Urban non-point source pollutants increases the biotic homogenization of the structure and function in river microbiome

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Keywords: river network, non-point source pollution, urbanization, biotic homogenization.

Abstract

Human activities were believed to affect the assembly and biogeographic pattern of river microorganisms, while the underlying processes are not sufficiently understood yet. Comparing the river microbial communities in highly urbanized rivers and undisturbed natural rivers could expand our understanding of the river ecosystem responses to human activities. Here, 28 urban and 24 nature reserve river surface sediment samples were collected in a river network spanning ~400 km located in the Qinling Mountains, Northwest China, and their geobiochemistry profiles and microbial populations were investigated. The data showed human activities resulted in significant increases in organic/inorganic nutrients, microbial population size and diversity in urbanized rivers. NMDS and db-RDA analysis together indicate that varying types of urban water pollutants were the primary drivers that governed the microbial community structure and function in the river network. The neutral community model and null model further indicated urban water pollutants act as the

dominant selective force to alter the microbial communities and increased the biotic homogenization in terms of both taxonomic and functional diversities. Further, biotic homogenization promotes urban river local biodiversity but reducing regional biodiversity. Overall, human modifications of the environment are reducing the biological differences that exist among river ecosystems.

1. Introduction

Rivers have been a preferred location for human settlements throughout history [1]. However, in recent decades, human activities such as rapid urbanization, industrialization and agriculture have discharged multiple anthropogenic contaminants and non-native (exotic) microorganisms into the river through industrial discharge, agricultural runoff, and municipal sewage [2, 3]. These contaminants can be either organic (e.g., antibiotics, polycyclic aromatic hydrocarbons, fertilizers, pesticides, manure) [4-7] or inorganic contaminants (e.g., nitrate, phosphorus, heavy metal) [8-10]. At the same time, river sediments often act as the recipients of reservoirs for these contaminants.

Extensive human activities and accelerated urbanization substantially influence the community structure and function of aquatic organisms and has become a major threat to river ecosystems. For examples, centralized wastewater treatment plants (WWTPs) are one of the most common systems for the treatment of urban wastewater. Several recent studies have presented evidence that the WWTPs effluent have detrimental effects on the river ecosystem functionality resulting in the degradation of habitats, deterioration in water quality, and reduces the abundance and diversity of benthic and sediment microbial communities [11-14].

WWTPs effluent are a common source of point-source pollution in the river ecosystem. In contrast, the immense industrial, domestic and agricultural activities sewage are considered as the primary contributors to non-point source pollution [2, 15]. Point-source pollution is easy to identify, but non-point source pollution is harder to identify and harder to address. United States Environmental Protection Agency reports that non-point source pollution is the leading remaining cause of water quality problems, yet there is little information available on the effects of widespread non-point source pollution on benthic microbial communities in river ecosystems [16, 17]. The potential ecological and health influences of non-point source pollution in river ecosystems require further research and monitoring.

Here, we focus on a large scale river network, which flows out of the Qinling Mountains, where it eventually empties into the Yangtze River (Fig. 1). The Qinling Mountains in Northwest China's Shaanxi Province are a geographically important north-south boundary in China, and an important natural barrier for the eastward flow of moisture from the Tibetan Plateau, providing a significant source of freshwater for downstream human communities. The distinctive feature of the river network is that it passes numerous cities (a current population of about 11.06 million) [18] and national nature reserves. Establishing nature reserves is an effective measure to avoid human activity disturbances [19, 20]. This river network provides a useful model for understanding the impact of non-point source pollution on the river ecosystem.

River sediments are considered as the major habitat of microbes in the aquatic environment [21, 22]. Therefore, sediment-associated microorganisms drive essential ecological processes and contribute significantly to river function [23]. In this study, the collected river surface sediment samples (0–2 cm deep) were located in eutrophic waterbodies (highly urbanized rivers) and oligotrophic waterbodies (undisturbed nature reserve rivers), based on trophic state index (TSI) (Fig. 1, Dataset 1B). Here, these collected surface sediments represent diverse non-point source pollution gradients, and creating multiple niches in this river network. We obtained millions of 16S rRNA tags from the river network sediments by deep sequencing, and attempted to elucidate the relationship between the microbial community and environmental factors in the river ecosystem.

2. Materials and methods

2.1 Study area, sampling procedure, and physicochemical analysis

Sampling was conducted in August-September 2022 within the river network. From each sampling site on three sampling occasions, water and sediment samples were collected within approximately 2 square meters at each site. The geographical data (longitude, latitude, and distance between sampling sites) were recorded by a GPS device (Dataset 1A). The water quality parameters, including water temperature, pH, dissolved oxygen (DO), water clarity of Secchi depth, transparency (SD), turbidity (TUB), total nitrogen (TN), nitrate-nitrogen (NO_3^-), ammonium-nitrogen (NH_4^+), total phosphors (TP), total dissolved phosphorus (TDP), phosphate, permanganate index, volatile phenol, *Chlorophyll*-a concentration were measured according to Environmental Quality Standards for Surface Water (Ministry of Ecology and Environment of People’s Republic of China, GB3838-2002).

Sediment total organic carbon (TOC) was measured by the Vario E1-III Elemental Analyzer interfaced with a Thermo Finnigan DELTA plus XL stable isotope ratio mass spectrometer. Each sample was performed in triplicate, and the values were expressed as the mean (n = 3).

2.2 Sediment cell counts

Immediately upon returning to the laboratory, composite sediment samples were prepared by the pooling and mixing of 3 replicates (10 g of each replicate) taken from each site (3 composite samples in total). Cell densities were investigated using catalyzed reporter deposition-fluorescent *in situ* hybridization (CARD-FISH) based on our previous studies [24, 25]. Cell counting was performed in triplicate, and the cell abundance were expressed as the mean (n = 3).

2.3 High-throughput sequencing

The frozen sediment samples (n=3) at each site were mixed for DNA extraction according our previous study method [26]. The V3–V4 hypervariable region of the 16S rRNA gene was amplified by PCR using bacterial and archaeal universal primers (515F and 806R). 16S rDNA amplicon samples were sequenced using Illumina MiSeq platform (Illumina Inc.). The raw data were analyzed according to our previous methods [24, 25].

Here, the “niche breadth” approach [27] was used to quantify habitat specialization. The formula is described below:

$$B_j = \frac{1}{\sum_{i=1}^{N} P_{ij}^2}$$

where B_j is the habitat niche breadth of OTUj in a metacommunity; and P_{ij} is the proportion of OTUj in a given site i, and N is the total number of sites. A higher B value of OTUs indicates a wider habitat niche breadth. In contrast, lower *B* values indicate that the OTUs occurred in fewer habitats and were unevenly distributed [28]. The average of B values from all OTUs in one community was regarded as the community-level B-value (*Bcom*) [29]. We calculated the *B* value based on species abundance and evenness for urban and nature reserve samples [30].

2.4 Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences

The Phylogenetic Investigation of Communities by construction of Unobserved States (PICRUSt2) software package was used to infer the metagenomics content of each sample, based on the 16S rRNA-based taxonomy and abundance of each OTU [31, 32]. The OTU table was imported into PICRUSt on the Galaxy web platform for functional gene prediction with reference to the KEGG (Kyoto Encyclopedia of Genes and Genomes) database [33]. These metagenomic interpretations were categorized into known functions associated with sequenced communities. Detailed descriptions of the process have been published previously [32]. In order to further identify potential microbial ecological functions at the studied sites, the obtained OTUs were compared with the FAPROTAX database [34] to predict potential metabolic functions of the microbial community.

