (TBD), an anxiolytic (diazepam (DZP)), a plasticizer (BPA), two antibiotics (sulfamethoxazole (SFZ) and SMT), two analgesic/anti-inflammatory drugs (indomethacine (IDT) and diclofenac acid (DFA)) (Table S7). Additionally, several factors, such as NH_4-N, acetaminophen (ATP, an analgesic/anti-inflammatory drug), gemfibrozil (GBL, a lipid regulator), caffeine (CAF, a stimulant), and sulfadiazine (SDZ, an antibiotic), alone explained considerable variance (adjusted R^2 > 10%, $P < 0.05$) in several DistLM models (Table S7).

Riverine co-occurrence network is structured by physico-chemical factors and PPCPs

Considering that there were a few strong correlations between physicochemical variables and PPCPs: temperature and IDT ($\rho = -0.83$, $P < 0.01$), pH and SDZ ($\rho = -0.83$, $P < 0.01$), EC and IPF ($\rho = 0.82$, $P < 0.01$), and NH_4-N and IPF ($\rho = 0.78$, $P < 0.01$) (Fig. S10), we further used partial db-RDA analysis to distinguish the relative contributions of physico-chemical variables and PPCPs on the variance of species abundance in the microbiome, network, modules, and central communities. Moreover, spatio-temporal (space and season, i.e. spatial eigenvectors and seasonal dummy factors) variables selected through step-wise DistLM were also incorporated into the partial db-RDA models (Table S7), given the pronounced spatio-temporal variation of the riverine microbial community (Fig. 1a and b). The results indicated that the explanatory power of the variables measured (physico-chemical parameters, PPCPs, spatio-temporal factors) increased from total to central communities and that therefore a large proportion of the variance in network (adjusted $R^2 = 56.0\%$) and central (adjusted $R^2 = 64.6\%$) communities was explained by the models (Fig. 6). It is noteworthy that the unique effect of PPCPs was notably greater (up

to 5.2 times) than that of physico-chemical factors (i.e. Ph-Ch) in most partial db-RDA models, including those for central species and modules I, IV, V, VII, VIII and X (Figs. 6 and S11b-k). Furthermore, the variation explained by spatial and temporal factors was comparable or larger than that of Ph-Ch or PPCPs, especially for the former one (Fig. 6 and S11).

The effects of physico-chemical variables, PPCPs and spatio-temporal factors on riverine communities were also evaluated by using boosted regression trees (BRT) models. Generally, spatio-temporal and physico-chemical factors had higher relative effects on total, abundant, rare and network communities than PPCPs (Fig. S12). However, the relative importance of PPCPs (37.7 ± 11.0%) increased and effects were greater than those of physico-chemical (12.6 ± 8.5%) or temporal (12.8 ± 16.4%) factors in BRT models for central and module communities with few exceptions, although a considerable amount of explained variance was contributed by spatial factors (36.9 ± 22.6%) (Fig. S12).

Discussion

Although the variation in microbial community composition in lake and ocean surface waters over temporal scales has been well documented (Yannarell et al., 2003; Shade et al., 2007; Gilbert et al., 2009), riverine microbial communities have received comparatively less attention (Zeglin, 2015). In the Jiulong River investigated here, although the two unconnected tributaries, NR and WR, represented sub-ecosystems with different water chemistry and anthropogenic stressors (livestock production vs. agricultural activity) (see supplementary results), the tributary-specific variation in microbial communities was less pronounced than that of seasonal variation. This result was in concordance with previous findings, demonstrating that lotic microbial communities had recurrent annual seasonal patterns in two temperate (Crump and Hobbie, 2005) and six Arctic rivers (Crump et al., 2009) respectively, the dynamics of which may be closely associated with seasonal changes in water chemistry (temperature, pH, DO, nutrients and organic matter), hydrology (river flow) and external climatic factors. When considering only samples from Columbia River, a clear seasonal shift of microbial community corresponding to river

discharge was uncovered in the study of Fortunato et al. (2012), implying that climatic and hydrological conditions may be one of the dominant factors structuring the composition and dynamics of microbial communities in running water systems (Fortunato et al., 2012). The variation in river discharge among hydrological seasons could result in great changes, not only in water chemistry and chemical pollutants, but also as a result of allochthonous inputs (e.g. from sediment re-suspension and erosion) (Savio et al., 2015). Further studies are needed to address the complex mechanisms determining the composition of microbial communities in different lotic ecosystems.

The application of network analysis allowed us to study not only the composition of microbial communities, but also the underlying network of ecological dependencies, and how these are affected by the environment. Consistent with the observations by Horner-Devine et al. (2007) and later studies (Barberan et al., 2012; Ju et al., 2014), we showed that microorganisms tend to co-occur less than expected by chance (i.e. they display species segregation) in an anthropogenic-impacted river. The high water flow during wet or flood periods could have resulted in increasing the mass effect and homogenization of environmental conditions, resulting in decreasing strength of species segregation, whereas opposite trends may appear during low water flow in the dry season (Ortega et al., 2015). However, our results based on C-score and C_{var}-score analysis offer an alternative hypothesis. Our results suggest that the degrees of species segregation during wet and dry seasons were highly comparable, but were both lower than that during normal season (Table 1). Moreover, by comparing the SES values of C_{var}-score for positively correlated and negatively correlated OTUs, we suggest that both positive and negative biotic interactions contributed significantly to species segregation (i.e. SES values: negative > positive > all interactions), with more a pronounced contribution from the negative interactions (e.g. competition). However, the roles of other processes, such as resource partitioning and neutral processes, cannot be resolved or excluded by our data (Horner-Devine et al., 2007).

