A highly connected river network continuum is not resilient to non-point source pollution from anthropogenic activity

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Highlight

Increased bacterial and archaeal diversity was provoked by multiple anthropogenic pollutants in the river network.
Sewage discharge and high nitrogen loadings reshaped the microbial composition in urban areas.
Homogeneous selection imposed by anthropogenic pollution stress primarily shaped assembly of bacterial and archaeal.
Environmental factors contribute more to the microbial variation than dispersal in the study area.

Abstract

Rivers have been a preferred location for human settlements throughout history. Comparing the bacterial and archaeal communities in highly human-impacted areas and undisturbed pristine nature reserves could expand our understanding of the mechanisms of river ecosystem responses to environmental changes resulting from anthropogenic contaminants. In this study, 24 urban and 28 nature reserve river sediment samples were collected in a highly connected river network spanning ~400 km, and their geobiochemistry profiles and microbial populations were investigated. The data showed that human activities had discharged significant amounts of nutrients and even exotic microorganisms into the river, resulting in significant changes in the microbial community and potential function, including an increase in pathogens, antibiotic-resistant bacteria and PAHs-degrading bacteria. Environmental selection was more important than dispersal limitation in the river network, and environmental factors were the best predictors of the variation observed between microbial communities. Specifically, homogeneous selection imposed by anthropogenic pollution stress lead to higher microbial diversity but a lower habitat niche breadth in urban river areas. Collectively, the study suggests that community composition and diversity dynamics of the river network are not random but driven by environmental factors. The natural buffering capacity of highly connected river network ecosystems is unable to fully resist the impacts from human activity pressures.

Introduction

Rivers play a crucial role in aquatic ecosystems as they host the highest levels of biodiversity on Earth (Widder et al., 2014). Rivers create hierarchic dendritic networks, shape the Earth's surface and, in their sediments, provide a significantly expansive habitat for microorganisms. Hence, sediments often harbor the majority of biomass within the river (Gibbons et al., 2014; Battin et al., 2016), drive essential ecological processes and contribute significantly to global biogeochemical cycles (Battin et al., 2016; Xie et al., 2016). In the river ecosystems, water flow and related deliveries of nutrients and organic matter to sediments constitute major constraints on sedimentary microbial life.

River networks represent a continuum of physical interlinked habitats (Doretto et al., 2020). A well-connected river network enhances the cycling of nutrients and energy, thereby promoting the dynamics and assembly of microbial communities (Phillipsen et al., 2015; Shao et al., 2019). However, human activity such as rapid urbanization, industrialization and agriculture that dumps multiple anthropogenic contaminant and non-native (exotic) microorganisms into the river network ecosystems, which drastically change the river biogeochemical cycle and poses an important threat to river functions (Wang et al., 2012; Beattie et al., 2020). These contaminants can be either organic (e.g., antibiotics, PAHs, fertilizers, pesticides) (Singh et al., 2019; Patel et al., 2020; Xu et al., 2023) or inorganic contaminants (e.g., nitrate, phosphorus) (Pruden et al., 2012; Manyi-Loh et al., 2016).

The urban river microbial communities are under a range of anthropogenic pollutant stressors (Saxena et al., 2015). Thus, urban rivers constitute a distinctive ecosystem characterized by persistent anthropogenic contamination (Devarajan et al., 2015). Human-induced contamination creates more diverse but physically connected local habitats (Isabwe et al., 2019). However, the microbial community response to environmental changes in urban river ecosystems under high anthropogenic activity is poorly understood, potentially leads to the significant reshaping of microbial community.

Here we focus on a large scale river network, which flows out of the Qinling Mountains, where it eventually empties into the Yangtze Rivers. The distinctive feature of the river network is that it passes numerous cities and national nature reserves. The establishment of nature reserves is effective and direct measures to protect biodiversity (He, 2009), and to keep nature reserve free from human activities (Dudley, 2008; Xiao et al., 2022). However, with the increasing population and the acceleration of urbanization, the conflict between human activities and nature reserves has become increasingly frequent. Hence, this river network provides a useful model for understanding relationships between the human land-use practices and microbial diversity.

In this study, the collected river sediment samples were located in areas with various levels of human activity, ranging from highly human-impacted urbanized rivers to undisturbed nature reserve

rivers. These sites could represent environmental gradients emanating from human activity, thereby creating potential niches in this river network. We obtained millions of 16S rRNA tags from the river network sediments by deep sequencing, and attempted to elucidate changes in bacterial and archaeal community while evaluating relationships among environmental factors, microbial community composition, spread of pathogens, and drug resistance in the river ecosystem.

Results

Biogeochemical properties of overlying water and sediment samples

A total of 28 human-impacted urban sites and 24 undisturbed pristine nature reserve sediment samples were collected. They covered a wide range of water depths (0.2-20 m) (Fig. 1, Dataset 1A), and a wide range of horizontal distance (0.72-376 km) (Dataset 3). The environmental chemical profiles of overlying water showed remarkable differences between urban and nature reserves samples (Fig. 2). 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 ($P<0.01$). Ammonia content and permanganate index of the water do not differ significantly, but they are higher in urban samples. Ammonium and nitrate were the dominant forms of dissolved nitrogen within the rivers. The total organic carbon (TOC) content in sediment also showed a same increasing trend from the nature reserve to urban areas (Fig. 2).

Microbial community abundance, richness and diversity in the river network.

The cell densities in the sediment samples of the extensive river network were investigated using CARD-FISH. 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 nature reserve samples, respectively (Fig. 3A, details in Dataset 2A). The sample from urban sites exhibited significantly higher abundance of microorganisms than those from the nature reserve samples ($P<0.01$), indicating 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). 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. 3, Dataset 2A). Notably, both the Chao 1 and Shannon demonstrated that microbial richness and diversity were considerably higher in the urban samples compared to the nature reserve samples ($p < 0.05$). Furthermore, the niche breadths (Bcom) (Fig. 3D) exhibited the narrowest Bcom values in urban areas, rather than those in nature reserve areas.

Microbial community structure and composition in the river network

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 (Fig. 4A). The ten most prevalent phyla in all of sites collectively constituted 95.48% of the total sequences (Fig. 4 A, Dataset 2C), matching the classical abundance-range relationship (Amend et al., 2013; Ruff et al., 2015). 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). It's worth noting that Archaea constituted only a minor fraction of the river sediment communities.

Although the microbial community of these sites exhibited similarities at the phylum level, 38 order in samples showing 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 2D). Furthermore, 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). This data demonstrates that urban and nature reserve habitats exhibited different microbial community structure at the broad taxonomic resolution level.

A heatmap was generated to represent the 50 most prevalent species (with relative abundances exceeding 1% in individual samples) (Fig. 5), 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.

Further, the presumed functional taxa associated with nitrogen transformations (i.e., nitrification and denitrification) were detected within these samples. Their relative abundances and occurrence frequency were notably higher in urbanized river than in nature reserve river areas (Dataset 2F). Three taxa of nitrite-oxidizing bacteria (NOB), ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) including *Nitrosomonas, Nitrospira,* Candidatus *Nitrocosmicus, Candidatus Nitrososphaera, Nitrosarchaeum, Candidatus Nitrosotenuis,* and *Nitrosospira* (Dataset 2E-F). Among them, *Nitrospira* exhibited the highest relative abundance, averaging 0.04%, and this prevalence was more pronounced in urbanized areas compared to nature reserve samples

(Dataset 2E). Additionally, the sequencing results found denitrifying bacteria, including *Rhodobacter*, *Paracoccus*, *Hyphomicrobium, Clostridium*, *Sphingomonas an*d others. *Rhodobacter*, *Hyphomicrobium*, *Sphingomonas* were the dominant taxa within the denitrifying bacterial community, constituting percentages of 1.55 %, 1.01 % and 0.39 %, respectively. Similarly to nitrifying bacteria, the relative abundance of denitrifying bacteria was higher in urban river areas in comparison to nature reserve river areas (Dataset 2E).

