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

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Highlight

A increasing bacterial and archaeal diversity were observed in the studied urban rivers.
Sewage discharge and high nitrogen loading changed urban community structure and assembly process.
Pathogens, antibiotic-resistant bacteria and PAHs-degrading bacteria enriched in in highly urbanized rivers.
The deterministic process mainly governed the microbial community assembly.

Abstract

Rivers have been a preferred location for human settlements throughout history. Human activity were believed to affect geographic pattern and assembly process of river microorganisms, which have not been widely investigated. Comparing the bacterial and archaeal communities in highly human-impacted urban areas and undisturbed pristine nature reserves could expand our understanding of the mechanisms of river ecosystem responses to environmental changes resulting from anthropogenic contaminants. Here, 28 urban and 24 nature reserve river sediment samples were collected in a highly connected river network spanning ~400 km located in Qinling Mountains, and their geobiochemistry profiles and microbial populations were investigated. The results showed that microbial community all exhibited significant correlation with degrees of human activity, and the population increased with increasing human activity. Human activities resulting in an increase in pathogens, antibiotic-resistant bacteria and PAHs-degrading bacteria in urban areas. Redundancy analysis and correlation analysis together indicated that Nitrogen were the major environmental attributes that affected the structure and assembly process of microbial community. The neutral community model and null model reveal the stochastic process and deterministic process both shaped the microbial community with greater influence of deterministic process (homogeneous selection), and the non-point source pollution led to increased biotic homogenization. Collectively, the study suggests that community composition and diversity dynamics of the river network are not random and mainly 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.

1. 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. Sediments often harbor the majority of biomass within the river (Battin et al., 2016; Gibbons et al., 2014), drive essential ecological processes and contribute significantly to global biogeochemical cycles (Xie et al., 2016). Water flow and related deliveries of nutrients and organic matter to sediments constitute major constraints on sedimentary microbial life.

In recent decades, human activity such as rapid urbanization, industrialization and agriculture that dumps multiple anthropogenic contaminant and non-native (exotic)

microorganisms into the river ecosystems through industrial discharge, agricultural runoff, and municipal sewage, which drastically change the river sediment biogeochemical cycle and poses an important threat to river functions (Beattie et al., 2020; Wang et al., 2012). These contaminants can be either organic (e.g., antibiotics, PAHs, fertilizers, pesticides) (Patel et al., 2020; Singh et al., 2019; Xu et al., 2023) or inorganic contaminants (e.g., nitrate, phosphorus) (Manyi-Loh et al., 2016; Pruden et al., 2012).

In highly urbanized areas, centralized wastewater treatment plants (WWTPs) are one of the most common systems for the treatment of domestic wastewater. Several recent studies have presented evidence that WWTPs high-concentration effluent reduces the abundance and diversity of benthic and sediment bacterial communities in urban and suburban rivers (Dai et al., 2023; Drury et al., 2013; Pascual-Benito et al., 2020). WWTPs are common source of point-source pollution (Kiedrzyńska et al., 2014). In contrast, the immense industrial, domestic and agricultural activities sewage are considered as the primary contributors to non-point source (NPS) pollution (Beattie et al., 2020; Ding and Liu, 2019). Point-source pollution is easy to identify, but NPS pollution is harder to identify and harder to address. United States Environmental Protection Agency (EPA) report that NPS pollution is the leading remaining cause of water quality problems, yet there is little information available on the effects of widespread NPS pollution on benthic microbial communities in river ecosystems (EPA, 2022). The potential ecological and health influences of NPS pollution in river ecosystems require further research and monitoring.

Here we are focusing on a large scale river network, which flows out of the Qinling Mountains, where it eventually empties into the Yangtze Rivers (Fig. 1). The Qinling Mountains in Northwest China's Shaanxi Province are a geographically important north-south boundary in China, act as a natural barrier for the eastward flow of moisture from the Tibetan Plateau, and providing a significant source of freshwater for downstream human communities. The distinctive feature of the river network is that it passes numerous cities areas 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). Most of nature reserves are located within Qinling Mountains, upstream of human communities. The various cities along the river contribute to different levels of NPS pollution. This river network provides a useful model for understanding the impact of human land-use practices lead to non-point source (NPS) pollution on river 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 represent NPS pollution 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.

2. Materials and methods

2.1 Study area, sampling procedure, and physicochemical analysis

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).

2.2 Sediment cell counts

Cell densities were investigated using catalyzed reporter deposition-fluorescent *in situ* hybridization (CARD-FISH) based on our previous study (Li et al., 2020; Li et al., 2023). Cell counting was performed in triplicate using ImageJ, and the cell abundance were expressed as the mean (n = 3).