2.5 Screening for antibiotic resistant bacteria from river sediments

The approach that has been used to evaluate the status of antibiotic resistance in the environment involves the calculation of resistance percentage, which is based on the ratio between the number of bacteria that are able to grow on culture media that are supplemented with antibiotics at doses close to the minimal inhibitory concentration (MIC) and the number of bacteria that grow on antibiotic-free media [35, 36]. The approach of heterotrophic plate count (HPC) method [37] was used to determine the concentration of antibiotic resistant bacteria in the collected river surface sediments. 0.1 g sediment samples were dissolved in 10 mL of 0.85% sterilized normal saline, and diluted 10 × fold for immediate processing. 100 μL diluted samples were cultured onto plate count agar medium. Medium contained four-class plates, 50.4 μg/mL sulfamethoxazole, 16 μg/mL tetracycline, 15 μg/mL azithromycin and no antibiotics. The media was added antifungal agent 200 μg/mL cycloheximide according to Gao el al., (2012) and Brooks et al. (2007) method. Total heterotrophic cultivable bacterial population was determined by plate count agar medium without antibiotics. The selected concentrations of antibiotics on the media were determined according to the criteria of the Clinical and Laboratory Standards Institute (CLSI) guidelines (2015) and the previous study [37-39]. Plates were incubated for 48–72 h at 37 °C [37, 40]. Duplicate counting was performed, and colony-forming units (CFU) were calculated.

2.6 Real-time quantitative PCR (qPCR)

Absolute quantification standard curve method was used to quantify the function genes in the sediment samples. The real-time quantitative PCR (q-PCR) (ABI7500, USA) using SYBR 2×RealUniversal PreMix (Tiangen Biotech). qPCR was performed to assess the abundance of the macrolide, tetracycline and sulfonamide antibiotic resistant genes (ARGs). The sequences of the primers and the reaction conditions used in this study are presented in Table S1 in the Supporting Information (SI) section.

The PCR reactions were performed in a 20-μL reaction mixture that comprised 10 μL of SYBR RealUniversal PreMix (2 ×), 0.4 μL of each type of primer (10/20 μM), and 1 μL of a DNA template (5 ng). The thermocycling protocol consisted of an initial denaturation for 1 min at 95 °C, followed by 40 cycles of denaturation (95 °C, 10 s) and annealing at the temperatures in Tables S1. The specificity of the PCR products was checked by a melting curve analysis. All qPCR assays were performed in triplicate for each gene,

The q-PCR standards were synthesized by cloning target genes into *Escherichia coli* DH5a using a PMD18-T vector Cloning Kit (TaKaRa Biotechnology, China). Vector concentrations were measured using NanoDrop, and the quantities of target genes per μL of solution were obtained basing on the length of plasmid and target gene sequence. Six-point calibration curves were generated by 10-fold serial dilutions of the plasmid carrying ARGs. All PCR reactions were run in parallel with serially diluted q-PCR standards and DNA-free water as the negative controls.

2.7 Statistical analysis

Null model analysis was carried out using the framework described by Stegen et al. (2012, 2013) to classify community pairs into underlying drivers of species sorting (or selection), dispersal limitation, homogeneous dispersal, and drift. The variation of both phylogenetic diversity and taxonomic diversity was measured using null model-based phylogenetic and taxonomic *β*-diversity metrics, namely *β*-nearest taxon index (*β*NTI) and Bray–Curtis-based Raup–Crick (RC_{Bray}). A significant deviation (i.e., $|\beta NTI| > 2$) indicates the dominance of selection processes. $\beta NTI < -2$ indicates significantly less phylogenetic turnover than expected (i.e., homogeneous selection) while $\beta NTI > +2$ indicates significantly more phylogenetic turnover than expected (i.e., variable selection). Subsequently, RC_{Bray} was used to further partition the pairwise comparisons that were not assigned to selection (i.e., $|\beta NTI| < 2$). The relative influence of homogenizing dispersal was quantified as the fraction

of pairwise comparisons with $|\beta NTI| < 2$ and $RC_{Bray} < -0.95$. Dispersal limitation was quantified as the fraction of pairwise comparisons with $|\beta NTI| < 2$ and $RC_{Bray} > 0.95$. The fractions of all pairwise comparisons with $|\beta NTI| < 2$ and $|RC_{Bray}| < 0.95$ were used to estimate influence of "undominated" assembly, which mostly consists of weak selection, weak dispersal, diversification, and/or drift.

A neutral community model was used to determine the contribution of stochastic processes to microbial community assembly by predicting the relationship between the frequency with which taxa occur in a set of local communities and their abundance across the wider metacommunity [41]. The model predicts that abundant taxa are more likely to be dispersed by chance and widespread across metacommunity, while rare taxa would be lost in different local communities due to ecological drift. In the model, the estimated migration rate is a parameter for evaluating the probability that a random loss of an individual in a local community would be replaced by dispersal from the metacommunity, and, therefore, is a measure of dispersal limitation. Higher *m* values indicate that microbial communities are less dispersal limited. The formula is as follows:

$$Freq_i = 1 - I(1/N \mid N*m*p_i, N*m*(1 - p_i))$$

where $Freq_i$ is the occurrence frequency of taxon *i* across communities; *N* is the number of individuals per community; *m* is the estimated migration rate; p_i is the average relative abundance of taxon *i* across communities; and *I*() is the probability density function of beta distribution. R^2 indicates the fit of the parameter based on nonlinear least-squares fitting.

2.8 Definition of trophic site groups

To evaluate the trophic status of each site, we calculated a trophic state index (TSI) based on the concentrations of TN, TP, Chl-a and SD in the water environment [42, 43]. Specifically, the calculation of the TSI was as follows:

$$TSI=0.326*TSI(Chl\text{-}a)+0.219*TSI(TN)+0.230*TSI(TP)+0.225*TSI(SD) \quad (1)$$

$$TSI(Chl\text{-}a) =10*[2.5+1.086*\ln(Chl\text{-}a)] \quad (2)$$

$$TSI(TP)=10*[9.436+1.624*\ln(TP)] \quad (3)$$

$$TSI(TN)=10*[5.453+1.694*\ln(TN)] \quad (4)$$

$$TSI(SD)=10*[5.118-1.94*\ln(SD)] \quad (5)$$

where TSI(Chl-a), TSI(TN), TSI(TP), and TSI(SD) are the trophic state index in relation to Chl-a (mg/L), TN (mg/L), TP (mg/L), and SD (m), respectively.

Five trophic levels were defined: oligotrophic (TSI < 30), mesotrophic (30 ≤ TSI ≤50), slightly eutrophic (50 < TSI ≤ 60), moderately eutrophic (60 < TSI ≤70) and highly eutrophic (TSI >70) [42]. For detailed information on the environmental variables of the trophic groups, see Dataset 1B.

2.9 Data available

Sequencing data are stored on the National Genomics Data Center (NGDC) Sequence Read Archive (PRJCA018972).

3. Results

3.1 Biogeochemical properties of river water and sediment samples

A total of 28 urban river and 24 undisturbed pristine nature reserve river surface (0-2 cm) sediment samples were collected (Fig. 1). Samples from all 52 locations covered a wide range of horizontal distance (0.72-376 km). The environmental chemical profiles of river water showed remarkable differences between the two regions (Fig. 2, Dataset 1A). The differences mainly included significantly higher concentration of total nitrogen (TN), ammonium, nitrite, nitrate, total phosphorus (TP), total dissolved phosphorus (TDP), phosphate, volatile phenol, Chlorophyll *a* in urban river samples (P<0.01). Ammonium and nitrate were the dominant forms of dissolved nitrogen within the rivers. The total organic carbon (TOC) content in sediment also showed a significant increasing trend from the natural river to urban river (P<0.0001) (Fig. 2).

The urban river sampling sites had a mean TSI of 53.07, ranging from 41.97 to 69.32, and most sites were eutrophic according to a TSI threshold larger than 50 (Zhang et al., 2018b). The nature reserve river sampling sites had a mean TSI of 36.23, ranging from 20.84 to 53.95, and most sites were oligotrophic and mesotrophic (TSI < 50). We found that the environmental variables related to eutrophication or nutrient levels generally showed significant relationships with TSI in both water and sediment habitats (P < 0.05). For instance, chlorophyll a, total nitrogen and dissolved total nitrogen in water tended to increase with increasing TSI, while the transparency decreased.

3.2 Microbial community abundance, richness and diversity

Microbial abundance ranged from 1.3×10^7 to 1.1×10^9 cells g-1 and 8.0×10^6 to 7.1×10^8 cells g-1 for urban and natural river samples, respectively (Fig. 3A, details in Dataset 2A). The abundance of microorganisms increased with increasing TSI, and the urban sites exhibited a significantly higher abundance of microorganisms than those from the natural river samples ($P<0.01$), indicated that the human activities have a great impact on microbial population.