Key influencing factors and ecological driving processes

We found that 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). To determine the underlying factors governing community dynamics across various spatial scales, the null model (Stegen et al., 2012) and neutral community model (NCM) (Sloan et al., 2006) were used to quantify the relative influences of deterministic and stochastic processes.

The null model indicated a prevalence of stochastic processes, particularly in the form of homogeneous selection (|βNTI| < 2, RCbary >+0.95) (Fig. 6B, Dataset 3), in governing the community assembly (details in Materials and Methods). Simultaneously, NCM (Fig. 6C) showed the relative contribution of stochastic processes exhibited a gradual decline with the escalation of human activity, elucidating 58.5% and 48.3% of the community variance within the nature reserve and urban samples, respectively.

To further assess the interaction between sediment microbial diversity and water quality, Spearman's rank correlation analysis were used to investigate the relationships between microbial diversity and geochemistry parameters. The Spearman's rank correlation analysis revealed noteworthy associations between nitrate, total nitrogen, phosphate, and both Chao1 richness and Shannon index (Table S1). To further investigate how environmental variables influence in microbial community, distance-based redundancy analysis (dbRDA) was performed to determine the correlation.

The db-RDA using pH, ammonium, ammonia, nitrite, total nitrogen, phosphate, total phosphorus in water as constraining variables further, 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). Ammonium exhibited the strongest influence on bacterial and archaea community composition within the river network (*P*□=□0.001).

Prediction of the functional diversity and metabolic pathways

Microbial community metabolic predictions guided by the FAPROTAX database (Louca et al., 2016) revealed distinctions in the metabolic potential related to nitrogen, carbon, and sulfur cycling between human-impacted urban river areas and nature reserve river areas (Dataset 4A-D). For instance, the microorganisms involved in aerobic ammonia oxidation, aerobic nitrite oxidation, nitrogen fixation, nitrification, nitrate ammonification at urban river was higher than that at nature reserve (ANOVA, $p < 0.001$). For carbon transformation, urban areas exhibited higher abundances of microbial groups associated with anoxygenic photoautotrophy, oxygenic photoautotrophy, chloroplasts, and photoautotrophy, in contrast to relatively lower abundances in the nature reserve. A similar trend was observed in the distribution of Cyanobacteria. Furthermore, the urban river areas displayed higher abundance of taxa involved in degradation of aromatic compound compared to the nature reserve (ANOVA, $p < 0.05$) (Fig. 3).

The sulfur cycling displayed heterogeneity among the samples. As depicted in Fig. 4, the urban river areas exhibited abundant taxa associated with the dark oxidation of sulfur compounds, in contrast to the nature reserve (ANOVA, $p < 0.05$). Furthermore, the abundance of taxa involved in sulfate respiration was higher in urban areas than at the nature reserve. These predictions collectively underscore the dissimilarities in nitrogen, carbon transformation and sulfur cycling metabolic potentials between urban and nature reserve samples.

Additionally, the PICRUSt2 database (Douglas et al., 2020) revealed the dominant ecological function were linked to metabolism, cellular processes, environmental information processing, genetic information processing, human diseases (Dataset 4C-E). 414 functional KEGG pathways at level 3 were annotated (Dataset 4F). The metabolism associated with carbon fixation, nitrogen, sulfur, phosphate, antibiotic biosynthesis and xenobiotic biodegradation were investigated. These data indicated that the ecological function related to biosynthesis of the antibiotics beta-lactam, penicillin, cephalosporin, macrolide, tetracycline, streptomycin, carbapenem were present in all sites. 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 suggests an increase in pollutants discharge from urban areas. Styrene degradation emerged as the predominant process among polycyclic aromatic hydrocarbon (PAH) degradation (Dataset 4G).

Antibiotic resistance rates

Tetracycline and sulfonamide are widely used to treat human and animal diseases worldwide (Gao et al., 2012; Li et al., 2015b). Majority of the river network examined in this study contained tetracycline and sulfonamide resistance bacteria. Fig. 8 illustrates the antibiotic resistance rates

that were resistant to tetracycline, sulfamethoxazole on plating count agar. The bacterial resistant rate within the urban samples ranging 0.40%- 11.80 % for anti-tetracycline and 1.6 %- 26.9 % for anti-sulfonamide, were generally higher than that in the nature reserve ranging 0.20% - 2.80 % and 1.25 % - 14.20 %. The sulfonamide resistance rate was consistently 2 to 6 times higher than that of the tetracycline resistance rate. While no significant differences were observed among the nature reserve samples, the antibiotic-resistant rates exhibited variability among the samples from urban areas.

Discussion

Over the past decades, human activities have caused significant changes in the biogeochemical cycles of nutrients within atmospheric, terrestrial, and aquatic ecosystems (Xuan et al., 2022). Urbanization, in particular, has played a crucial role in driving these transformations. With approximately half of the global population residing in urban areas as of 2008, projections indicate that this figure is anticipated to rise to 66% by 2050, which will lead to a corresponding increase in the production of nutrient-rich urban wastewater (Atashgahi et al., 2015). However, understanding the potential effects of anthropogenic pollution effluents within receiving rivers remains a topic with unsatisfactory clarity. In this study, we conducted a comprehensive investigation of bacterial and archaeal community in a river network, encompassing both structural and functional aspects, to elucidate their responses to varying pollution levels.

Anthropogenic activity induces river eutrophication and enhance river microbial diversity

Considerable variation exists within the bacterial and archaeal communities in terms of microbial abundance, composition, diversity across different river sites (Fig. 3-5). A significant increase in the microbial abundance and diversity was observed in the urban river areas, accompanied by significant niche differentiation (Fig. 3A-D). This change can be attributed to the continuous influx of industrial, domestic, and agricultural wastewater, which was caused by a large population of about 11.06 million in sampled regions. Due to persistent inputs of rich nutrients into the river (Fig. 2), leading to the rise in microbial population and diversity ($P < 0.01$).

Furthermore, the db-RDA analysis suggested that TN contributed significantly to the changes in the river sediment microbial community ($P < 0.05$; Fig. 3F). Specifically, the high nitrogen loading, often in the form of nitrate and ammonium (Fig. 2), is a common consequence of human activities. Excessive nitrogen inputs can lead to eutrophication, a process where the nutrient enrichment promotes the growth of algae (Chlorophyll *a* content had showed in Fig. 2) (Kuypers et al., 2018). The proliferation of algae leads to increased organic matter production and subsequent decomposition by bacteria and archaea. This increased availability of organic matter and the changed nutrient conditions can influence the community composition. The influx of different organic compounds and nutrients creates a more complex environment, allowing for the coexistence of a broader range of microbial species (Li et al., 2023). These changes in community

diversity can have cascading effects on ecosystem functioning and overall microbial processes in the river network.

However, the observed increases in microbial diversity within urban river areas contradicted findings from previous studies. Several investigations have documented the response of microbial communities to high-concentration effluents from Wastewater Treatment Plants (WWTPs). For instance, Drury et al. (2013) demonstrated notable reductions in diversity and population among sedimentary microbial communities within the Chicago metropolitan rivers (Drury et al., 2013). Similarly, Pascual-Benito et al. (2020) reported that the discharge of WWTP effluents led to reductions in the diversity of planktonic bacteria(Pascual-Benito et al., 2020). Furthermore, Dai et al. (2023) revealed that WWTPs effluent discharge was linked to decreases in microbial community diversity and ecology complexity within urban coastal sediment (Dai et al., 2023).