2.3 High-throughput sequencing

5g of frozen sediment sample was aseptically subsampled for DNA extraction. The sequencing steps were conducted by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai,

China) using Illumina MiSeq platform (California, USA), and the raw data analysis according our previous study (Li et al., 2020; Li et al., 2023).

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).

2.4 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).

2.5 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., 2015) and the previous study (Gao et al., 2012; Munir et al., 2011). 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.

2.6 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 (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:

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

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

200 **2.7 Data available**

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

203 **2. Results**

204 **2.1 Biogeochemical properties of river water and sediment samples**

205 A total of 28 human-impacted urban sites and 24 undisturbed pristine nature reserve
206 surface sediment samples were collected (Fig. 1). They covered a wide range of
207 horizontal distance (0.72-376 km). The environmental chemical profiles of river water
208 showed remarkable differences between the two regions (Fig. 2, Dataset 1). The
209 differences mainly included significantly higher concentration of total nitrogen (TN),
210 ammonium, nitrite, nitrate, total phosphorus (TP), total dissolved phosphorus (TDP),
211 phosphate, volatile phenol, Chlorophyll *a* in urban river samples ($P<0.01$). Ammonia
212 content and permanganate index of the water also are higher in urban samples.
213 Ammonium and nitrate were the dominant forms of dissolved nitrogen within
214 the rivers. The total organic carbon (TOC) content in sediment also showed a significant
215 increasing trend from the nature reserve to urban areas ($P<0.0001$) (Fig. 2).

216 **2.2 Microbial community abundance, richness and diversity**

217 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
218 cells g^{-1} for urban and nature reserve samples, respectively (details in Dataset 2). The
219 sample from urban sites exhibited significantly higher abundance of microorganisms than
220 those from the nature reserve samples ($P<0.01$), indicating the human activities have a
221 great impact on microbial population.
222
223 Using universal primers for bacteria and archaea, 3,309,566 high-quality sequences were
224 generated throughout the river network (Dataset 2, SI Appendix Fig. S1). These
225 high-quality sequences were clustered into 18,038 operational taxonomic units (OTUs,
226 97% similarity level) (Dataset 2). A range of 1,628–6,104 chao1 OTUs and 1.89–6.68
227 Shannon index were observed in the urban samples, and a range of 1,321–5,471 chao1

228 OTUs and 1.88–6.31 Shannon index were observed in nature reserve samples (Fig. 3B-C).
229 Notably, both the Chao 1 and Shannon demonstrated that microbial richness and diversity
230 were considerably higher in the urban samples compared to the nature reserve samples ($p <$
231 0.05). Furthermore, the niche breadths (*Bcom*) (Fig. 3D) exhibited the
232 narrowest *Bcom* values in urban areas, rather than those in nature reserve areas.

233 **2.3 Microbial community structure and composition**

234 80 microbial phyla and 3,570 species were identified in the urban samples, and 74 phyla
235 and 3,371 species were identified in nature reserve samples (Dataset 2). At the phylum level,
236 the urban and nature reserve samples investigated here shared a high proportion of taxa
237 (Fig. 4A). The ten most prevalent phyla in all of sites collectively constituted 95.48% of the
238 total sequences (Fig. 4 A), matching the classical abundance-range relationship (Amend et
239 al., 2013; Ruff et al., 2015). The dominant microbial phyla included Proteobacteria (28.69%),
240 Firmicutes (28.32%), Actinobacteriota (17.79%), Chloroflexi (4.99%), Bacteroidota (4.51%),
241 Acidobacteriota (3.51%), Planctomycetota (3.51%), Verrucomicrobiota (2.43%),
242 Deinococcota (1.01%), Cyanobacteria (0.72%) (Fig 4A, Dataset 2). Archaea constituted only
243 a minor fraction of the river sediment communities (0.02~4.96%).
244
245 Although the microbial community of these sites exhibited similarities at the phylum level, 38
246 order in samples showing significant differences ($P<0.01$) between urban and nature
247 reserve river ecosystems (Fig. 4B, SI Appendix Fig. S1B). For example, Bacillales,
248 Clostridiales, Steroidobacterales were dominant in urban areas, while Erysipelotrichales,
249 Deinococcales, Alicyclobacillales, Pyrinomonadales, Burkholderiales were dominant in
250 nature reserve areas (SI Appendix Fig. S2B, Dataset 2). Furthermore, the analysis of
251 similarities showed that the urban samples harbored significantly dissimilar microbial
252 communities at OTU level (Fig. 3E; PERMANOVA pseudo $F = 2.1455$, $p.adj = 0.024$, 999
253 permutations; ANOSIM R sqr = 0.0597, $P = 0.03$).
254
255 A heatmap was generated to represent the 50 most prevalent species (with relative
256 abundances exceeding 1% in individual samples) (Fig. 5), and most of these dominant
257 OTUs were classified as unclassified species. Commonly observed with relatively high
258 abundances in all samples were *Exiguobacterium-sibiricum*, *Exiguobacterium* sp., and
259 *Acinetobacter lwoffii*. In urban samples, the dominant taxa included Unclassified
260 Planococcaceae, Unclassified Steroidobacteraceae, Unclassified Anaerolineaceae, and
261 Unclassified Rhizobiales. On the other hand, the unclassified *Acinetobacter*, unclassified