Regarding overall network topology, our results demonstrated that, similar to microbial guilds in lakes (Eiler et al., 2012; Kara et al., 2013), soils (Barberan et al., 2012), marine water columns (Steele et

al., 2011), activated sludges (Ju et al., 2014) and anaerobic digesters (Rui et al., 2015), the co-occurrence network of the lotic microbial community exhibited power-law degree distribution, "small-world" properties, and a modular structure. Consistent with the findings of Kara et al. (2013) and Ma et al. (2016), the complexities of the microbial ecological networks in Jiulong River were the highest during normal season while being lowest during wet season, as indicated by the values of CC and APL, confirming again that microbial co-occurrence relationships vary across hydrological seasons.

Nevertheless, by integrating taxonomic assignments of OTU nodes and network structure, we found the assembly of the lotic microbial community was non-randomly determined by taxonomic relatedness, i.e. closely related taxa tended to be inter-connected and clustered together (Fig. 2), which may be attributed either to strong niche overlap in closely related species or to synergistic relations. This is consistent with the widespread phenomenon of phylogenetic clustering of environmental microbiomes reported elsewhere, which reflects the effects of environmental filtering and niche differentiation among species within a taxon(Chaffron et al., 2010; Ju and Zhang, 2015). This pattern was also evident in the network modules (Fig. 2), which demonstrated seasonal variation (Fig. 3). For example, the modules I (abundance higher in normal and dry seasons) and V (abundance higher in dry season) were assembled by OTUs affiliated with Actinomycetales, which are well known as "ultramicrobacteria" that prefer oligotrophic niches (Hahn et al., 2003; Warnecke et al., 2005; Allgaier and Grossart, 2006), whereas copiotrophic Burkholderales (genera *Acidovorax*, *Limnohabitans* and *Rhodoferax*) and Flavobacteriales (genus *Flavobacterium*) dominated in module IV (abundance higher in dry season), providing evidence for the existence of distinct and discrete ecological niches over temporal scales (i.e. modules) in the river ecosystem that are preferentially occupied by different groups of microbial taxa.

Moreover, habitat preference of microorganisms may also play a vital role in determining their co-occurrence patterns (Chaffron et al., 2010). Thus, it was reasonable to find that TFB (indigenous population) and FIB (exogenous population) tended to form distinct modules (Figs. 3 and S8). The FIB, mainly made up by Clostridiaceae (module II), likely entered into Jiulong River water due to

sewage/feces pollution rather than being released from river sediments (Hu et al., 2014; Hu et al., 2016). On the one hand, although the drastic changes in living environments (e.g. oxygen, light, and temperature, etc.) could prevent the growth and survival of FIB, the observation of prolonged persistence and survival (days to weeks) of typical FIB (fecal Bacteroidales, *coliforms*, *enterococci* and *Escherichia coli*) in fresh waters has been reported in numerous previous studies (Harwood et al., 2014). The divergent proportion of *Turicibacter* to central community may mirror active interaction between FIB components of module II. On the other hand, Clostridiaceae have the ability to form endospores to resist harsher environmental conditions (Wunderlin et al., 2014), which may be another reason for the high abundance of Clostridiaceae detected here. Therefore, the positive associations between fecal bacteria species may be attributable to a similar environmental fate and / or to an origin at the same spatially or temporally restricted discharge sources.

It has recently been suggested that nutrients and emerging contaminants have an interactive effect on shaping invertebrate, algal and microbial communities in aquatic environments (Corcoll et al., 2014; Aristi et al., 2016; Sabater et al., 2016). Our results indicated that most of the nutrient variables measured here had negative correlations with the richness and diversity of microbial communities from Jiulong River, providing further evidence for our earlier findings based on 16S rRNA gene amplicon pyrosequencing that showed a similar relationships between nutrients and α-diversity of a bacterial community from the same watershed during the normal hydrological season (Hu et al., 2014). This may be due to the detrimental effects of high concentrations of nutrients on community biodiversity (Song et al., 2015). Furthermore, in agreement with negative relationships between microbial diversity and chemical pollutants that have previously been reported in soils (Ager et al., 2010) , marine sediments (Sun et al., 2013) and lakes (Pomati et al., 2017), we found that four PPCP compounds and total concentrations of PPCPs were negatively associated with α-diversity of riverine microbial community while only two PPCPs showed positive correlations. Such patterns suggest that the co-occurrence of various PPCPs in surface waters of Jiulong River may tend to cause an overall detrimental effect on community diversity.

One possible explanation is attributed to the stress effect (antimicrobial toxicity) of PPCPs (Luo et al., 2014), which likely causes the proliferation of tolerant or resistant populations at the expense of sensitive ones (Corcoll et al., 2014). MPB, PPB, and TCS are in fact well known for their antimicrobial properties (Brausch and Rand, 2011). Although BPA is less toxic for microorganisms, and may serve as a carbon and energy source for some microbial functional groups involved in BPA mineralization (Michalowicz, 2014), the concentration of BPA may be too low (average 0.2 μg/L) to initiate degradation of this compound in the presence of sufficient nutrients in Jiulong river. On the other hand, the positive correlations between TBD, SMT and biodiversity implied the existence of a possible linkage between the taxonomic diversity of microbial communities and micropollutant biotransformations (biodegradation or co-metabolism) on large spatio-temporal scales (Johnson et al., 2015). Nevertheless, further studies are required to prove this possibility at such low environmental concentrations (ng/L).