The anthropogenic pollution sources resulting from industrial, domestic and agricultural activities are considered as the primary contributors to non-point source (NPS) pollution (Ding and Liu, 2019; Beattie et al., 2020). In contrast, WWTPs primarily constitute the point source pollution (Kiedrzyńska et al., 2014). While increased diversity is one outcome in our study, the specific response of bacterial and archaeal communities to anthropogenic contamination can vary depending on the magnitude and duration of the pollution, local environmental conditions, and the resilience of the microbial communities present. Hence, the potential ecological and health influences of NPS pollution in river ecosystems require further research and monitoring.

Composition of sedimentary microorganisms were significantly influenced by anthropogenic pollution effluents.

Nonmetric multidimensional scaling (NMDS) analysis, based on OTU, demonstrated distinct different between the microbial communities of urban river sediments and those of the nature reserve river ecosystems (Fig. 3D, OTUs details in Dataset 4). This differentiation persisted even when considering the exclusion of "rare biosphere" taxa (< 0.1% total sequence). Remarkably, the urban river ecosystems exhibited 511 endemic genera (Fig. S2).

Allochthonous sources have the potential to introduce microbiological contaminants and disrupt the river microbial community diversity and composition, as observed in previous studies (Drury et al., 2013; Xuan et al., 2022). Simultaneously, given the long history of pollution in the river network, sewage carrying a myriad of organic and inorganic contaminants can profoundly change the environmental conditions of riverine ecosystems, the changed nutrient conditions can potentially favor specific bacterial and archaeal taxa, leading to shifts in community composition. Some bacterial and archaeal communities might have adapted to these altered conditions over time (Archana et al., 2016; Eckert et al., 2018).

Nitrogen-enriched urbanized rivers exhibited a notably higher relative abundance of key functional bacterial groups (Dataset 2), including nitrifying bacterial community (such as *Nitrosomonas, Nitrosospira*, *Nitrospina, Nitrospira*), denitrifying bacteria (such as *Paracoccus, Hyphomicrobium*, and *Gemmobacter*) (Xuan et al., 2022). *Nitrospira* are ubiquitous participants in the nitrogen cycle, playing essential roles such as oxidation of nitrite and reduction of nitrate (Lücker et al., 2010). Therefore, inputs of ammonia from manure runoff can lead to increased abundance of *Nitrospira*, consequently elevating nitrate concentrations in waters (Daims et al., 2015; Henson et al., 2018). Elevated nitrate levels contribute to freshwater eutrophication and pose a potential threat to human health when present in drinking water supplies.

This observation is further supported by the presence of taxa associated with specific functions, including *Rhodoferax*, *Thiobacillus*, *Methylotenera*, *Crenothrix*, *Planktothrix, Microcystis*, *Anabaena*, and *Flavobacteria,* which exhibited higher abundance in urban areas (Dataset E-F). While these taxa are generally found in freshwater, their prevalence in elevated abundances often links to the presence of human-impacted pollutants (Beattie et al., 2020). Genera like *Rhodoferax* and *Thiobacillus* are identified for their capabilities in iron reduction and sulfur oxidation, respectively (Finneran et al., 2003; Zhi-Hui et al., 2010). *Methylotenera* and *Crenothrix* play important roles in freshwater carbon cycling by utilizing reduced carbon sources such as methane as their primary carbon source (Oshkin et al., 2014; Oshkin et al., 2015; Oswald et al., 2017). Additionally, previous studies showed that increases in Cyanobacteria, particularly genera such as *Planktothrix*, *Anabaena*, and *Microcystis* are linked to occurrences of freshwater eutrophication (Havens, 2008; Dolman et al., 2012; Graham et al., 2012; Domingues et al., 2014). *Flavobacteria* are abundant and common heterotrophs in freshwater environment (García-Armisen et al., 2014; Wang et al., 2018), playing a crucial contribution in various carbon cycles, such as the degradation of algal organic matter and other forms of biodegradation (Kirchman, 2002; Teeling et al., 2012; Williams et al., 2013). The higher abundance of such taxa observed in the urban areas could potentially be attributed to the contamination resulting from human activities.

Strikingly, an increase in the diversity and proportion of the archaea was also identified under the anthropogenic pollution effluent. Krzmarzick et al. (2018) propose 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 (Krzmarzick et al., 2018). This finding could be further supported by the study that Yang et al., (2022a), 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 (Yang et al., 2022).

Overall, this interplay between microbial dynamics and nutrient influx underscores the complex nature of microbial responses to anthropogenic influences in urban river environment. Urban rivers

represent significant hotspots where nitrogen loading escalates due to the inflow of nutrients from anthropogenic sources, thereby changing the aquatic microbial community composition.

Anthropogenic activity induced pathogens and antibiotic resistant microbes in the river network

The data showed that anthropogenic activity not only contribute to water quality degradation and changed ecosystem functioning but also to introduce microbes of fecal contamination and pathogens microorganism into river. Within the urban areas, the high abundance of Bacteroidetes and Firmicutes were observed (Dataset 2C). This finding is of significance as certain members of these phylum are recognized as good indicators of fecal contamination (Wéry et al., 2008; Wang et al., 2018). The elevated abundance of Bacteroidetes and Firmicutes in the urban river areas can likely be attributed to the presence of untreated wastewater from residential areas, which may contain fecal pollutants.

Additionally, the study also identified a marked increase in the abundance and diversity of potential pathogens (Beattie et al., 2020), including genera such as *Acinetobacter*, *Arcobacter*, *Exiguobacterium*, *Chryseobacterium*, along with well-known pathogens like toxic *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* within urban river environment (Dataset 2E). These findings highlight a distinct shift in microbial communities due to anthropogenic activities leading to fecal contamination (Drury et al., 2013; García-Armisen et al., 2014). Remarkable, these genera have also demonstrated a remarkable capacity to degrade and tolerate a wide array of organic compounds found in sewage water (Kasana and Pandey, 2018; Zhang et al., 2020). This dual capability underscores their adaptability to environments influenced by anthropogenic activities and suggests their involvement in sewage degradation processes (Zhang et al., 2009; VandeWalle et al., 2012).

The impact of human activities on river microbial communities is also evident in the predicted metabolic functions of these communities. Functions linked to drug and antimicrobial metabolism were identified in urban river and nature reserve river sediment samples (Dataset 4). Notably, higher antibiotic resistance rates were observed in human-impacted urban river areas (Fig. 8). The presence of antibiotics in the river is a widespread concern (aus der Beek et al., 2016). 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 through various wastewater treatment processes (Zhang et al., 2015). Urban water ecosystems have been identified as potential hotspots for the spread of ARBs in China (Hou et al., 2021; Reddy et al., 2022). 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 (Rizzo et al., 2013).

Notably, antibiotic resistance rates varied in the urban samples with various levels of human activity, despite no significant differences in nature reserve samples (Fig. 8). This emphasizes 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 exert selective pressure on bacteria, contributing significantly to antibiotic resistance development (Baker-Austin et al., 2006).

Ubiquitous PAHs contamination in the river network

High relative abundances of ecological function linked to xenobiotic biodegradation were detected in both urban and nature reserve sites (Dataset 4G). The presence of biodegradation genes could serve as indicators for the existence of xenobiotics and their corresponding metabolites (Pagé et al., 2015; Kansole and Lin, 2016). The observed high abundance of genes related to xenobiotic biodegradation in the urban river is consistent with the findings of a previous study (Bai et al., 2014; Wang et al., 2018). However, the high abundance of PAHs-degrading bacteria such as *Sphingobium*, *Rhodococcus*, *Novosphingobium*, *Pseudomonas*, *Sphingomonas*, *Brevibacterium*, *Arthrobacter*, *Nocardioides,* and *Mycobacterium* were observed in the whole river network (Datset 2E) (Ghosal et al., 2016), may suggest the accumulation of PAHs in the whole river network.