262 Pseudomonas, unclassified Sphingomonadaceae, unclassified *Deinococcus*, *Pseudomonas*
263 *parafulva* had high abundances in nature reserve samples.

264 **2.4 Nitrogen transformation microorganisms**

265 The presumed functional taxa associated with nitrogen transformations (i.e., nitrification and
266 denitrification) were detected within these samples. Nitrite-oxidizing bacteria (NOB),
267 ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA)
268 including *Nitrosomonas, Nitrospira,* Candidatus *Nitrocosmicus, Candidatus Nitrososphaera,*
269 *Nitrosarchaeum, Candidatus Nitrosotenuis,* and *Nitrosospira* (Dataset 2E-F) were identified.
270 Among them, *Nitrospira* exhibited the highest relative abundance, averaging 0.04%, and this
271 prevalence was more pronounced in urbanized areas compared to nature reserve samples
272 (Dataset 2E). Additionally, the sequencing results found denitrifying bacteria,
273 including *Rhodobacter*, *Paracoccus*, *Hyphomicrobium, Clostridium*, *Sphingomonas an*d
274 others. *Rhodobacter*, *Hyphomicrobium*, *Sphingomonas* were the dominant taxa within the
275 denitrifying bacterial community, constituting percentages of 1.55 %, 1.01 % and 0.39 %,
276 respectively. Similarly to nitrifying bacteria, the relative abundance of denitrifying bacteria
277 was higher in urban river areas in comparison to nature reserve river areas (Dataset 2E).

278 **2.5 Prediction of the functional diversity and metabolic pathways**

279 Microbial community metabolic predictions guided by the FAPROTAX database (Louca et al.,
280 2016) revealed distinctions in the metabolic potential related to nitrogen, carbon, and sulfur
281 cycling between urban and nature reserve areas (Dataset 4A-D). For nitrogen
282 transformation, the microorganisms involved in ammonia oxidation, nitrite oxidation, nitrogen
283 fixation, nitrification at urban river was higher than that at nature reserve (ANOVA,
284 $p < 0.001$). For carbon transformation, urban areas exhibited higher abundances of
285 microbial groups associated with anoxygenic photoautotrophy, oxygenic photoautotrophy,
286 chloroplasts, and photoautotrophy. A similar trend was observed in the distribution of
287 Cyanobacteria. Furthermore, the urban areas displayed higher abundance of taxa involved
288 in degradation of aromatic compound compared to the nature reserve (ANOVA, $p < 0.05$)
289 (Fig. 6). The sulfur cycling displayed heterogeneity among the samples. As depicted in Fig. 6,
290 the urban areas exhibited abundant taxa associated with the dark oxidation of sulfur
291 compounds (ANOVA, $p < 0.05$). Furthermore, the abundance of taxa involved in sulfate
292 respiration was higher in urban areas.
293
294 Additionally, the PICRUSt2 database (Douglas et al., 2020) annotated 414 functional KEGG
295 pathways (Dataset 4C-F). The metabolism associated with carbon fixation, nitrogen,

296 sulfur, phosphate, antibiotic biosynthesis and xenobiotic biodegradation were investigated.
297 The presence of antibiotic resistance proteins was also detected (Dataset 4B, D, F). In all
298 samples, biodegradation pathways encompassing 21 types of xenobiotic compounds were
299 determined (Dataset 4F-G). The ecological function of xenobiotic biodegradation exhibited a
300 noticeable increasing trend from the nature reserve to the human-impacted sites. This trend
301 suggests an increase in pollutants discharge from urban areas. Styrene degradation
302 emerged as the predominant process among polycyclic aromatic hydrocarbon (PAH)
303 degradation (Dataset 4G).