Using universal primers for bacteria and archaea, 3,309,566 high-quality sequences were generated throughout the river network (Dataset 2A, SI Appendix Fig. S1). These high-quality sequences were clustered into 18,038 operational taxonomic units (OTUs, 97% similarity level) (Dataset 2B). Diversity within each samples was calculated at an equivalent sequencing depth across all samples using OTU-level based on three sediment composite samples per site (Materials and Methods). A range of 1,628–6,104 chao1 OTUs and 1.89–6.68 Shannon index were observed in the urban samples, and a range of 1,321–5,471 chao1 OTUs and 1.88–6.31 Shannon index were observed in nature reserve samples (Fig. 3B-C). The Chao 1 and Shannon in sediments increased with increasing TSI ($P < 0.05$). Both the Chao 1 and Shannon demonstrated that microbial richness and diversity were considerably higher in the urban river compared to the natural river samples ($P < 0.05$). Furthermore, the niche breadths (*Bcom*) (Fig. 3D) exhibited the narrowest *Bcom* values in urban areas, rather than those in natural river.

3.3 Microbial community structure and composition

80 microbial phyla and 3,570 species were identified in the urban samples, and 74 phyla and 3,371 species were identified in nature reserve samples (Dataset 2C-F). At the phylum level, the urban and nature reserve samples investigated here shared a high proportion of taxa. The ten most prevalent phyla in all of sites collectively constituted 95.48% of the total sequences (Fig. 4A), matching the classical abundance-range relationship [44, 45]. The dominant microbial phyla included Proteobacteria (28.69%), Firmicutes (28.32%), Actinobacteriota (17.79%), Chloroflexi (4.99%), Bacteroidota (4.51%), Acidobacteriota (3.51%), Planctomycetota (3.51%), Verrucomicrobiota (2.43%), Deinococcota (1.01%), Cyanobacteria (0.72%) (Fig 4A, Dataset 2C).

Archaea constituted only a minor fraction of the river sediment communities (0.02~4.96%), and an increase in the diversity and proportion of the archaea was identified in urban areas. In the urban river, proportion of the archaea exhibited 1.58 times higher than those in natural river, and archaeal Chao1 richness and Shannon diversity exhibited 1.47 and 2.36 times higher than those in natural river, respectively (Fig. 4A).

Although the microbial community of these sites exhibited similarities at the phylum level, 38 order in samples showed significant differences (P<0.01) between urban and nature reserve river ecosystems (Fig. 4B, SI Appendix Fig. S1B). For example, Bacillales, Clostridiales, Steroidobacterales were dominant in urban areas, while Erysipelotrichales, Deinococcales, Alicyclobacillales, Pyrinomonadales, Burkholderiales were dominant in nature reserve areas (SI Appendix Fig. S2B, Dataset 2C).

Further, a heatmap was generated to represent the 50 most prevalent species (Fig. S3), and most of these dominant OTUs were classified as unclassified species. Commonly observed with relatively high abundances in all samples were *Exiguobacterium-sibiricum*, *Exiguobacterium* sp., and *Acinetobacter lwoffii*. In urban samples, the dominant taxa included Unclassified Planococcaceae, Unclassified Steroidobacteraceae, Unclassified Anaerolineaceae, and Unclassified Rhizobiales. On the other hand, the unclassified *Acinetobacter*, unclassified Pseudomonas, unclassified Sphingomonadaceae, unclassified *Deinococcus*, *Pseudomonas parafulva* had high abundances in nature reserve samples.

The analysis of similarities showed that the urban samples harbored significantly dissimilar microbial communities at OTU level (Fig. 3E; PERMANOVA pseudo F = 2.1455, p.adj = 0.024, 999 permutations; ANOSIM R sqr = 0.0597, P = 0.03). Along the trophic gradient, we observed taxonomic homogenization for bacteria and archaea (Fig. 3F). Bacterial and archaea communities in the river network generally became more clustered in highly eutrophic urban sites, measured by the distance to group centroid (Fig. 3F).

3.4 Prediction of the functional diversity and metabolic pathways

Microbial community metabolic predictions guided by the FAPROTAX database [34] revealed distinctions in the metabolic potential related to nitrogen, carbon, and sulfur cycling between urban and nature reserve areas (Dataset 4A-D). For nitrogen transformation, the microorganisms involved in ammonia oxidation, nitrite oxidation, nitrogen fixation, nitrification at urban rivers was higher than that at nature reserve (ANOVA, P < 0.001). For carbon transformation, urban areas exhibited a higher abundances of microbial groups

associated with anoxygenic photoautotrophy, oxygenic photoautotrophy, chloroplasts, and photoautotrophy. Furthermore, the urban areas displayed a higher abundance of taxa involved in degradation of aromatic compound compared to the nature reserve (ANOVA, $P < 0.05$) (Fig. 3I). The sulfur cycling displayed heterogeneity among the samples. As depicted in Fig. 3I, the urban areas exhibited abundant taxa associated with the dark oxidation of sulfur compounds (ANOVA, $P < 0.05$). Furthermore, the abundance of taxa involved in sulfate respiration was higher in urban areas.

Additionally, the PICRUSt2 database [31] annotated 414 functional KEGG pathways (Dataset 4C-F). The metabolism associated with carbon fixation, nitrogen, sulfur, phosphate, antibiotic biosynthesis and xenobiotic biodegradation were investigated. The presence of antibiotic resistance proteins was also detected (Dataset 4B, D, F). In all samples, biodegradation pathways encompassing 21 types of xenobiotic compounds were determined (Dataset 4F-G). The ecological function of xenobiotic biodegradation exhibited a noticeable increasing trend from the nature reserve to the human-impacted sites. This trend suggested an increase in pollutants discharge from urban rivers. Styrene degradation emerged as the predominant process among polycyclic aromatic hydrocarbon (PAH) degradation (Dataset 4G). Combined the microbial community structure and functional diversity indicated that more sensitive species may be replaced by more tolerant species following environmental change, leading to increase of the similarity in species features, i.e., functional homogenization (Fig. 3I).

3.5 Antibiotic resistance rates and genes

Three kinds of antibiotic, macrolides, tetracycline and sulfonamide, were selected for antibiotic resistance rates (ARRs) test. Macrolides and tetracyclines are among the most widely used antibiotics globally for both human and agricultural applications [46, 47]. Sulfonamides were the first synthetically produced antibiotic for widespread use and extensive resistance has since emerged among clinical isolates [48].

All of collected samples in this study showed macrolide, tetracycline and sulfonamide resistance activity. Fig. 5A illustrated the macrolides, tetracycline and sulfamethoxazole resistance rates on plating count agar. The urban samples antibiotic resistant rate ranged 11.2% - 29.5 % for azithromycin, 13.0-29.0 % for tetracycline, 11.0-26.9 % for sulfonamide. The natural river samples antibiotic resistant rate ranged 2.4-18.2% for azithromycin, 4.0-16.0 % for tetracycline, 2.4-16.0 % for sulfonamide. The antibiotic resistant rate in urban samples were higher than those in natural river samples. In the natural river samples, no

significant differences in antibiotic resistance rate were observed, but variability in antibiotic resistance rates were observed in the urban samples.

Five antibiotic resistance genes (ARGs) representing three classes of resistance (two sulfonamide: *sul*1 and *sul*2; two tetracycline: *tet*O and *tet*W; and one macrolide: *erm*F) (Fig. 5B) were selected for quantification in all samples to characterize how loads of ARGs change between urban and natural rivers. The five target ARGs were selected due to their documented prevalence in watersheds impacted by anthropogenic and agricultural runoff [49]. All five of these genes were among those identified as candidate indicators of the extent of the impact of antibiotic resistance in the environment by the European Cooperation in Science and Technology Action [35].