Polycyclic aromatic hydrocarbons (PAHs) comprise a group of organic priority pollutants that pose significant environmental and public health risks due to their toxic, genotoxic, mutagenic, and potentially carcinogenic properties. These compounds are widely distributed and are known for their persistence in the environment (Mojiri et al., 2019). PAHs are globally widespread, primarily originating from long-term anthropogenic pollution sources linked to rapid industrialization and urbanization (Kuppusamy et al., 2017). 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 (Lu et al., 2011; Kuppusamy et al., 2017; Patel et al., 2020). Numerous urban rivers in China have been identified as containing PAHs (Cao et al., 2010; Qian et al., 2017; Wang et al., 2018), and significant biodegradation of PAHs capacity has also been observed in other rivers (Muangchinda et al., 2017). In this study, the data confirmed that PAHs pollution has impacted the whole river network ecosystems.

Environmental gradients emanating from human activity create potential niches in the river network.

River network sediment communities exhibit high diversity, encompassing thousands of OTUs across major taxa that inhabit various habitats within the river network (Fig. 3-5). Intriguingly, the Distance-Decay Relationship (DDR) (Fig. 6A) reveals a distinct biogeographical pattern within this extensively interconnected river system. Microbial migration is constrained by geographical distance, signifying limitations due to dispersal. These findings underscore the coexistence of

simultaneous niche-based deterministic and neutral-based stochastic dispersal processes that jointly shape this biogeographical signature.

Disentangling the factors that influence microbial community structure and function in response to environmental changes remains a pivotal concern in microbial ecology (Dini-Andreote et al., 2015; Jia et al., 2018). The application of the null model and neutral community model (Fig. 6B-C) revealed that deterministic processes predominantly shaped the microbial communities in the intricately interconnected river network. This suggests that local environments wield more influence in shaping microbial population composition within the river network than dispersal limitations. Past research has demonstrated that persistent wastewater contamination can exert robust deterministic influences on microbial community assembly (Yang et al., 2022). Furthermore, the differentiation of microbial communities between urban and nature reserve rivers is underscored by their narrower niche breadths in the urban setting. This implies that deterministic processes play a stronger role in shaping microbes with limited niche breadths, rather than those with broader ones. Significantly, homogeneous selection emerges as the principal deterministic force governing microbial community assembly in urban areas, likely due to the relatively consistent rich nutrient habitat environment for microorganisms across various urban sampling sites.

Conclusions

In summary, our study reveals a distinctive biogeographical signature within the interconnected river network, wherein microbial abundance and diversity exhibit significant correlations with anthropogenic activity. Despite the rivers' inherent capacity for dilution and self-purification, the extensive connectivity of the river network were vulnerable to non-point source pollution from anthropogenic activities. Anthropogenic pollutants stand out as the principal drivers altering microbial communities, consequently augmenting the role of homogeneous selection in shaping urban microbial community assembly. Furthermore, pathogens, antibiotic tolerant bacteria and PAHs degradation bacteria also were promoted by anthropogenic activities contamination. These findings demonstrate that microorganisms developed typical structural and functional survival strategies to adapt to combined anthropogenic activities contaminants. This newfound understanding holds substantial potential for informing effective strategies in riverine ecological management, particularly in mitigating the ecological risks posed by urbanization-related effluents.

Materials and methods

Study area, sampling procedure, and physicochemical analysis

The present study focused on the reaches of the Yangtze River that run through southern of Shaanxi Provinces (Fig. 1). Sampling was conducted in August-September 2022 within the river network. At each sampling site, geographical data (longitude, latitude, and distance between sampling sites) were recorded by a GPS device (Dataset 1). The areas of the rivers sediment sampled were surveyed, while their overlying water quality parameters, including temperature, pH,

dissolved oxygen, water clarity of Secchi depth, turbidity, total nitrogen, 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 standard test methods were measured for water samples according to Environmental Quality Standards for Surface Water (GB3838-2002).

Sediment TOC was measured by the Vario E1-III Elemental Analyzer interfaced with a Thermo Finnigan DELTA plus XL stable isotope ratio mass spectrometer. Analyses were normalized relative to an acetanilide standard. The concentrations of TOC were expressed as percent dry precipitates. Each sample was performed in triplicate, and the values were expressed as the mean (n = 3).

Sediment Cell counts

The CARD-FISH protocol was based on our previous study (Li et al., 2020). The probe-labelling peroxidases were EUB338 (bacteria: 5'-GCW GCC WCC CGT AGG WGT-3') and Arch915 (archaea: 5'-GTG CTC CCC CGC CAA TTC CT-3'). Probe NON338 (5'-ACT CCT ACG GGA GGC AGC-3') was used as a control. 5g frozen sediment samples were subsampled. The frozen sediment samples were unthawed and then were fixed with 4% paraformaldehyde for 24 h at room temperature. The fixed sediments were washed three times by centrifugation (8 000 × *g* for 10 min) using phosphate buffered saline (PBS) at 4°C and stored in an ethanol/PBS buffer (1:1) at −20°C for further processing. After that, 100 μL of fixed sediment was diluted with 900 μL ethanol/PBS buffer (1:1) and dispersed using ultrasound. Next, 20 μL of dispersed sediment was diluted in 20 mL of Milli-Q filtered water. The suspended sediment was filtered on polycarbonate filters, and 0.1% low melting point agarose was dripped onto the filters and dried at 46°C in an incubator. The microbes were permeabilized using 15 μg/mL proteinase K. Then, 3% H_2O_2 was used to inactivate the endogenous peroxidases (Li et al., 2018).

For hybridization, filters were placed in a tube and mixed with 500 μL hybridization solution (10% dextran sulfate, 2% blocking reagent (Roche, Germany), 0.1% (w/v) sodium dodecyl sulfate, 20 mM Tris–HCl [pH 8.0], 0.9 M NaCl and formamide) and 1 μL of probe working solution (final concentration, 0.028 μM) (90). Microorganisms were hybridized for at least 60 min on a rotor at 46°C, and then the filters were washed twice using washing solution (90) (0.01% SDS, 5 mM EDTA [pH 8.0], 20 mM Tris–HCl [pH 8.0] and 3 mM NaCl) at 48°C for 20 min. After washing, filters were mixed with 1,000 μL of amplification solution (0.0015% H_2O_2, 1×PBS [pH 7.4], 0.1% (w/v) blocking reagent) and 1 μL of Alexa 488-labelled tyramides (Life Technologies™, Thermo Fisher, USA). The probes were incubated at 46°C in amplification solution for at least 30 min in the dark. Finally, all microorganisms were stained using DAPI and mounted with ProLong Gold Antifade reagent (Life Technologies, Carlsbad, California, USA). Cell counting was performed in triplicate using ImageJ (Fan and McKenney, 2023), and the cell abundance were expressed as the mean (n = 3).

High-throughput sequencing and data analysis

Five grams of frozen sediment sample was aseptically subsampled for DNA extraction. These samples were used for DNA extraction taken from the inner part of the core to reduce contamination from the sampling process. A negative control (5 mL of water) was included with each batch of subsamples and subjected to DNA extraction in the lab. DNA was extracted from each subsample using the E.Z.N.A.® soil DNA kit (Omega Bio-tek, Norcross, Georgia, USA), according to the manufacturer's instructions. The final DNA concentration was determined by a NanoDrop 2000 (Thermo Scientific, Waltham, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The sequencing steps were conducted by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The bacterial and archaeal universal primers were 515F and 806R (91), targeting the 16S rDNA V4 region. The polymerase chain reaction (PCR) protocol was performed according to previously described methods (Li et al., 2015a; Li et al., 2023). Triplicate amplifications products were extracted from a 2% agarose gel and further purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA) and quantified using Quanti Fluor™-ST (Promega, USA). Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, California, USA).