304 **2.6 Antibiotic resistance rates**

305 Tetracycline and sulfonamide are widely used to treat human and animal diseases
306 worldwide (Gao et al., 2012; Li et al., 2015). Majority of the river network examined in this
307 study contained tetracycline and sulfonamide resistance bacteria. Fig. 7 illustrates the
308 antibiotic resistance rates that were resistant to tetracycline, sulfamethoxazole on plating
309 count agar. The bacterial resistant rate within the urban samples ranging 0.40%- 11.80 % for
310 anti-tetracycline and 1.6 %- 26.9 % for anti-sulfonamide, were generally higher than that in
311 the nature reserve ranging 0.20% - 2.80 % and 1.25 % - 14.20 %. The sulfonamide
312 resistance rate was consistently 2 to 6 times higher than that of the tetracycline resistance
313 rate. While no significant differences were observed among the nature reserve samples, the
314 antibiotic-resistant rates exhibited variability among the samples from urban areas.

315 **2.7 Key influencing factors and ecological driving processes**

316 The distance-based redundancy analysis (dbRDA) using environment parameters as
317 constraining variables, highlighted the potential significance of these chemical parameters
318 in elucidating microbial community compositions (Fig. 3F). Ammonium (r sqr= 0.3551,
319 *P*=0.001), pH (r sqr= 0.2293, *P*=0.005), Nitrite (r sqr=0.2211, *P*=0.003), Nitrate (r sqr=0.188
320 *P*=0.006), TN (r sqr= 0.17, *P*=0.006), TOC (r sqr= 0.1367, *P*=0.032), phosphate (r sqr=
321 0.0615, *P*=0.010) were more crucial in shaping the microbial community (Fig. 3F).
322 Ammonium exhibited the strongest influence on bacterial and archaea community
323 composition within the river network ($P = 0.001$).
324
325 Across the large river network spanning a geographic distance of ~400 km, the microbial
326 community increased in dissimilarity with increasing spatial distance, showed a
327 distance-decay relationships (DDRs) (Fig. 8A). To further assess the underlying factors
328 governing community dynamics in the river network, the null model (Stegen et al., 2012) and
329 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 environmental selection processes, particularly in the form of homogeneous selection (|βNTI| < 2, RCbary >+0.95) (Fig. 8B, Dataset 3), in governing the community assembly (details in Materials and Methods). Simultaneously, NCM (Fig. 8C) 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.

3. Discussion

Over the past decades, human activities have resulted in an influx of nutrient-rich wastewater into river, causing significant changes in the biogeochemical cycles within aquatic ecosystems (Atashgahi et al., 2015; Xuan et al., 2022). 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 composition and functional aspects, to elucidate their responses to varying pollution levels.

3.1 Human activity enhance river microbial diversity

Considerable variation exists within the bacterial and archaeal communities in terms of microbial abundance, 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 human-impacted river sediment microbial community (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 in Fig. 2) (Kuypers et al., 2018). The proliferation of algae leads to increased organic matter production and subsequent decomposition by bacteria and archaea (TOC content in Fig. 2). This increased availability of organic matter and the changed nutrient conditions can influence the community composition. 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 (Ibarbalz et al., 2019). The influx of different organic compounds and nutrients into river creates a more complex environment, allowing for the coexistence of a broader range of microbial species. These changes in nutrient can have cascading effects on ecosystem functioning and overall microbial processes in the river network.

However, the observed increases in microbial diversity within urban 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. Similarly, Pascual-Benito et al. (2020) reported that the discharge of WWTP effluents led to reductions in the diversity of planktonic bacteria. 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.

The pollution resulting from industrial, domestic and agricultural activities are considered as the primary contributors to NPS pollution (Beattie et al., 2020; Ding and Liu, 2019). In contrast, WWTPs primarily constitute the point source pollution (Kiedrzyńska et al., 2014). The response of microbial communities to contamination depends on factors such as pollution magnitude, duration, and local environmental conditions. We suggest that high-concentration point source pollution tends to reduce microbial diversity, while relatively low-concentration NPS pollution can increase microbial diversity in the river ecosystem.

3.2 Structure of sedimentary microorganisms were significantly influenced by anthropogenic pollution effluents.

3.2.1 Key functional taxa. Nitrogen-enriched urbanized rivers exhibited a notably higher relative abundance of nitrogen-transformation taxa groups (Dataset 2), including nitrifying community (such as *Nitrosomonas, Nitrosospira*, *Nitrospina, Nitrospira*), denitrifying bacteria (such as *Paracoccus, Hyphomicrobium*, and *Gemmobacter*). These taxa are ubiquitous participants in the nitrogen cycle (Lücker et al., 2010). The inputs of nitrogen from human activity can lead to increased abundance of nitrogen-transformation taxa groups, consequently elevating nitrate concentrations in waters (Daims et al., 2015; Henson et al., 2018).