Another object of our study was to evaluate the relative importance of Ph-Ch and PPCPs on riverine microbial communities and its co-occurrence network. The DistLM and partial-db RDA analyses showed that several physico-chemical variables (i.e. temperature, pH and nutrients) and a subset of PPCPs (i.e. TBD, DZP, BPA, SFZ, SMT, IDT and DFA) were the main drivers determining abundance variations of microbial community OTUs by explaining a considerable fraction of variance in community composition, especially for network (modules) and central communities. The results of BRT models further confirmed the essential role of PPCPs in structuring the abundance variations of modules and central communities. Remarkably, the partition of the variance showed that although the concentrations of most PPCP compounds in river waters (at ng/L level, i.e. sub-minimal inhibitory concentration (sub-MIC)) were far below the threshold of toxicological concern (Table S1), a surprisingly large potential effect of PPCPs on the central community was revealed. The PPCPs at sub-MIC levels may disturb transcriptional regulatory and signaling systems, and subsequently affect microbe-microbe communication (Andersson and Hughes, 2014). If our interpretation of central species as being of central functional importance for the microbial component of the ecosystem is correct, this would reveal an important mechanism for how PPCPs may

affect ecosystem services in polluted rivers. A recent study employing a novel GSA-QHTS screening method provided conclusive evidence that the activity of riverine microbial biofilm community was indeed affected by low concentrations of PPCPs (Rodea-Palomares et al., 2016). Considering the considerable variability in community composition explained by temperature, pH and nutrients, our findings support previous observations that organic micropollutants, excessive nutrients and other critical physico-chemical variables have combined effects on the structure of freshwater biota.

Despite the significant effects of PPCPs on modules and central communities revealed here, a large amount of variation in community composition was explained by spatial and temporal factors, especially the former (Figs. 6, S11 and S12). This may reflect either the consequence of unmeasured environmental factors or the influence of stochastic processes (e.g. dispersal limitation, ecological drift). For example, other chemical pollutants (polycyclic aromatic hydrocarbons (PAHs) and pesticides) were reported to impose stresses on the structure and function of aquatic microbiomes (Ager et al., 2010; Sun et al., 2013; Jeanbille et al., 2016). On the other hand, the importance of dispersal limitation should be considered for our dataset because of the geographic isolation (watercourse non-connection between NR and WR) (Heino et al., 2015). Nevertheless, our results highlight the previously unrecognized role of organic micropollutants such as PPCPs on the riverine microbial co-occurrence relationship and thus on fundamental ecological structures within the habitat. Further experimental/toxicological studies are, however, required to establish a definitive causal link between chemical micropollutants and their ecological effects.

Experimental procedures

Study area

Jiulong River is the second largest river in Fujian Province, China. It has a catchment area of 1.47×10^4 km^3. Jiulong River watershed consists of two major tributaries, NR (North River) and WR (West River). Previous investigations showed that long-term excess nutrient loading (> 20 years) resulted in the

eutrophication of Jiulong River (Chen et al., 2013), while diverse chemical micropollutants such as PAHs (polycyclic aromatic hydrocarbons) (Maskaoui et al., 2002), pesticides (Zheng et al., 2016) and PPCPs (pharmaceuticals and personal care products) (Lv et al., 2014) were detected with high frequencies and in ecotoxicologically relevant concentrations. Moreover, several FIBs (fecal indicator bacteria) (Clostridiaceae, *Methanobrevibacter* and *Methanosphaera*) appeared in considerable abundance in the surface waters of Jiulong River (Hu et al., 2014; Hu et al., 2016).

Sample collection and physico-chemical measurements

A total of 20 sampling sites were chosen from NR (11 sites) and WR (9 sites). The surface water samples (~ 0.5 m sampling depth) were collected during normal (6^{th}-7^{th}, September, 2012), dry (14^{th}-15^{th}, January, 2013) and wet (6^{th}-7^{th}, June, 2013) hydrological seasons, respectively (Fig. S1). The average water discharges of the Jiulong River, which were obtained from the Bureau of Hydrology, Ministry of Water Resources, China (http://xxfb.hydroinfo.gov.cn), were 364 m^3/s, 171 m^3/s and 985 m^3/s during September, 2012, and January and June, 2013, respectively. Temperature, pH, electric conductivity (EC), DO and DO saturation of surface waters were determined *in situ* using a YSI-650 MDS meter with multi-probe (YSI, Yellow Springs, Ohio, USA). For nutrient analysis, ~ 300 mL surface water was filtered through 0.45 μm cellulose membranes, and frozen at -20°C until analysis. The concentrations of NH_4-N, NO_2-N, NO_3-N, SRP, and TP were determined using a flow injection analyzer (QC8500, Lachat®, Loveland,Co., USA). DIN was defined as the sum of NH_4-N, NO_2-N, and NO_3-N. Moreover, water samples were also collected for the measurement of the concentration of PPCPs. A total of 51 PPCP compounds were determined based on liquid chromatography/triple quadrupole mass spectrometry (Table S1), as described in our previous work (Lv et al., 2014). For molecular microbiological analysis, about 500 to 1,000 mL water samples were pre-filtered through 20 μm mesh (Millipore, Bedford, MA, USA) to remove large particulate matter, and subsequently filtered onto 0.22 μm Sterivex-GP filters (Millipore, Bedford, MA, USA). All samples were stored at -80°C until analysis.

Nucleic acid extraction and 16S rRNA gene amplicon sequencing

DNA was extracted in duplicate in accordance with a previously published enzyme/phenol-chloroform protocol (Hu et al., 2014). The V4-V5 region of the prokaryotic 16S rRNA gene was amplified using a universal primer pair 515F (5'-GTGYCAGCMGCCGCGGTA-3')/907R (5'-CCGYCAATTYMTTTRAGTTT-3') (Quince et al., 2011). The PCR condition was 95°C for 3 min, 30 cycles of 95°C for 45s, 50°C for 60s, 72°C for 90s and finally 72°C for 10 min. Triplicate PCR reactions were performed for each sample and pooled for subsequent purification and Illumina MiSeq sequencing. MiSeq sequencing was performed at the Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) with a paired-end strategy (2 × 250bp). The raw sequence data generated in this study were deposited into the NCBI short reads archive (SRA) database under BioProject number PRJNA328778.