Raw fastq files were demultiplexed, quality filtered by Trimmomatic, and merged by FLASH. OTUs were clustered with a 97% similarity cutoff using UPARSE. Chimaeric sequences were identified and removed using UCHIME. To remove the effects of sampling intensity (number of reads) on downhole or intersite comparisons of richness estimates, we randomly subsampled the 10,000 reads in each sample and calculated Bray–Curtis (read count) dissimilarity matrices using QIIME. Taxonomic assignment was performed using the SILVA 16S rRNA database (version 138). The distance-based maximum likelihood was used for phylogenetic analysis. Bootstrap analysis was performed using 1,000 replications. Alpha-diversity metrics (observed OTU richness, Chao1 richness, Shannon and Simpson diversity indices, and equitability index) were calculated using mothur. The resulting beta-diversity matrices were used for 2-dimensional non-metric multidimensional scaling (NMDS) ordinations with 20 random starts. Beta-diversities of microbial communities were estimated based on Bray–Curtis distances between samples, and the distances were calculated using OTU tables resampled to a minimum number of sequences from each sample.

The "niche breadth" approach (Levins, 1968) was used to quantify habitat specialization. The formula is described below:

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

where *Bj* is the habitat niche breadth of OTUj in a metacommunity; and Pij 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 that a wider habitat niche breadth. In contrast, lower *B* values indicate that the OTUs occurred in fewer habitats and were unevenly distributed (Pandit et al., 2009). The average of B values from all OTUs

in one community was regarded as the community-level B-value (*Bcom*) (Jiao et al., 2020). We calculated the *B* value based on species abundance and evenness for urban and nature reserve samples (Salazar, 2020).

Prediction of microbial functions groups

In order to identify potential microbial ecological functions at the studied sites, the obtained OTUs were compared against the FAPROTAX database to predict potential metabolic functions of the microbial community. The Functional Annotation of Prokaryotic Taxa v.1.0 (FAPROTAX, http://mem.rcees.ac.cn:8080/) pipeline was used to extrapolate microbial community functions (Sansupa et al., 2021). Furthermore, potential functions of bacterial and archaeal communities also were predicted from 16 S rRNA gene-derived OTUs using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) against the Kyoto Encyclopedia of Genes and Genomes database (KEGG) (Douglas et al., 2020).

Screening for antibiotic resistant bacteria

The conventional approach of heterotrophic plate count (HPC) method was used to determine the concentration of antibiotic resistant bacteria in the collected samples. 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 Plating count agar medium. Medium contained three-class plates, 50.4 μg/mL sulfamethoxazole, 6 μg/mL tetracycline and no antibiotics. Total heterotrophic culturable bacterial population was determined by plating samples on media without antibiotics. The selected concentrations of antibiotics on the media were determined according to the criteria of the Clinical and Laboratory Standards Institute (CLSI) (Li et al., 2015b) and the previous study (Munir et al., 2011; Gao et al., 2012). The media were added antifungal agent 200 μg/mL cycloheximide according to Gao el al., (2012) and Brooks et al. (2007) method. Plates were incubated for 48–72 h at 37 °C (Brooks et al., 2007; Munir et al., 2011). Duplicate counting was performed, and colony-forming units (CFU) were calculated. The resistance rates defined as the number of bacteria that grew on antibiotic plates divided by the total number of bacteria that grew on plates containing no antibiotics.

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 null model expectation was generated using 999 randomizations. 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 (Sloan et al., 2006). 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; $_{pi}$ 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.

Data available

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

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Competing Interest Statement

The authors declare no competing interests.

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Figure

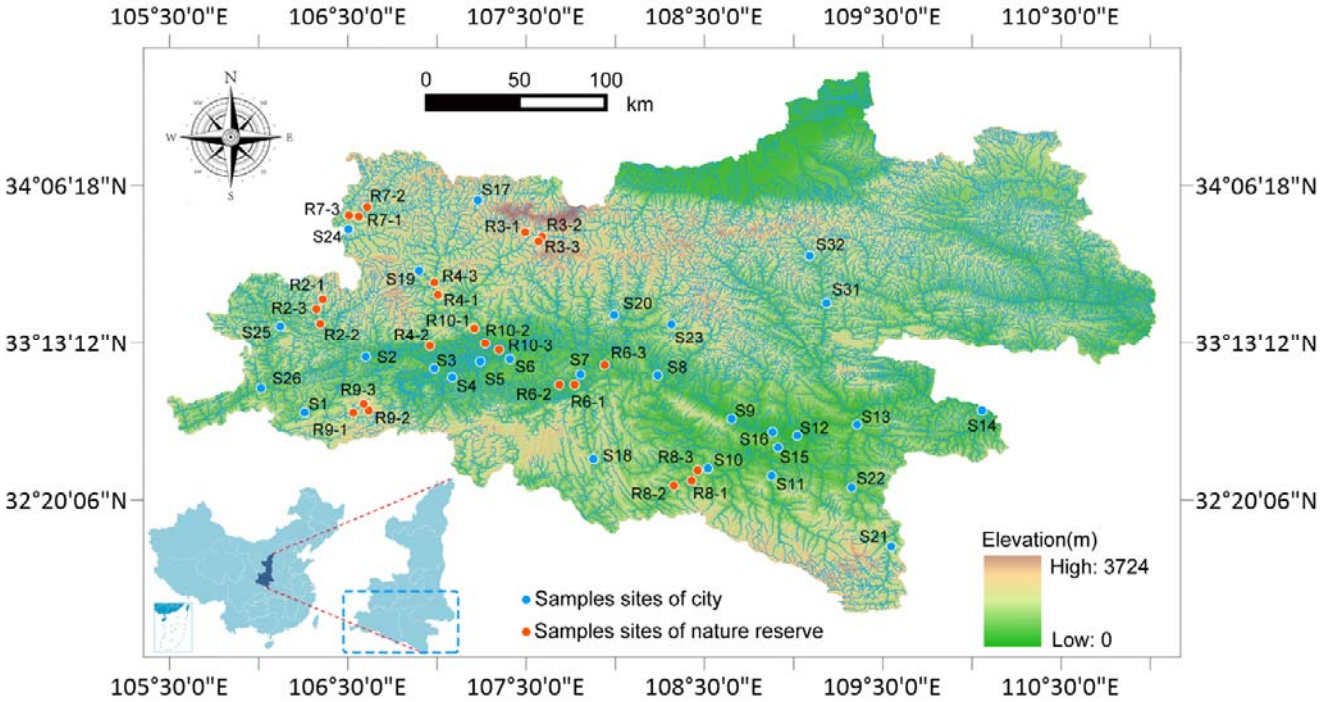

Figure 1. Location of the collected river network sediment samples. Blue circles indicate the urban sites, and orange circles indicate the nature reserve sites, respectively. The *Inset* map shows the extent of the study area relative to China. Maps were drawn using ArcGIS Desktop 10.4.