This observation is further supported by the presence of taxa associated with specific functions, such as *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., 2015; Oshkin et al., 2014; 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 (Dolman et al., 2012; Domingues et al., 2014). *Flavobacteria* are abundant and common heterotrophs in freshwater environment (García-Armisen et al., 2014), playing a crucial contribution in various carbon cycles, such as the degradation of algal organic matter and other forms of biodegradation (Williams et al., 2013). The higher abundance of such taxa observed in the urban areas can be attributed to the contamination resulting from human activities.

3.2.2 Pathogens. 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 (Wang et al., 2018; Wéry et al., 2008). The elevated abundance of Bacteroidetes and Firmicutes in the urban river areas suggested 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 Shigella* and *Staphylococcus aureus* within urban river environment (Dataset 2E-F). These findings highlight a distinct shift in microbial communities due to human 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 (VandeWalle et al., 2012; Zhang et al., 2009).

3.2.3 Antibiotic resistant microorganisms. 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).

3.2.4 PAH-degrading bacteria. High relative abundances of ecological function linked to xenobiotic biodegradation were detected in urban sites (Dataset 4G). 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). The presence of biodegradation genes could serve as indicators for the existence of xenobiotics and their corresponding metabolites (Kansole and Lin, 2016; Pagé et al., 2015). Furthmore, 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 urban river sediment. PAHs primarily originating from long-term anthropogenic pollution sources linked to rapid industrialization and urbanization (Kuppusamy et al., 2017; Mojiri et al., 2019). 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 (Kuppusamy et al., 2017; Lu et al., 2011; Patel et al., 2020). Numerous urban rivers in China have been

identified as containing PAHs (Cao et al., 2010; Qian et al., 2017), and significant biodegradation of PAHs capacity has also been observed in other rivers (Muangchinda et al., 2017).

3.2.5 Archaea. Strikingly, an increase in the diversity and proportion of the archaea was also identified under the anthropogenic pollution effluent. In the urban areas, proportion of the archaea exhibited 1.58 times higher than those in nature reserve, and archaeal Chao1 richness and Shannon diversity exhibited 1.47 and 2.36 times higher than those in nature reserve, respectively. 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).

3.2.6 Microbial composition structure. 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).

3.3 Non-point source pollution led to increased biotic homogenization

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, suggest the effluent led to increased biotic homogenization, and to our knowledge, likely due to the relatively consistent rich nutrient habitat environment for microorganisms across various urban sampling sites. This result provides an excellent illustration of the concept of biotic homogenization, which suggests 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 (McKinney, 2006). Further investigations are needed to explore the universality of biotic homogenization due to human activity effluent across a range of river ecosystems.

4. Conclusions

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 human activities. Microbial abundance and diversity exhibit significant correlations with different degrees of human activity. 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.

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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