The ARGs were found in all samples, and Fig. 5B shows the occurrences and quantities of ARGs in the sediments of the urban and natural rivers. The concentrations of ARGs varied greatly, ranging from 1.04×10^3 copies/g (*sul2*, Natural River) to 4.10×10^8 copies/g (*tetO*, urban river). The quantity of ARGs in the urban river ranged from 1.36×10^4 to 1.63×10^8 copies/g for *sul1*, 2.27×10^4 to 3.00×10^8 copies/g for *sul2*, 5.36×10^3 to 4.10×10^8 copies/g for *tetO,* 2.52×10^3 to 2.17×10^8 copies/g for *tetW,* 1.72×10^3 to 2.02×10^8 copies/g for *ermF*. The quantity of ARGs in the natural river ranged from 1.42×10^3 to 9.49×10^6 copies/g for *sul1*, 1.04×10^3 to 1.9×10^7 copies/g for *sul2*, 1.49×10^3 to 2.5×106 copies/g for *tetO,* 2.34×10^3 to 9.45×106 copies/g for *tetW, 2.12* $\times 10^3$ to 5.52×10^6 copies/g for *ermF*. The quantity of ARG abundances in the sediments of the urban river were higher than those in the sediments collected from the natural river. A wide concentration range for each class of resistance genes was observed in the urban river in the tetracycline, macrolide and sulfonamide resistance genes. In contrast, this trend was not observed in the natural river.

3.6 Key influencing factors and ecological driving processes

Across the large river network spanning a geographic distance of ~400 km, the microbial community increased in dissimilarity with increasing spatial distance, showed a distance-decay relationships (DDRs) (Fig. 6A, Dataset 2G). However, we observed microbial communities in the natural river sites had a steeper DDR slope between sampled sites than those in urban sites (Fig. 6A), indicated the environmental selection have more strong effect on the urban river sites. The distance-based redundancy analysis (dbRDA) using environmental parameters as constraining variables, highlighted the potential significance of these chemical parameters in elucidating microbial community compositions (Fig. 3F). Ammonium (r sqr= 0.3551, *P*=0.001), pH (r sqr= 0.2293, *P*=0.005), Nitrite (r

sqr=0.2211, *P*=0.003), Nitrate (r sqr=0.188 *P*=0.006), TN (r sqr= 0.17, *P*=0.006), TOC (r sqr= 0.1367, *P*=0.032), phosphate (r sqr= 0.0615, *P*=0.010) were more crucial in shaping the microbial community (Fig. 3F).

To further assess the underlying factors governed community dynamics in the river network, the null model [50] and neutral community model (NCM) [41] were used to quantify the relative influences of deterministic and stochastic processes in our study. The null model indicated a prevalence of environmental selection processes, particularly in the form of homogeneous selection (|βNTI| < 2, RCbary >+0.95) (Fig. 6B, Dataset 3), in governing the urban river community assembly (details in Materials and Methods). Simultaneously, neutral community model (Fig. 6C) showed a gradual decline in the contribution of stochastic process with the increase of human activity and eutrophication, elucidating 58.5% and 48.3% of the stochastic process within the nature reserve and urban samples, respectively.

4. Discussion

4.1 Non-pointed source pollutions changed the river community diversity, compositions and function

Considerable variation exists within the bacterial and archaeal communities in terms of abundance, richness, diversity (Fig. 3A-C) and composition (Fig. 3E) between urban and natural rivers, accompanied by significant niche differentiation (Fig. 3D). Nonmetric multidimensional scaling (NMDS) (Fig. 3F) and network analysis (Fig. 3I), based on OTU, demonstrated distinct differences between the microbial communities of urban and those of the natural river ecosystems. The impact of human activities on river microbial communities was also evident in the predicted metabolic functions of these communities (Fig. 3I). This differentiation persisted even when considering the exclusion of "rare biosphere" taxa (< 0.1% total sequence). Here, we further investigated key function taxa, including nitrogen-transformation microorganisms, pathogens, Polycyclic Aromatic Hydrocarbons (PAHs)-degrading bacteria, antibiotic-resistant bacteria and the archaea in the urban river samples.

(i) Urbanized rivers exhibited a notably higher abundance of nitrogen transformation taxa groups (Dataset 2E-F, *P* < 0.01), including nitrogen-fixing bacteria (*Anaeromyxobacte*) [51], nitrifying bacteria (*Nitrosomonas, Nitrospina, Nitrosospira*, *Nitrospira*) and denitrifying bacteria (*Paracoccus, Hyphomicrobium*, and *Gemmobacter*) [52]. The 16S rDNA

sequencing-inferred physiology involvement nitrogen transformation function showed a similar pattern trend in the urban river (Fig. 3I, $P < 0.01$). Previous studies showed that high abundance of nitrogen transformation taxa was correlated with high nutrient availability [53, 54].The pronounced increase of nitrogen transformation taxa in direct proportion with the TP and inorganic nitrogen content (Fig. 2) in the urban rivers might be fueled by the high nutrient sources from sewage.

The db-RDA analysis further suggested that nitrogen (often in the form of nitrate and ammonium) and pH contributed significantly to the changes in the urban river sediment microbial community (Fig. 3G). The high nitrogen loading and lower pH (Fig. 2), are common consequences of the continuous input of industrial, domestic, and agricultural wastewater, which was caused by a large population of about 11.06 million in sampled regions [18]. Excessive nitrogen inputs can lead to eutrophication, a process where the nutrient enrichment promotes the growth of algae (Chlorophyll *a* content in Fig. 2) [55]. The proliferation of algae leads to increased organic matter production, which is subsequently decomposed by bacteria and archaea (TOC content in Fig. 2). The ‘‘productivity/resources hypothesis’’ posits that greater resource availability and/or primary production can support larger population sizes and limit local extinction, promoting species coexistence [56]. Therefore, the influx of organic compounds and nutrients into the urban river created a more uniform environment, allowing for the coexistence of a broader range of microbial species. The increased availability of organic matter and the changed nutrient conditions can profoundly influence the river microbial population, and enhanced the microbial population size and diversity in urbanized rivers.

(ii) High abundance of Bacteroidetes and Firmicutes were observed in the urban river (Dataset 2C, $P <0.01$). These taxa, in general, have been frequently found in WWTPs and wastewater-influenced environments [57], which are recognized as good indicators of fecal contamination [33, 58]. The elevated abundance of Bacteroidetes and Firmicutes in our collected urban river sediment samples suggested the presence of untreated wastewater from residential areas in these areas, which may contain fecal pollutants. Additionally, according to Pathogen–Host Interaction (PHI) database [59], our study also identified a marked increase in the abundance and diversity of pathogens like *Acinetobacter*, *Arcobacter*, *Exiguobacterium*, *Chryseobacterium*, *Escherichia Shigella* and *Staphylococcus aureus* within urban river (Dataset 2E-F, $P < 0.01$). These findings highlighted urban non-point source untreated water pollutants have resulted in fecal contamination in the river network. Furthermore, such pathogens also have demonstrated a remarkable capacity to

degrade and tolerate a wide array of organic compounds found in sewage water [60, 61]. This dual capability underscored their adaptability to environments influenced by urban non-point source water pollutants and also suggested their involvement in sewage degradation processes [62, 63].

(iii) High abundances of ecological function linked to xenobiotic biodegradation [64] were detected in urban sites (Dataset 4F). As we known that the presence of biodegradation genes could serve as indicators for the existence of xenobiotics and their corresponding metabolites [65, 66]. Furthermore, high abundance of aminobenzoate, benzoate, naphthalene were observed in the urban segment of the river (Dataset 3G), which was also accompanied by increased abundance of potential PAHs degrading bacteria, such as *Sphingobium*, *Rhodococcus*, *Novosphingobium*, *Pseudomonas*, *Sphingomonas*, *Brevibacterium*, *Arthrobacter*, *Nocardioides,* and *Mycobacterium* were observed in the urban river (Datset 2E, $P < 0.01$) [67]. It suggested the accumulation of PAHs in our collected urban river sediment samples. PAHs primarily originating from long-term anthropogenic pollution sources linked to rapid industrialization and urbanization [68, 69]. These PAHs pollutants, characterized by persistence and low solubility, tend to accumulate in river sediments due to their hydrophobic nature and strong adsorption onto sediment particles [5, 68]. Numerous urban rivers in China have been identified as containing high abundance of PAHs [70, 71], and significant high biodegradation of PAHs capacity has also been observed in urban areas [72, 73].