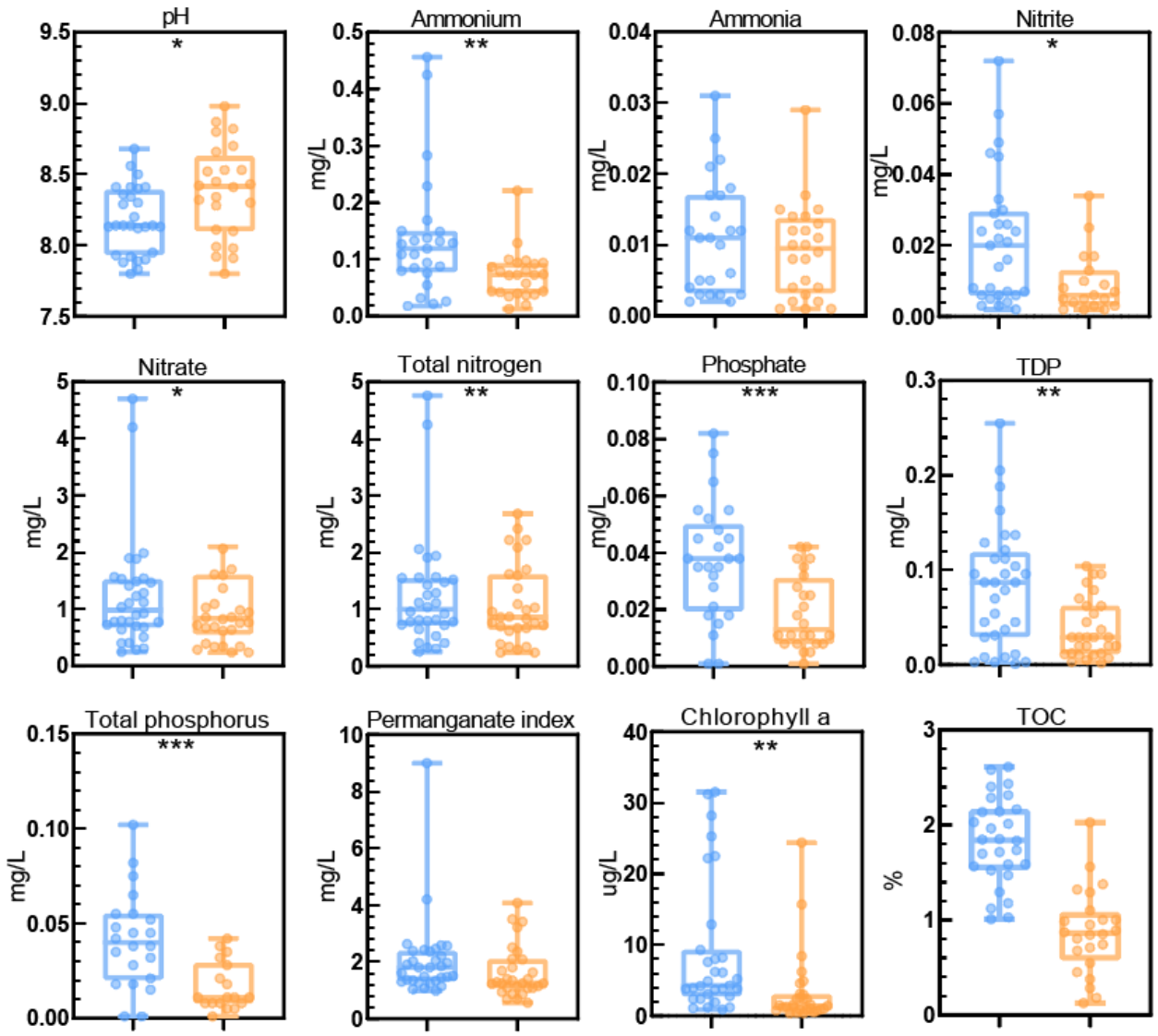

Figure 2. Comparison of geochemical characterization in human-impacted urban sites and undisturbed nature reserve sites in the river network. The blue circles indicate urban sites and orange circles indicate nature reserves sites, respectively.

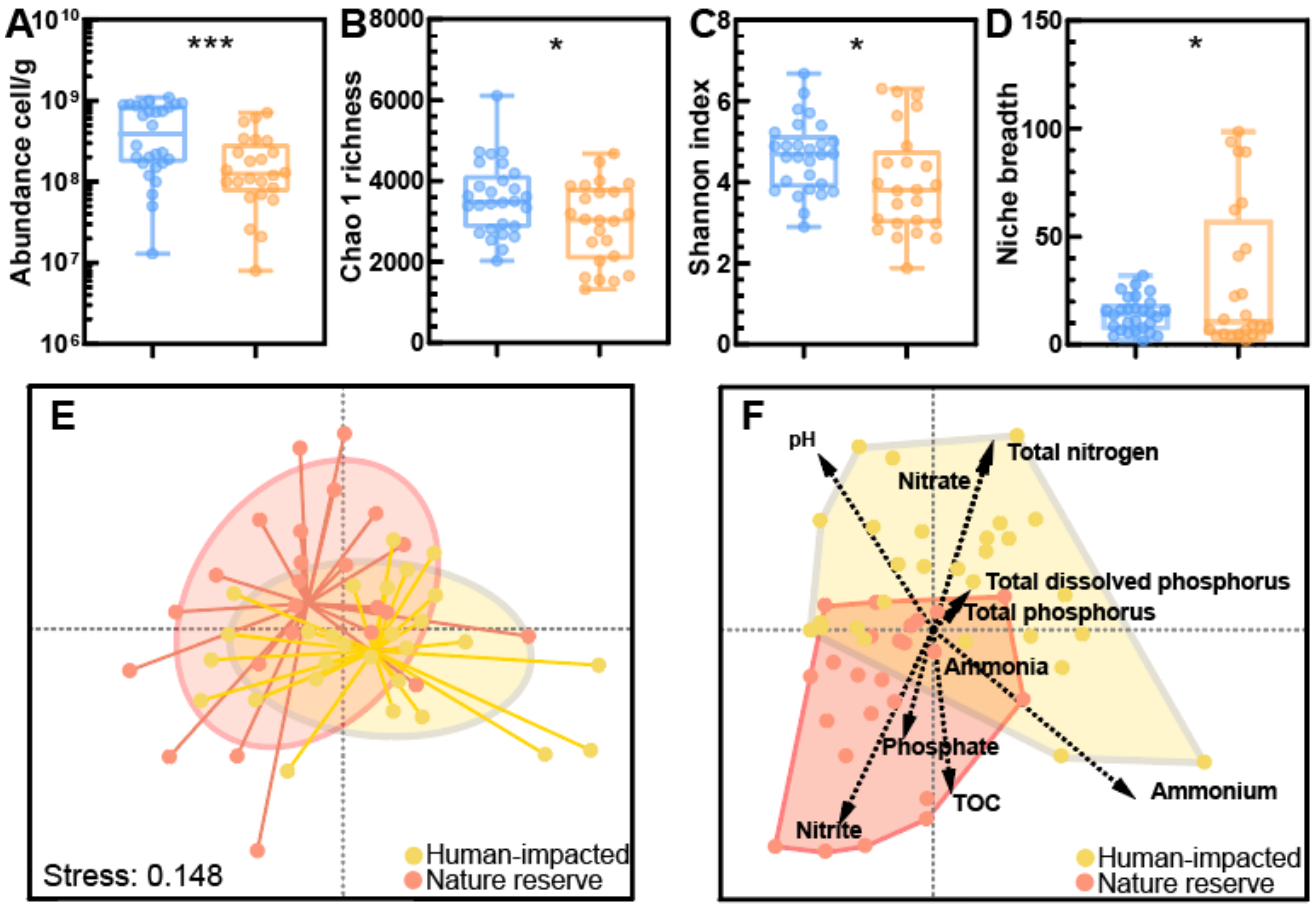

Figure 3. The microbiome in the large river network. (A-D), Microbial abundance, Richness estimates, Shannon index and Niches breadth were investigated. (E), Similarity of microbial communities visualized by NMDS. (F), A db-RDA showing microbial community similarity when environmental factors were used as constraining variables. 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.