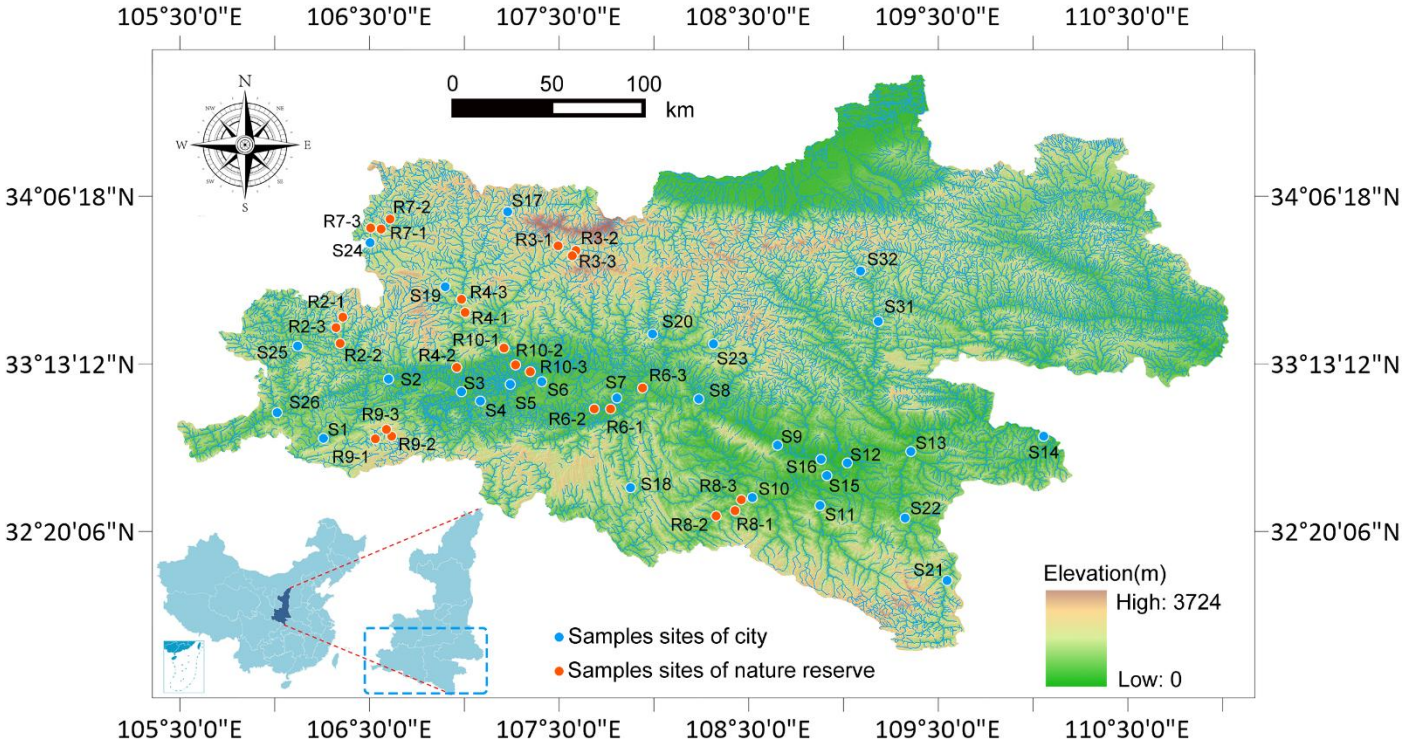


Figure 1. Location of the collected river network sediment samples. Blue circles indicate the urban sites, and orange circles indicate the nature reserve sites, respectively. The *Inset* map shows the extent of the study area relative to China.