(iv) Functions linked to drug and antimicrobial metabolism were identified in urban river and nature reserve river sediment samples (Dataset 4D). In our study, a higher ARRs and ARGs were observed in highly urbanized river areas (Fig. 6A-B). The presence of antibiotics in the river is a widespread concern. Zhang et al. (2015) reported that over 68% of total antibiotic usage in China in 2013 was excreted by humans and animals, leading to around 53,800 tons of antibiotics entering the environment [74]. Urban water ecosystems have been identified as potential hotspots for the spread of antibiotic resistant bacteria in China [75, 76]. The continuous selective pressure exerted by antibiotic residues, even at sub-inhibitory concentrations, in combination with the diverse and dense microbial communities sustained by nutrient-rich environments, creates favorable conditions for the dissemination and proliferation of antibiotic resistance [36].

Notably, the data showed that significant variation between cities in antibiotic resistance rates and genes, with no significant differences in natural river samples (Fig. 6). This

correlation was highly significant for antibiotic use and population. This feature emphasized the importance of the different levels of human activity contribution based on population size, potentially resulting in varied antibiotic resistance rates among urban regions. Additionally, pollutants from human activities, such as heavy metals and disinfectants, further exerted selective pressure on bacteria, contributing significantly to antibiotic resistance development [77].

(v) An increase in the diversity and proportion of the archaea was also identified under the impact of human activities pollution effluent. In the urban river areas, proportion of the archaea exhibited 1.58 times higher than those in natural river, and archaeal Chao1 richness and Shannon diversity exhibited 1.47 and 2.36 times higher than that in the natural river, respectively. This finding is similar to previous study revealed that in a high-contamination zone, archaeal Chao1 richness and Shannon diversity exhibited substantial increases, approximately 1.81 and 1.56 times higher than those in a low-contamination zone, respectively [10]. Krzmarzick et al. (2018) proposed that archaea have demonstrated a notable capacity for enduring harsh environments, leading to the possibility that numerous archaeal species possessing exceptional adaptability to severe anthropogenic pollution could flourish in urban river environments [78].

Overall, our research demonstrated that human population alongside contamination level serve as the predominant influences shaping urban river microbial communities in the river network ecosystem. Some bacterial and archaeal communities might have adapted to these altered conditions over time [79, 80]. Therefore, the changed environmental conditions can potentially favor specific bacterial and archaeal taxa, leading to shifts in community composition. These changes in nutrients have cascading effects on ecosystem functioning and overall microbial processes in the river network. Given the human activities that carry a myriad of exotic microorganisms and organic/inorganic contaminants into the urban river, urbanization can profoundly change the riverine ecosystems [11, 81].

4.2 Urbanization non-point source pollution increased biotic homogenization

In this study, river microbial communities exhibited high diversity, encompassing thousands of OTUs across major taxa that inhabit various habitats within the river ecosystem (Fig. 3-5). The distance-decay relationship (DDR) was observed for both urban and natural river samples (Fig. 6A). Understanding the mechanisms of interaction of diverse ecological processes in microbial community assembly is a central question in the field of river

biogeography [82]. Stochastic neutral models postulate that water mass transport must interact with dispersal limitation to create a distance-decay pattern [83-85]. However, recently, it has been generally accepted that both niche-based deterministic and neutral-based stochastic processes occur simultaneously in the assembly of microbial communities [86-88], and the relative contributions of different processes vary depending on geographic scales, strength of environmental gradients, and the type of organisms[89].

Previously, the investigation on the impacts of geographic distances and environmental conditions on microbial community assembly has been a research hotspot in river ecology [86, 87]. In large scale watersheds such as Yangtze River and Tingjiang River, geographic distance was proven key factor governing community succession [10, 90, 91]. However, we observed microbial communities in the natural river sites had a steeper DDR slope between sampled sites than those in urban sites (Fig. 6A). In other words, the geographic variation in microbial community composition is lower across cities than across natural river ecosystems. Furthermore, our analyses also show narrower niche breadths were witnessed for samples in urban river (Fig. 3D), and a greater homogenization in the community composition along the urban river than across the corresponding natural river ecosystems (Fig. 3F), suggested that persistent uniform non-point source pollution such as high nitrogen, PAHs, antibiotic and so on arises from urbanization human activity exert robust environmental selection deterministic influences on river system. Therefore, similar urban environmental condition could promote community fusion, contributing to a more similar community structure (Fig. 3F-H) and function (Fig. 3I), and indicating the taxonomic and functional homogenization occur simultaneously in the river network.

The application of the neutral community model (Fig. 6C) revealed that deterministic processes predominantly shaped the urban river microbial communities in the river network. Null model further uncovered the homogeneous selection dominated governs the urban river community assembly (Fig. 6B). This finding suggests that the accelerated eutrophication rise from non-point source pollution created a more uniformity environment in urban river system. As discussed by Olden and Poff (2003), functional homogenization occurs when the same kind of ecosystem becomes more uniformly widespread. As indicated by the trends noted above, intensively urbanized ecosystems are sometimes relatively simple, with uncomplicated food webs and dominated by abiotic controls and imported food subsidies. Thus, it seems likely that the spread of similar species and similar physical conditions in the intensively modified urban river habitats will tend to produce an increase in functional homogenization.

According to the United Nations, 68% of the global population will live in cities by 2050, increasing the environmental and social stresses for the billions of humans living in urban areas. Previous studies showed that human-induced land-use changes result in the biotic homogenization of soil microbial communities [92]. When measured by extent and intensity, urbanization non-pointed source pollutions is one of the most homogenizing of all major human activities [93]. Cities homogenize the physical environment because they are built to meet the relatively narrow needs of human.

Our results, emphasizing a convergence (reduced geographic variation) in the urban rivers microbiome and function, are consistent with the effects of urbanization on bird [94], fish [95], plant [96], invertebrate [97, 98], zooplankton [99], dust samples [100] communities. Together, our findings provide novel evidence that accelerated urbanization and eutrophication cause river microbial community and functional homogenization and lead to enhance the local (alpha) diversity of microbes.

4.3 Urbanization biotic homogenization promotes local biodiversity but reducing global biodiversity

The non-native species often enrich urban river local biodiversity [101]. Hence, the urban river microbial species richness and diversity is generally enriched relative to surrounding natural habitats (Fig. 3A-D), and promote 274 non-native species establishment (Fig. 3E). Urbanization alters the natural selection regime, often putting native species at a competitive disadvantage. Previous many studies show that the construction and expansion of towns and cities promote the loss of native species and their replacement by non-native species [93]. In our study, as indicated by the discussion 4.1 noted above, the massive non-pointed source pollutions created by city growth not only destroy the habitat of native species but they create habitat for species that are adapting to urban river conditions. This process of replacing localized native species with increasingly widespread non-native species promotes biotic homogenization on spatial scales.

However, urbanization biotic homogenization has the effect of increasing urban local biodiversity (Fig. 3B-C) but reducing regional biodiversity (3499 species in the urban river sites, 3736 species in natural river, Fig. 3E). The non-native biota becomes increasingly enriched in species that are spreading to many parts of the river network to replace many natural river locally unique, endemic native species. Hence, urbanization can drive biotic homogenization by reducing trait and phylogenetic differences in species assemblages between regions, thus diminishing the regional distinctiveness of biotas and likely have

negative impacts on key ecosystem functions. The global diversity is decreased by the subsequent extinction of unique regional species that are lost to the global species pool.

The problem for biodiversity conservation is that despite this high species richness in urbanizing areas, including the importation of non-native species, global biodiversity continues to decline. This pattern of local enrichment but global decline is a crucial one for conservation biology because it may divert public attention away from the more global problem of global species decline. Consequently, as cities expand across the planet, biological homogenization increases because the same “urban-adaptable” species become increasingly widespread and locally abundant in cities across the planet.

This result provided an excellent illustration of the concept of biotic homogenization, which suggested that human modifications of the environment are reducing the biological differences that exist among natural ecosystems. This process is predicted to result in a more homogenized biosphere on regional and global scales [93] and reducing global biodiversity. Further investigations are needed to explore the universality of biotic homogenization due to human activity effluents across a range of river ecosystems.