Sequencing analysis

The raw paired-end reads were quality trimmed using Trimmomatic v0.30 (Bolger et al., 2014), and combined using FLASH software (Magoč and Salzberg, 2011). The assembled contigs were subsequently analyzed using QIIME v1.8.0 as described elsewhere (Caporaso et al., 2010). The reads with mismatches in the barcode and primer sequences and chimeric characteristics were discarded. A table of OTUs was generated at an identity of 97% based on a UCLUST open-reference OTU picking pipeline (Edgar, 2010). Singletons were excluded from the downstream analysis to reduce biases caused by PCR and sequencing errors. Taxonomic assignment was performed using an RDP classifier with the Greengenes database v13.8 (McDonald et al., 2012) at a bootstrap cutoff of 80%. The OTUs were classified as indigenous taxa (i.e. TFB, Typical freshwater bacteria) in river waters using the taxonomic framework of TFB supplied at the freshwater microbial field guide site (https://github.com/mcmahon-uw/FWMFG) if the OTUs could be assigned to the 5^{th} taxonomic level (a few TFB have freshwater nomenclature at the 4^{th} taxonomic level) with more than 70% confidence. The exogenous taxa (i.e. FIB and sewer indicator bacteria) and other

functional prokaryotic taxa were identified based on the taxa list in Table S5. To clarify the potential environmental sources of Clostridiaceae (the highest relative abundance of FIB in Jiulong River), the correlation among FIB taxa was explored using spearman analysis, and the population composition of Clostridiaceae from water and sediment samples was analyzed using the Nonmetric Multidimensional Scaling ordination method.

Statistical analyses

All statistical analyses were performed in QIIME 1.8.0 or using R (Ihaka and Gentleman, 1996) with packages as listed below. The α-diversity metrics, including the observed OTUs, Chao1 (Chao1 richness), PD (phylogenetic diversity), Shannon (Shannon-Wiener diversity), R-Simpson (Simpson's reciprocal indexes), and Equitability (Pielou's equitability index), were calculated using the QIIME script "alpha_diversity.py". PCoA was conducted to compare similarities among samples based on Bray-Curtis or Jaccard distance metrics using R package phyloseq (McMurdie and Holmes, 2013). ANOSIM and Adonis tests were conducted to identify whether microbial communities differed significantly between different hydrological seasons (normal, dry and wet seasons) or locations (i.e. NR and WR) (Hu et al., 2016). Correlations between α-diversity metrics (i.e. richness and evenness) and physico-chemical variables or PPCPs (≥ 60% occurrence) were examined by means of a Spearman's rank correlation test using R package Hmis (Harrell, 2008). The false discovery rate (FDR) (Benjamini-Hochberg adjustment) method was used for multiple testing corrections of generated *P*-values (Benjamini and Hochberg, 1995). The relationship between β-diversity of microbial community and physico-chemical variables or PPCPs was examined using a distance-based linear model (DistLM) with a forward selection procedure (9,999 permutations) (Anderson, 2004). Furthermore, using R package vegan (Oksanen et al., 2013), partial distance-based redundancy analysis (partial db-RDA) was performed to evaluate the relative contributions of spatio-temporal and physico-chemical variables as well as PPCP concentrations on the β-diversity of total, co-occurring network (and the modules) and central communities. Before partial db-RDA, the

sequence abundance of OTUs was transformed by Hellinger distance, and the physico-chemical and PPCP parameters were normalized using z-score transformation. A set of spatial eigenvectors, which represented the directional spatial structure of the Jiulong River, was generated based on watercourse distances using the asymmetric eigenvector maps (AEM) approach (Blanchet and Legendre, 2010). To account for the effect of some unmeasured temporal factors (i.e. hydrological and external climatic parameters), different hydrological seasons (i.e. normal, wet and dry) were coded as dummy factors (0 and 1).

In addition, BRT (boosted regression trees) was used as a supplementary method to evaluate the relative influence of spatio-temporal, physico-chemical variables, and PPCP concentrations on microbial β-diversity variances. BRT is an advanced form of regression that incorporates interactions between explanatory variables and has the ability to model complex nonlinear functions (Elith et al., 2008). BRT models were fitted using a Gaussian loss distribution, a learning rate to 0.001, a tree complexity of 3, and a bagging size of 0.75. Models were developed with a 10-fold cross-validation approach using the R packages dismo (Hijmans et al., 2015) and gbm (Ridgeway, 2013). Principal component analysis (PCA) was used to extract the most important patterns of changes in physico-chemical variables and PPCPs. The PCA axes for physico-chemical variables and PPCPs were selected according to the Kaiser-Guttman rule. Spatial and temporal variabilities were represented by AEM eigenvectors and dummy factors, respectively. Moreover, the first two axes of PCoA ordination of microbial communities, which represented the main microbial β-diversity variance, were included in BRT models as the response variable.

C-score and network analysis

The C-score, which counts the number of checkerboard units (i.e. 2 × 2 matrix) where both OTUs occur once but at different sites, was calculated under the null model and used as the metric of community-wide non-random co-occurrence patterns (Stone and Roberts, 1990). The C-score and C_{var}-score (i.e. variance of C-score) tests were performed using R package EcoSimR (http://ecosimr.org/)

with constant row and column sums, a sequential swap randomization algorithm, and a burn in of 30,000 swaps. To facilitate comparison among different seasons, a SES was calculated as follows:

SES = (observed C-score- mean simulated C-score)/standard deviation of simulated C-scores (Gotelli and McCabe, 2002).