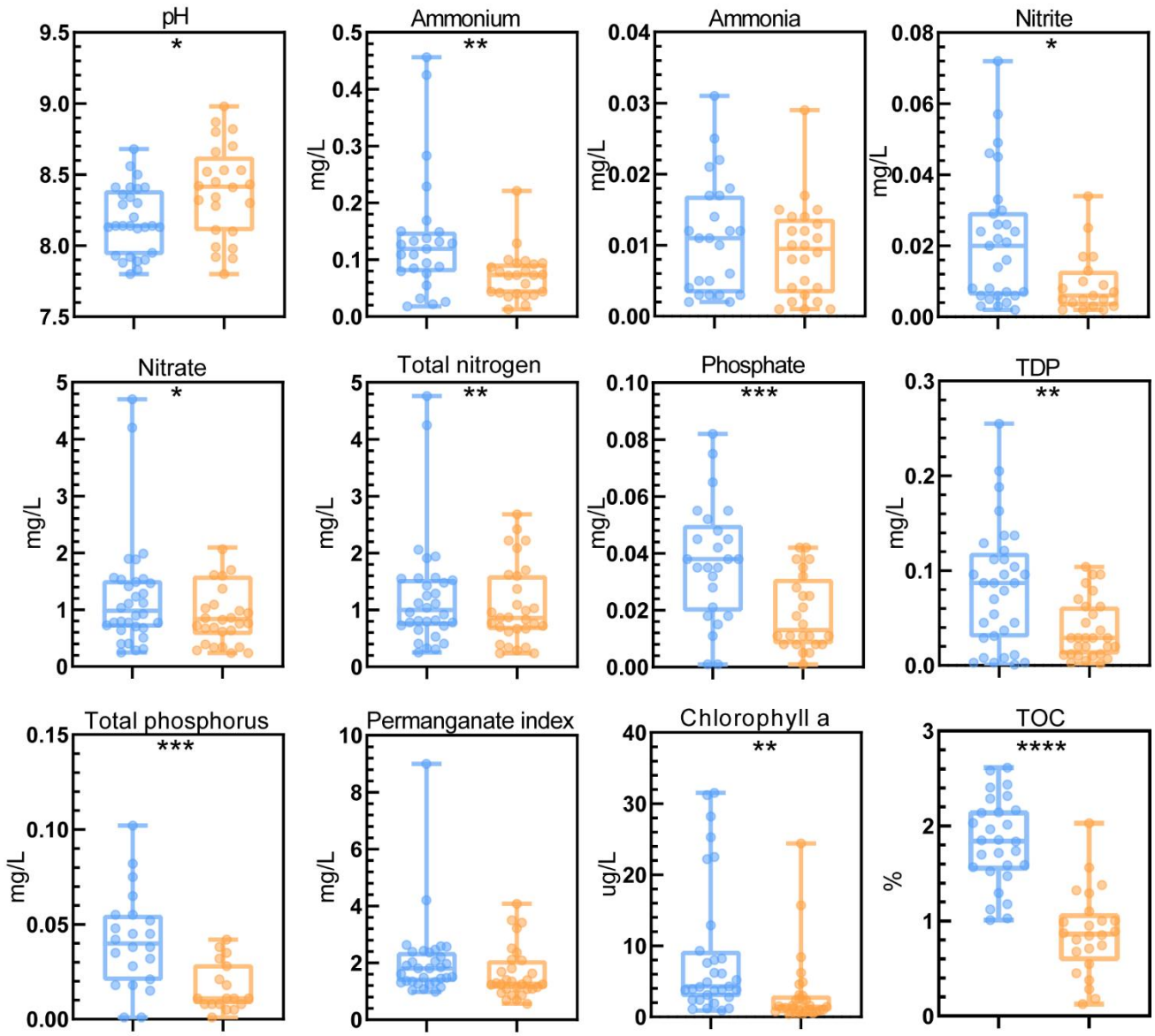


Figure 2. Comparison of geochemical characterization in human-impacted urban sites and undisturbed nature reserve sites in the river network. The blue circles indicate urban sites and orange circles indicate nature reserves sites, respectively.