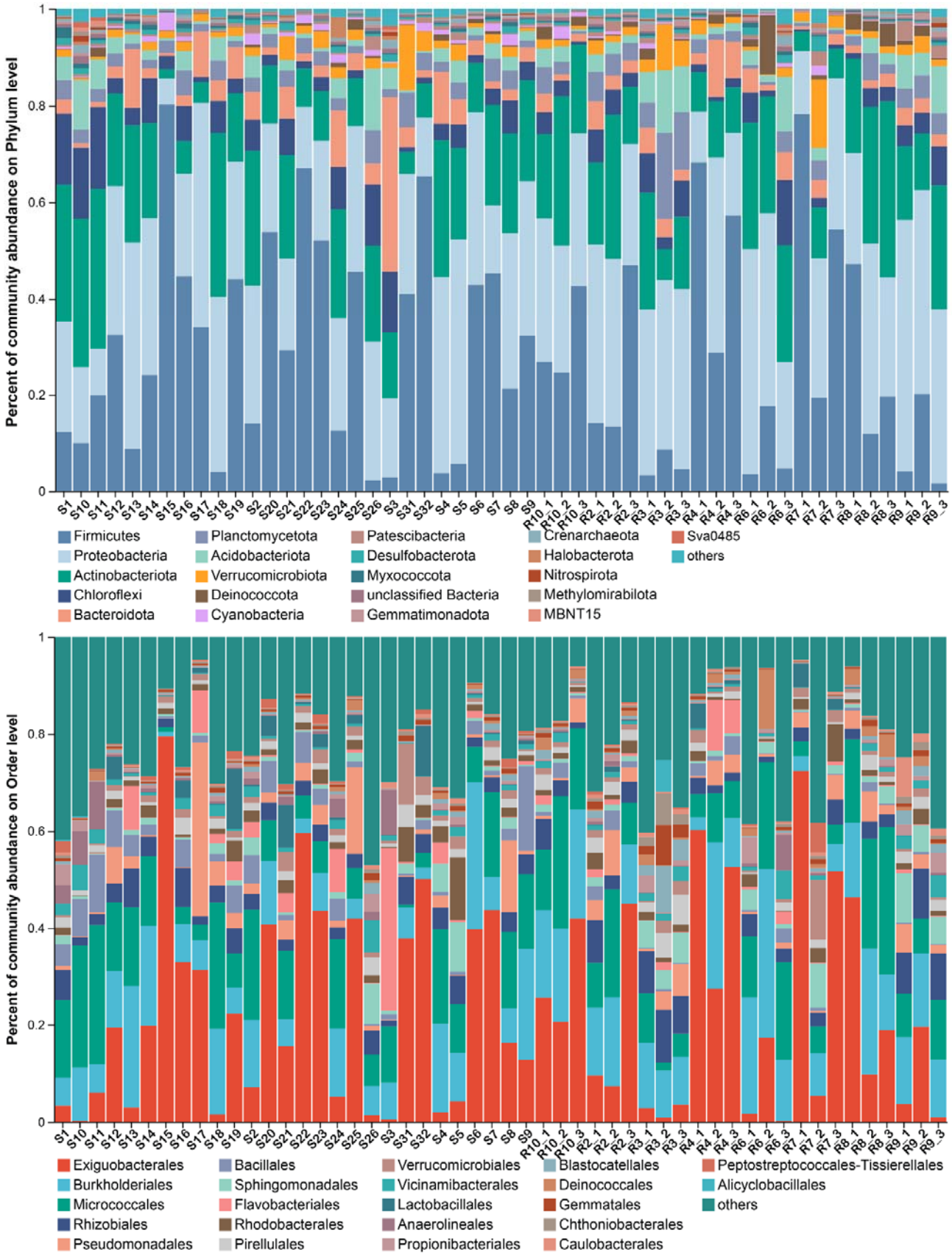

Figure 4. Relative sequence abundance of bacterial and archaeal. The graph shows the relative abundance of the most abundant bacterial and archaeal at phylum (A) and classes (B) level in urban river and nature reserve according to average relative abundance across all samples.

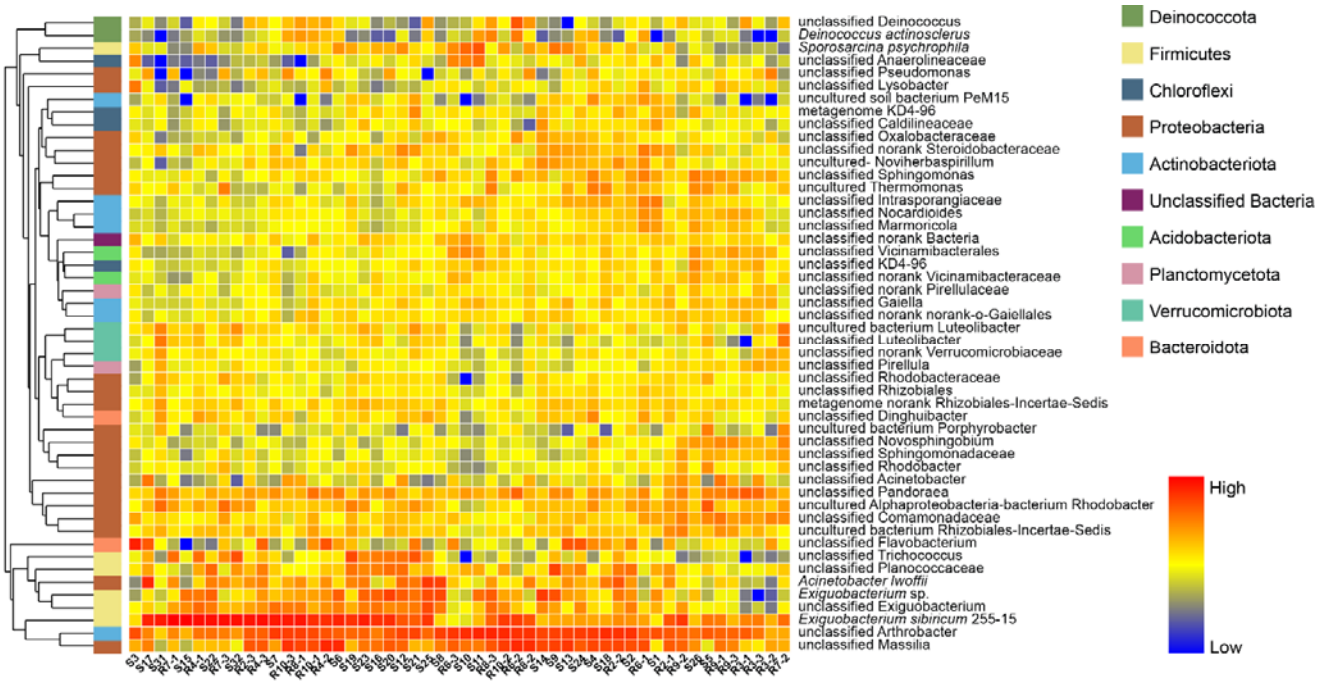

Figure 5. A heatmap was constructed for the 50 most dominant species.