5. Conclusions

Despite the rivers' inherent capacity for dilution and self-purification, the river network was vulnerable to non-point source pollution from human activities. We found that urban river is important hot spots of local microbial taxonomic and functional diversity but also support a more homogenization in the structure and function of the river microbiome. As a result, biotic homogenization promotes urban river local biodiversity but reducing regional biodiversity. More specifically, we show that urban river harbor more similar microbiomes than adjacent natural river ecosystems. The urban river is characterized by higher proportions of fast-growing bacteria, algae, nitrifiers, PAHs degradation bacteria, and important pathogens, fecal coliform bacteria and antibiotic resistant bacteria, which were particularly dominant in the nutrient rich, more population, and more intensively human activity urban river. Our analyses indicate that anthropogenic pollutants stand out as the principal drivers altering microbial communities, consequently augmenting the role of homogeneous selection in shaping urban microbial community assembly. Together, our study represents a comprehensive assessment of the structure and functional differences in the urban and natural river microbiome.

Acknowledgements

This work was supported by the Youth Program of National Natural Science Foundation of China (No. 32100081), Central Public-interest Scientific Institution Basal Research Fund, CAFS (NO.14).

Competing Interest Statement

The authors declare no competing interests.

Declaration of Generative AI and AI-assisted technologies in the writing process

Statement: During the preparation of this work the authors used CHATGPT in order to improve readability and language. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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Figure

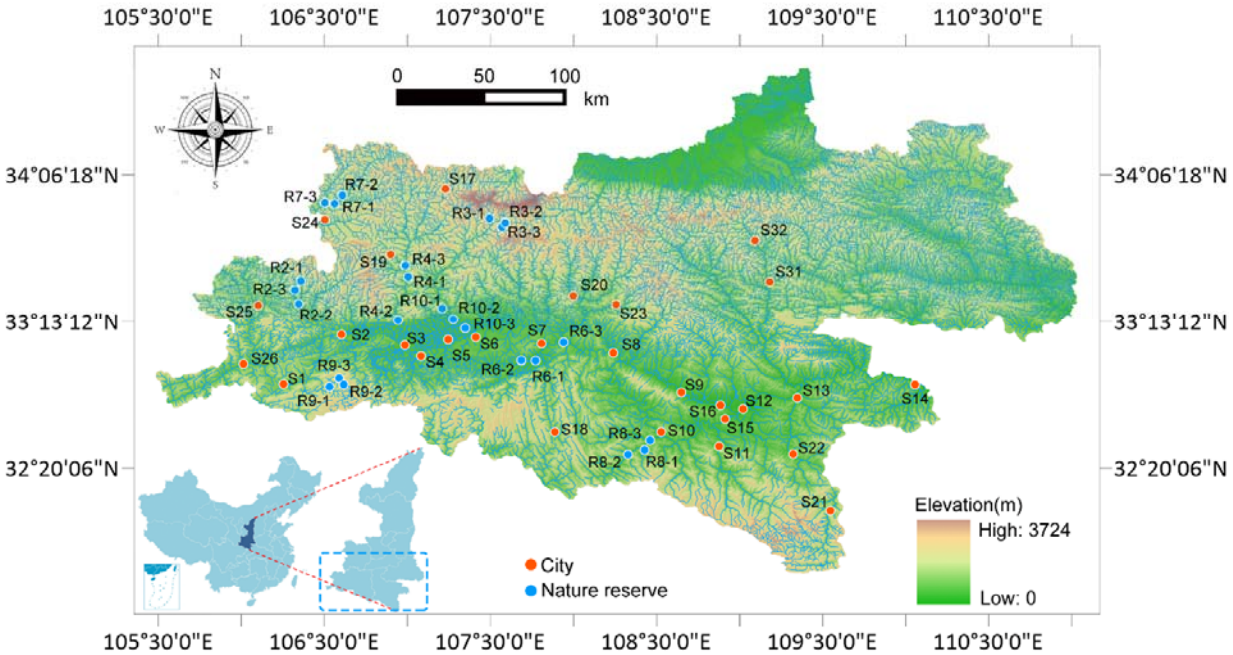

Figure 1. Location of the collected river network sediment samples. Orange circles indicate the urban river sites, and blue circles indicate the nature reserve river sites, respectively. The Inset map shows the extent of the study area relative to China.

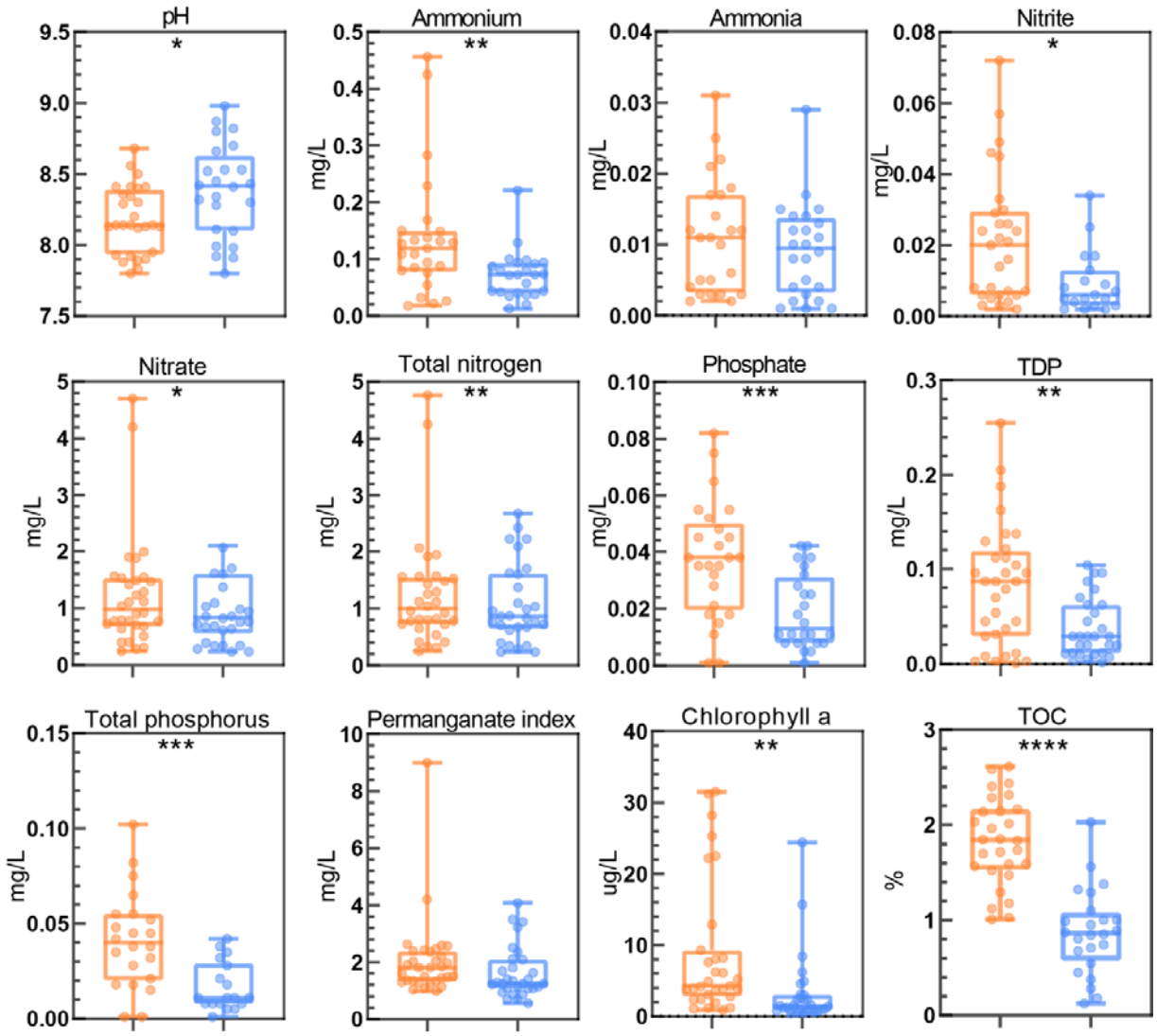

Figure 2. Comparison of geochemical characterization in human-impacted urban rivers and undisturbed natural rivers in the river network. The orange circles indicate urban rivers and blue circles indicate natural rivers, respectively.