For network analysis, Spearman's rank coefficients (ρ) between all 97%-cutoff OTUs with occurrence in at least 20% of samples and at least 60 reads (2,379 OTUs) were calculated pairwise using R package Hmisc (Harrell, 2008). Subsequently, those significant (FDR-adjusted *P*-value < 0.01) and robust ($\rho \geq 0.6$) correlations between OTUs were exported as a GML format network file using R package igraph (Csardi and Nepusz, 2006). Network visualization and modular analysis (modules were identified by using the Louvain algorithm) were conducted using Gephi (Bastian et al., 2009). Moreover, 10,000 Erdös-Réyni random networks, which had the same number of nodes and edges as the real co-occurrence networks, were generated with each edge having the same probability of being assigned to any node (Erdös and Rényi, 1960), and the average value of each network metric was reported. Topological properties of both real and random networks, including MD (modularity), CC (average clustering coefficient), and APL (average shortest path length), were compared to determine the degree of randomness of the real networks. The small-world coefficient (ó) was calculated to investigate the small-world property of the networks (i.e. the degree of clustering and shortness of paths between nodes) (Telesford et al., 2011). Nodes with high degree (> 100 connections to other nodes) and low betweenness centrality values (< 5000) in a co-occurrence network were defined as central species, which is similar to the designation of keystone species used elsewhere (Ma et al., 2016). The R scripts used for the above co-occurrence network analysis are available at https://github.com/RichieJu520/Co-occurrence-network-analysis (Ju et al., 2014). The observed incidence (O%) and random incidence (R%) of co-occurrence patterns between taxa were statistically checked, as described previously. In brief, O% was calculated as the number of observed edges (E_o) between two taxa divided by total number of edges (E) in a co-occurrence network; R% was theoretically calculated by

considering the frequencies of two taxa (n(N1) and n(N2)) and random associations (Ju et al., 2014; Ju and Zhang, 2015). Therefore, the degree of a lack of agreement between O% and R%, as reflected by the O/R ratio, can be used as a measure of the non-randomness of observed co-occurrence patterns.

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Author contributions

AH and CPY led in conceiving the ideas, with contributions from FJ and HB. FJ and HB contributed to data analytic tools. FJ performed the statistical and network analyses. AH, LH, JL, XY, HJ and SIM performed the field sampling, measurement of physico-chemical variables, and sample preparation for Illumina MiSeq sequencing. QS provided the PPCPs data. AH, FJ, HB and CPY integrated the data and wrote the manuscript.

Conflict of Interest: The authors declare that they have no competing interests.

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Table 1. Observed C-scores and C_{var}-scores, mean metric values under null models, standardized effect sizes (SES) for riverine microbial communities of 20 sampling sites over three hydrological seasons. Higher observed SES values of C-score suggest greater degrees of species segregation than would be expected by chance. Higher SES values of C_{var}-score indicate greater degrees of both species segregation and aggregation. All *P*-values are < 0.0001.

Season	Number of			C-score			C_{var}-score		
	Sites	OTUs	Cor.[a]	Obs.	Mean null	SES	Obs.	Mean null	SES
Normal									
All[b]	20	2353	22058	13.2	12.8	8.9	173.	132.8	9.0
Positive	20	1036	16912	7.6	7.3	15.0	95.1	68.3	13.7
Negative	20	664	5146	8.5	7.9	29.7	122.	76.5	26.3
Dry									
All	20	2339	9810	12.2	11.8	7.3	151.	119.6	7.3
Positive	20	869	9078	6.3	6.0	13.6	70.2	50.9	13.2
Negative	20	385	732	7.8	7.1	30.1	102.	59.4	29.2
Wet									
All	20	2369	8979	9.5	9.3	7.4	128.	107.4	7.3
Positive	20	1145	6879	5.2	5.0	10.5	62.6	50.5	10.6
Negative	20	674	2100	5.7	5.3	15.2	75.8	52.9	15.3

[a]. Cor., number of correlation

[b]. All, the communities after removing the OTUs with occurrence less than 20% samples and less than 60 reads; Positive, positively-correlated OTUs; Negative, negatively-correlated OTUs.

Table 2. Comparison of topological properties of co-occurrence networks of riverine microbial communities with identically sized Erdös-Rényi random network.

	N	E	MD	MD_r	CC	CC_r	APL	APL_r	σ
River network	2194	44680	0.672	0.087 (± 0.001)	0.511	0.019 (± 0.000)	3.918	2.442 (± 0.000)	17.1 (± 0.2)
Normal season sub-network	1036	16912	0.620	0.110 (± 0.002)	0.572	0.032 (± 0.001)	4.540	2.314 (± 0.001)	9.1 (± 0.1)
Dry season sub-network	869	9078	0.423	0.151 (± 0.003)	0.526	0.024 (± 0.001)	4.921	2.567 (± 0.001)	11.4 (± 0.3)
Wet season sub-network	1145	6879	0.549	0.205 (± 0.005)	0.415	0.010 (± 0.001)	6.107	3.093 (± 0.001)	20.3 (± 1.2)

Abbreviations: N, number of nodes; E, number of edges; MD, modularity; CC, average clustering coefficient; APL, average shortest path length; σ, small-word coefficient $\sigma = (CC/CC_r)/(APL/APL_r)$ and $\sigma > 1$ indicate 'small-world' properties, i.e., high interconnectivity and high efficiency (Telesford et al., 2011).Subscript r indicates the properties of the Erdös-Rényi random network. The standard deviation of MD_r, CC_r, APL_r and σ of 10,000 Erdös-Rényi random networks was provided in the brackets.

Figure Legends:

Figure 1. Spatio-temporal variations of microbial community structure in Jiulong River, China. Principal coordinate analysis with Bray-Cutris distance matrix of lotic microbial communities in normal (September, 2012), dry (January, 2013) and wet (June, 2013) hydrological seasons (a) and in the two tributaries (b). 95% ellipses were constructed using the "ordiellipse" function of vegan package to illustrate seasonal or tributary samples.