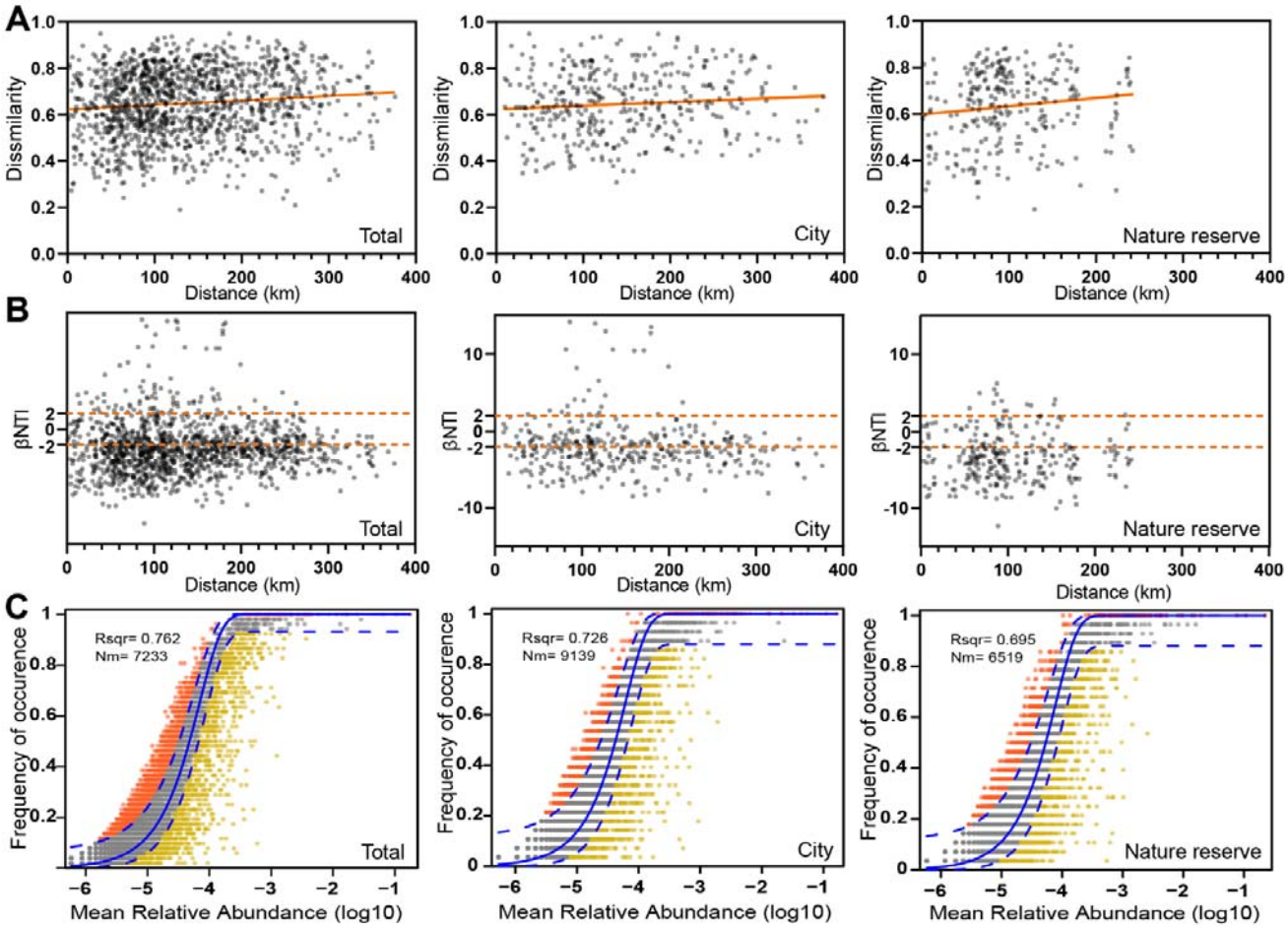

Figure 6. General patterns of microbial diversity in the river network system. (A) Distance-decay curves showing Bray–Curtis dissimilarity against geographic distances between sampling sites. Solid lines denote the ordinary least-squares linear regressions. (B) Scatter plot of βNTI values grouped by 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.

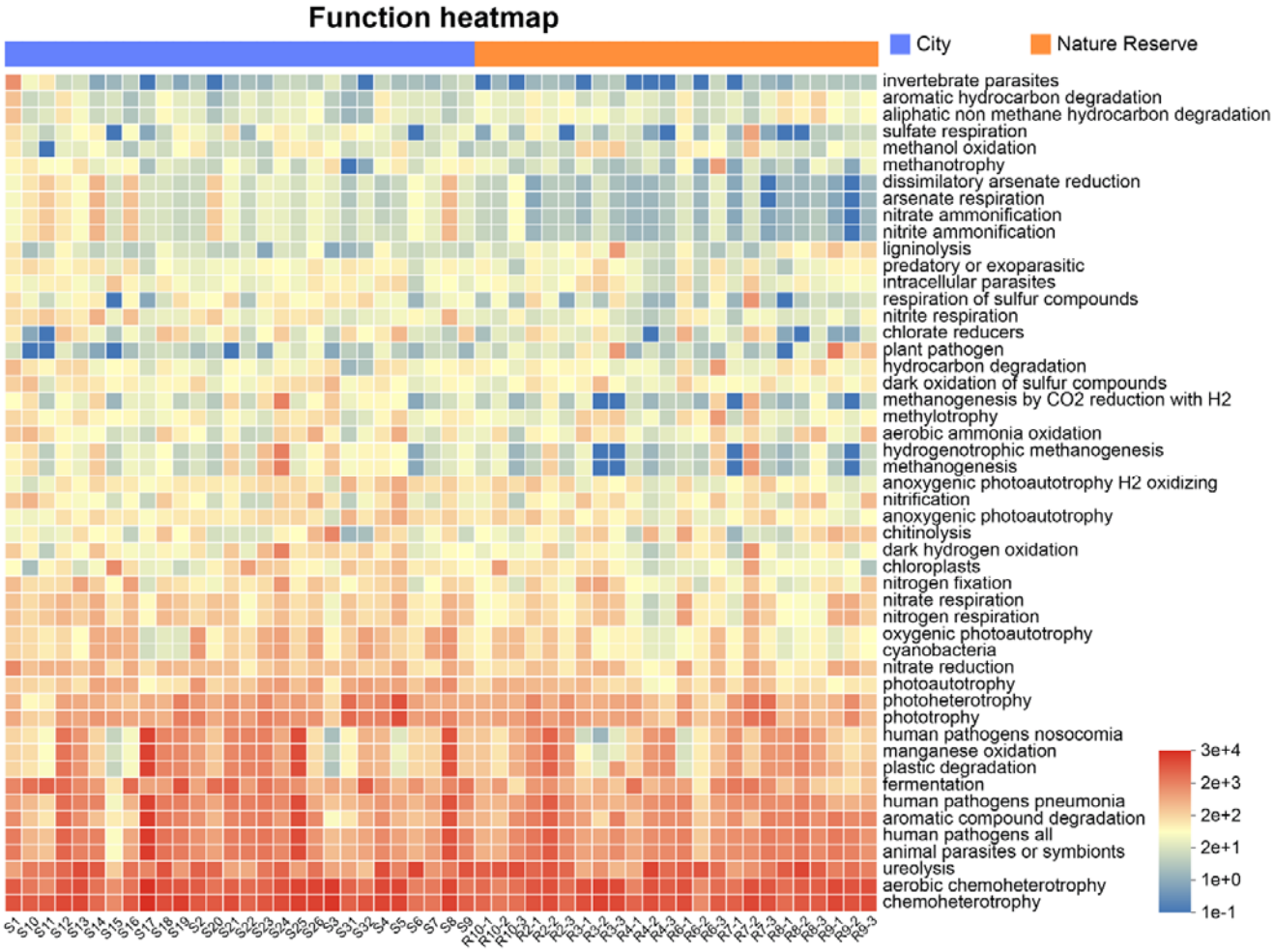

Figure 7. Prediction of the functional diversity and metabolic pathways between urban areas and nature reserve areas.

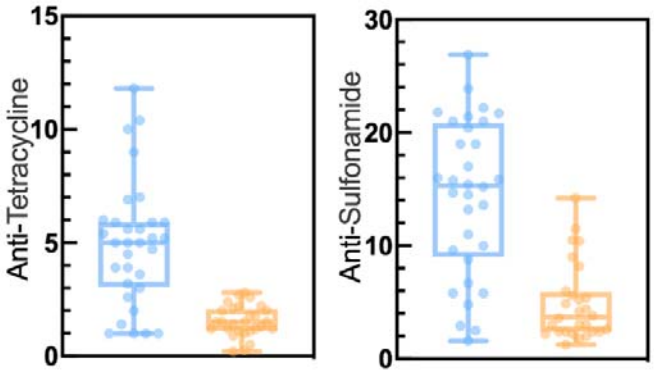

Figure 8. Antibiotic resistance rates. The blue circles indicate urban sites and orange circles indicate nature reserves sites, respectively. While no significant differences were observed among the nature reserve samples, the antibiotic-resistant rates exhibited variability among the samples of urban areas.