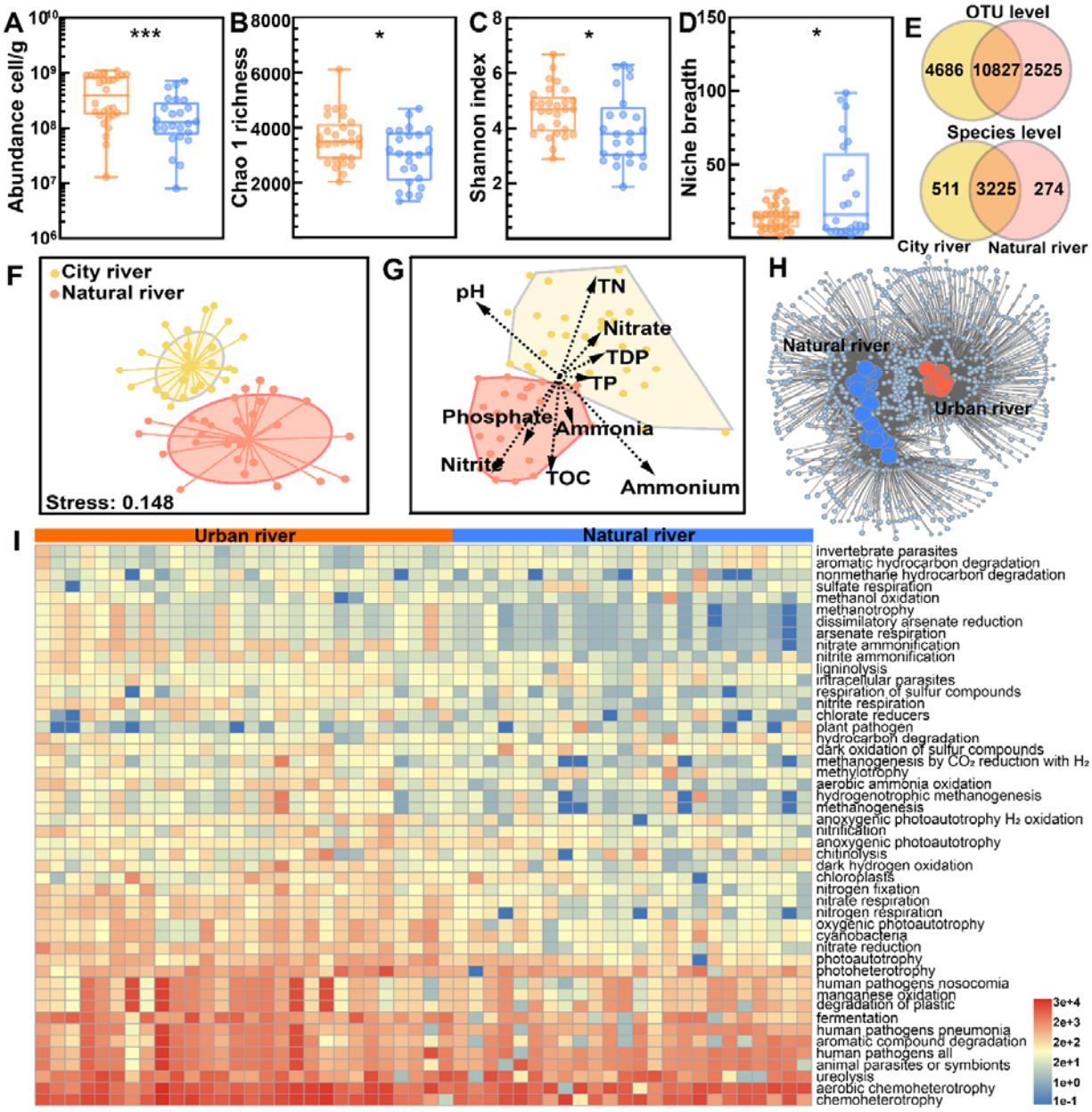

Figure 3. The microbiome in the large river network. (A-D) Microbial abundance, Richness estimates, Shannon index and Niches breadth were investigated in urban (orange circles) and natural (blue circles) river samples. (E) Venn analysis. (F) Similarity of microbial communities visualized by two-dimensional (2D) nonmetric multidi-mensional scaling (NMDS). (F) A distance-based redundancy analysis (db-RDA) showing microbial community similarity when environmental factors were used as constraining variables and were fitted onto the ordination as arrows. The length of each arrow indicates the multiple partial correlation of the variable to RDA axes and can be interpreted as an indication of that

variable's contribution to the explained community similarity. (I) Prediction of the functional diversity and metabolic pathways between urban and natural river samples.

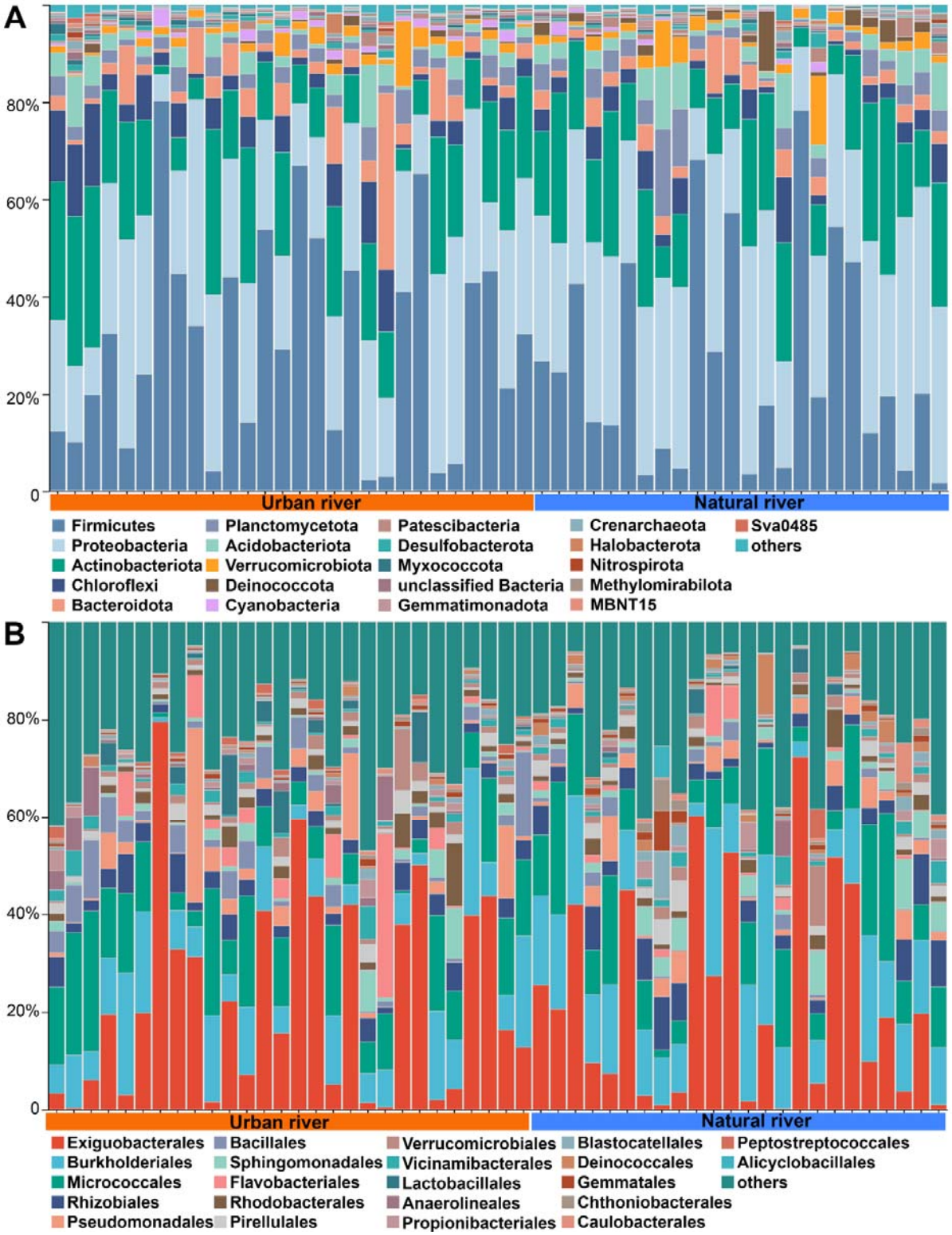

Figure 4. Relative sequence abundance of bacterial and archaeal. The graph shows the relative abundance of the most abundant bacterial and archaeal at phylum (A) and classes (B) level in urban and natural river according to average relative abundance across all samples.