Figure 2. Co-occurrence networks of 97%-cutoff OTUs within lotic microbial communities based on correlation analysis. A connection stands for a very strong (Spearman's $\rho \geq 0.8$) and significant (FDR-adjusted *P*-value < 0.01) correlation. The size of each node is proportional to the number of connections (i.e., degree). Left panel: OTUs colored by the order-level taxonomy; Right panel: OTUs colored by modularity class.

Figure 3. Ternary plot showing the seasonal distribution and relative abundance of OTUs from modules I-X. Modularity classes are indicated by colors, while the size of the circle indicates the relative abundance of each OTUs in total samples. Sep, September, 2012 (normal season); Jan, January, 2013 (dry season); Jun, June, 2013 (wet season).

Figure 4. (a) Degree-centrality plot of 97%-cutoff OTUs in the co-occurrence

network of lotic microbial communities. OTUs with high node degree (> 100) and low betweenness centrality values (< 5,000), which reflected that nodes are highly connected and centrally clustered within the network, are viewed as central species (Ma et al., 2016). Circles filled in red represent typical freshwater bacteria. (b) Relative proportions of microbial orders in the total microbiome, co-occurrence network and central species, based on read abundance (upper right panel) and richness (lower right panel) of 97%-cutoff OTUs. All OTUs of the total microbiome (109,827 OTUs), co-occurrence network (2,194), and central species (151) account for 100%, 80.3% and 8.6% of total 2,400,000 16S rRNA gene sequences obtained from 60 surface water samples of Jiulong River, respectively.

Figure 5. A heatmap showing the correlation between α-diversity metrics and physico-chemical (Ph-Ch) variables or PPCPs. The Spearman's correlation matrix only keeps correlations with FDR-adjusted $P < 0.05$. OTUs, observed number of OTUs; Chao1, Chao1 richness index; PD, phylogenetic diversity index; Shannon, Shannon-Wiener diversity index; R-Simpson, Simpson's reciprocal index and Equitability, Pielou's equitability index. TBD, Thiabendazole; SMT, Sulfamethazine; CAF, Caffeine; MPB, Methyl paraben; BPA, Bisphenol A; TCS, Triclosan; PPB, Propyl paraben; Total, the sum of all PPCPs, and Total*, the sum of all PPCPs without TBD and SMT.

Figure 6. Variation partitioning of microbial β-diversity variances among

physico-chemical (Ph-Ch), PPCPs, spatial (Space), and temporal (Season) factors as well as their joint terms. (A) General partitioning model. Within the model, a–d refer to the pure effect of each explanatory factor; e–j refer to the joint effect of two explanatory factors, k–n refer to the joint effect of three explanatory factors; o relates to the joint effect of all four explanatory factors, and u (Res, residuals) is the residual variance; (B)-(D) variance explained by each term in total microbiome, co-occurring network and central species.