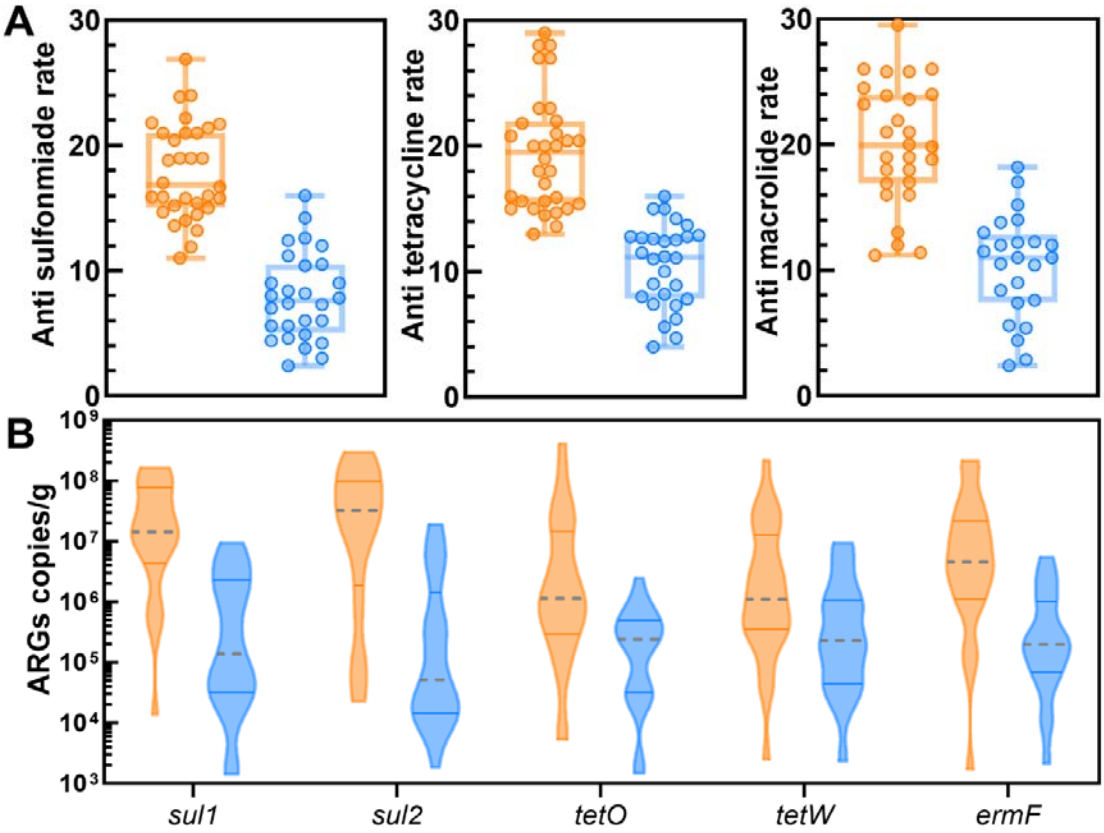

Figure 5. Different of antibiotic resistance rates (A) and antibiotic resistance gene abundance (B). The orange circles indicate the urban river and blue circles indicate the natural river, respectively. While no significant differences were observed among the natural river, the antibiotic-resistant rates exhibited variability among the samples of the urban river.

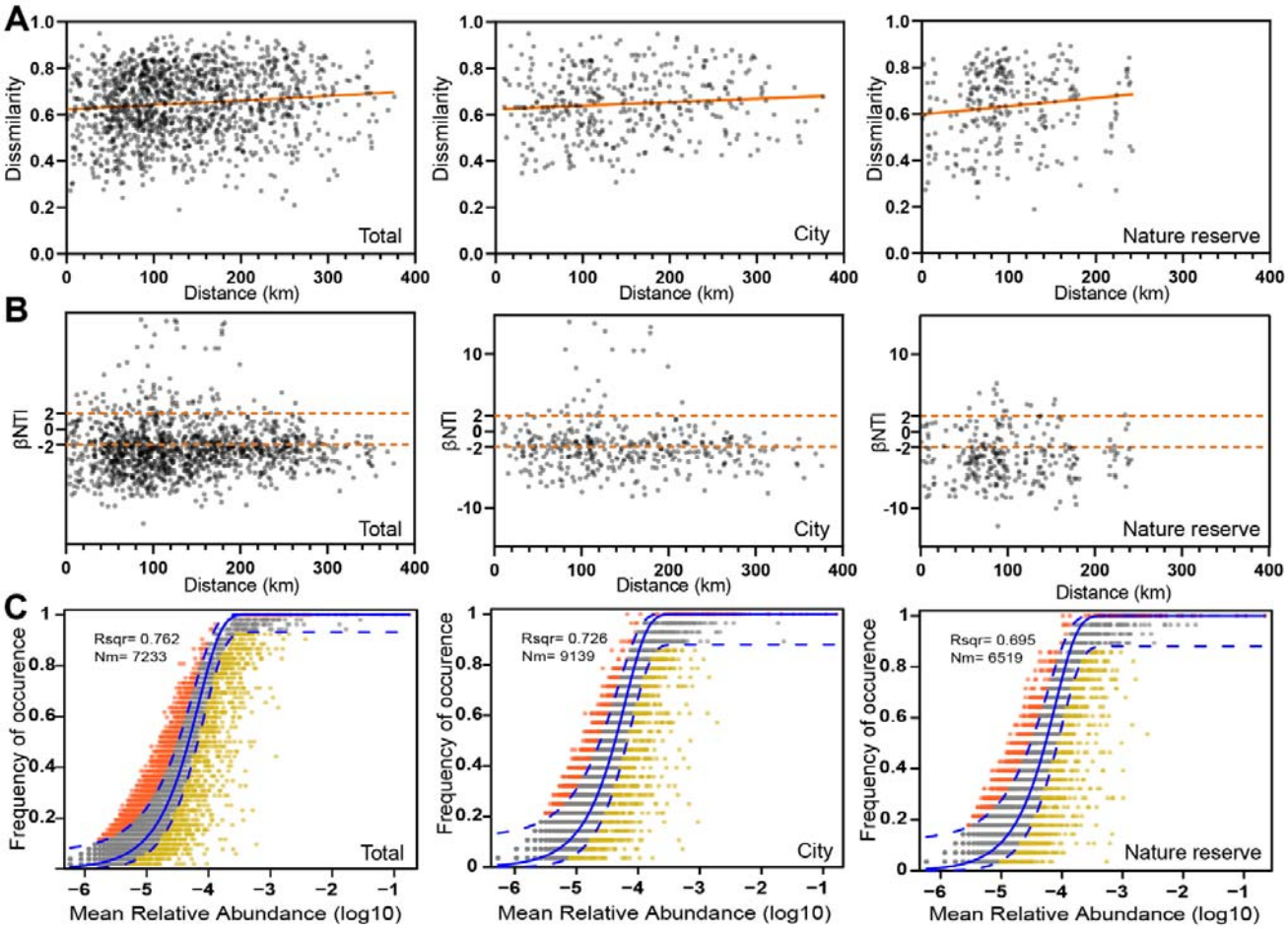

Figure 6. General patterns of microbial diversity in the river network system. (A) Distance-decay curves showing Bray–Curtis dissimilarity against geographic distances between sampling sites. Solid lines denote the ordinary least-squares linear regressions. (B) Scatter plot of βNTI values grouped by spatial scales of the river network. (C) Fit of the neutral community model (NCM) of community assembly. The solid blue lines indicate the best fit to the NCM as in Sloan et al. (2006), and the dashed blue lines represent 95% confidence intervals around the model prediction. OTUs that occur more or less frequently than predicted by the NCM are shown in different colors. Nm indicates the metacommunity size times immigration, R^2 indicates the fit to this model.