Fig. 1

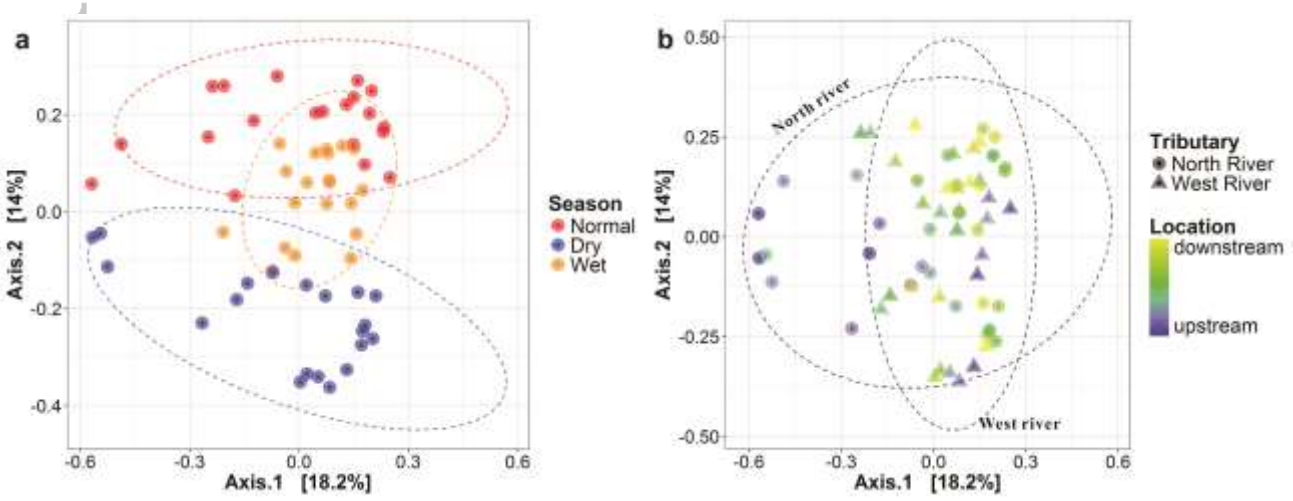

Fig. 2

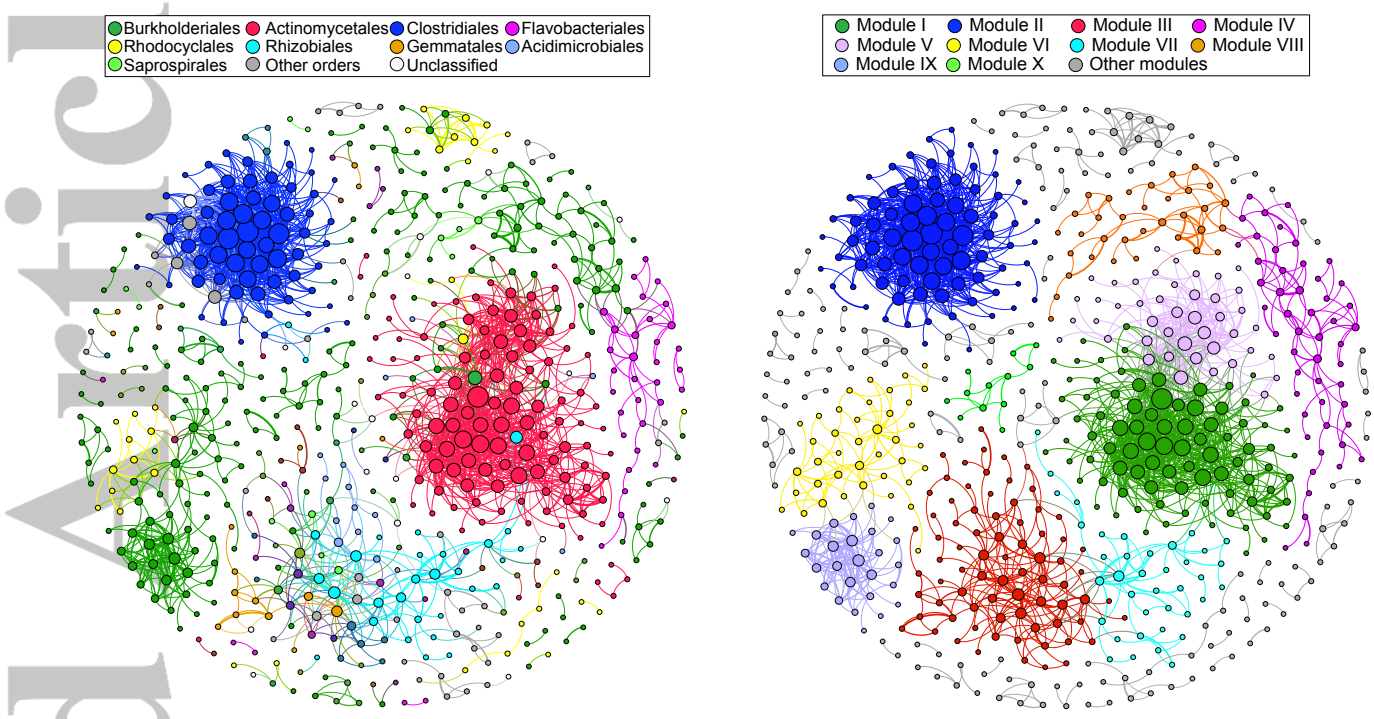

Fig.3

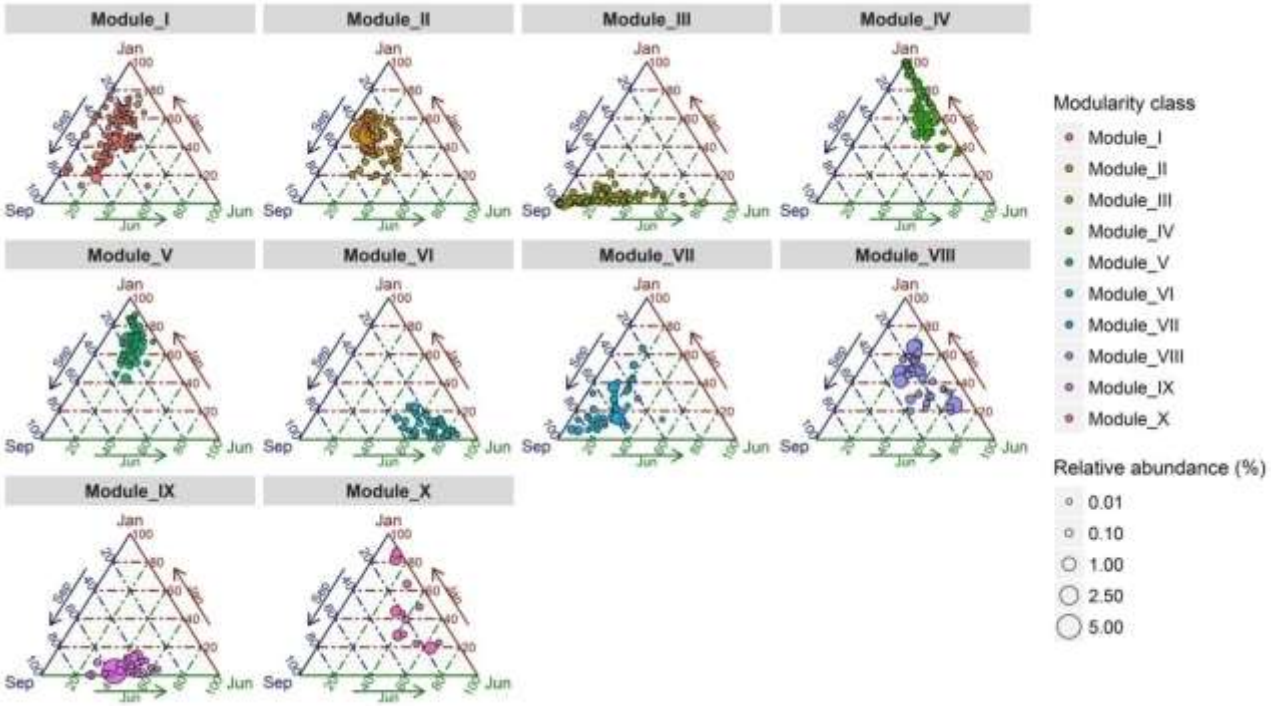

Fig. 4

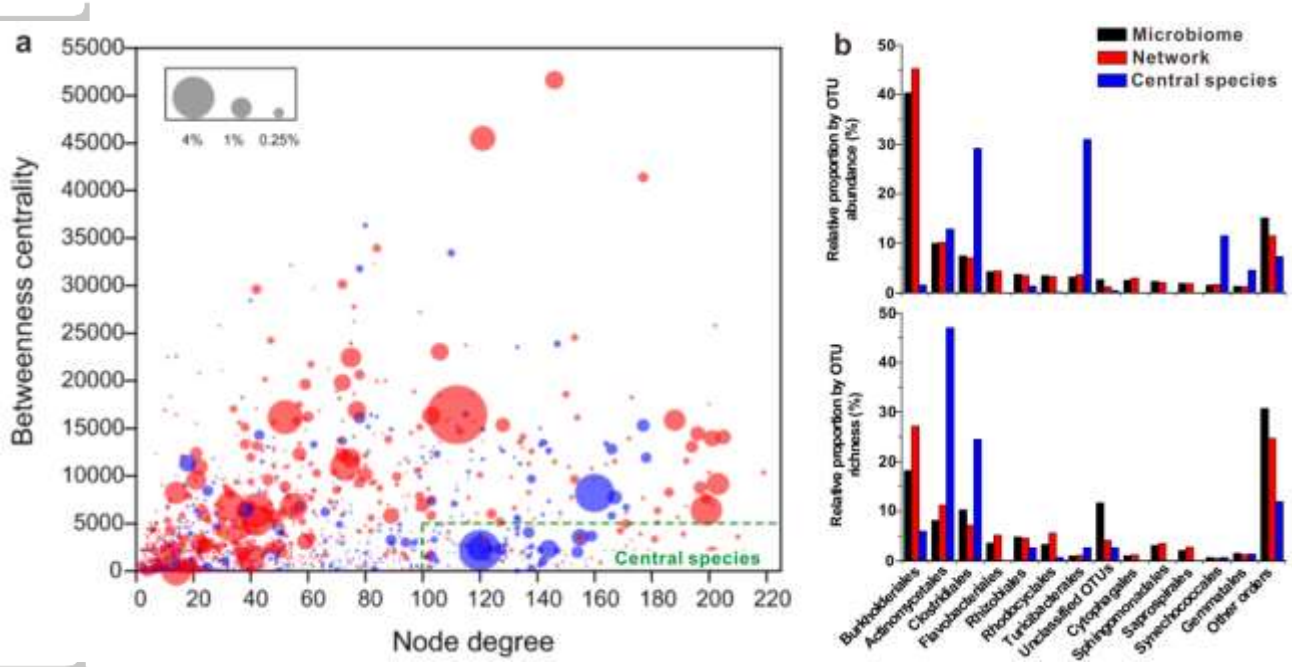

Fig. 5

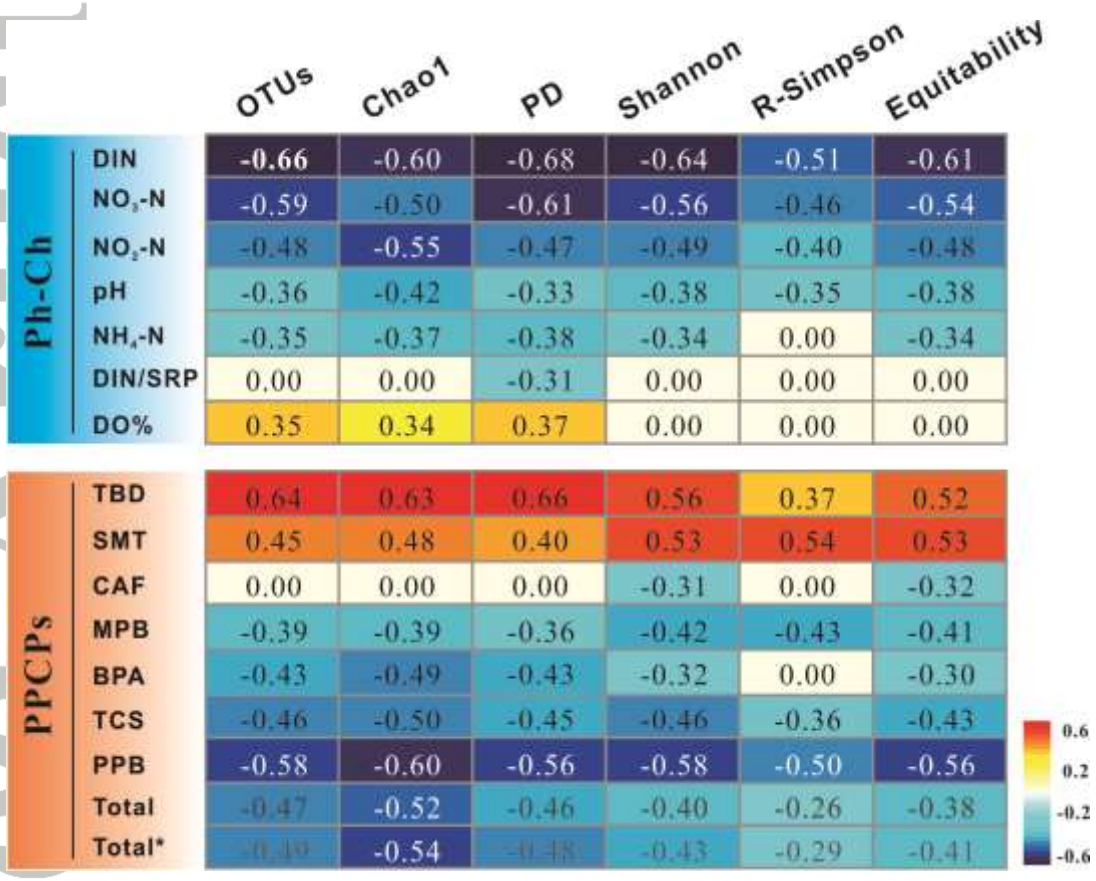

Fig. 6