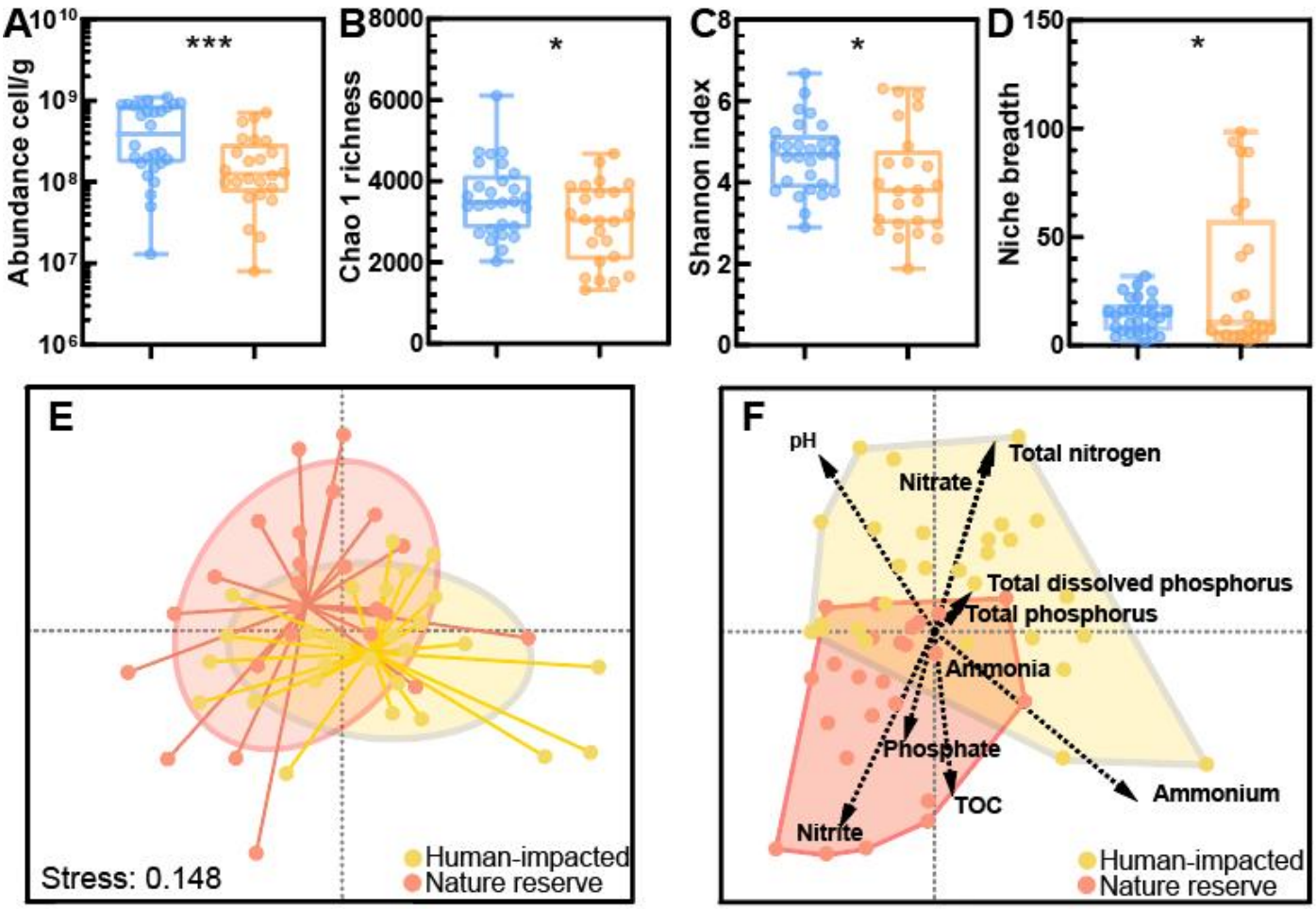
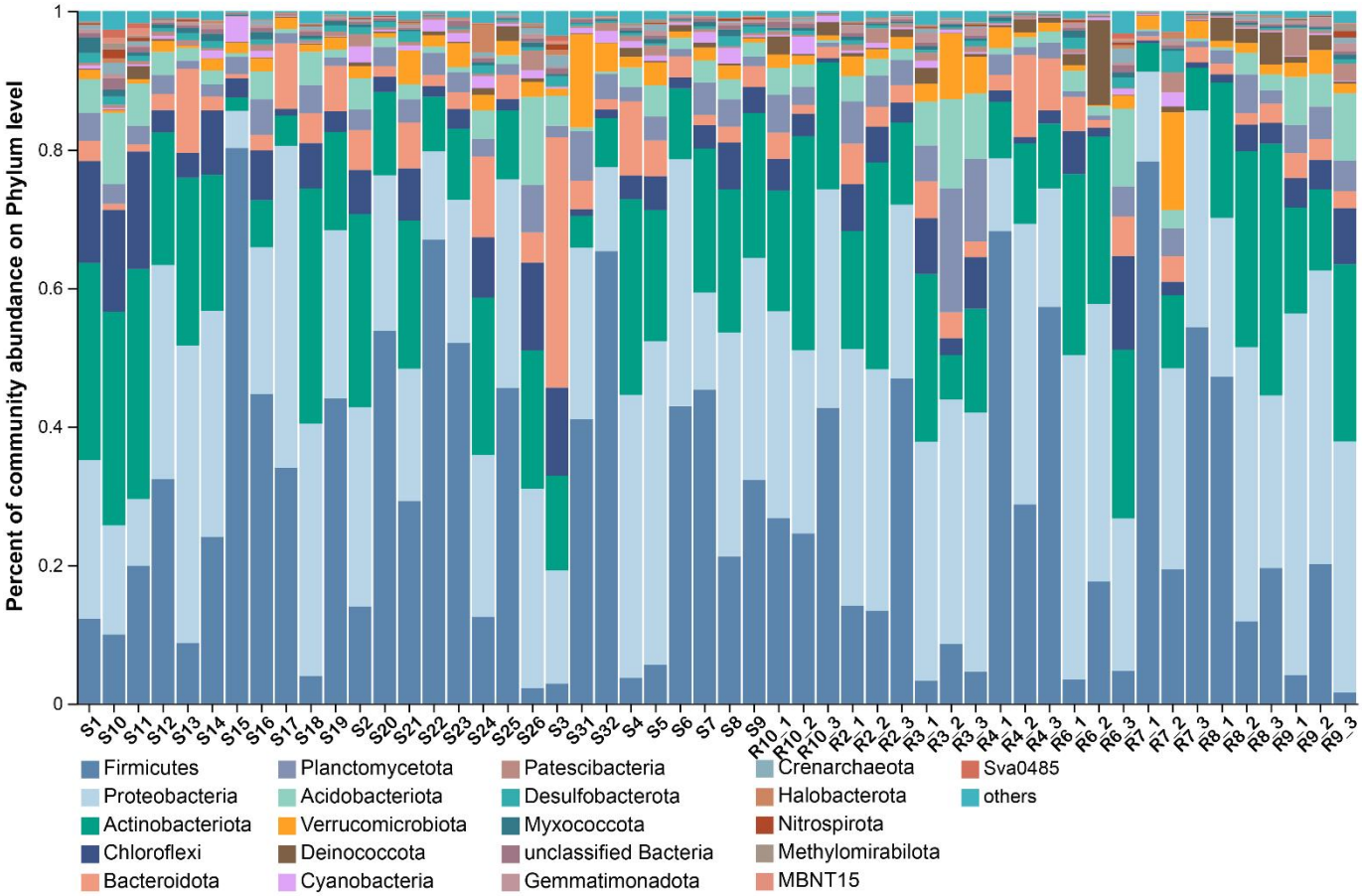


Figure 3. The microbiome in the large river network. (A-D), Microbial abundance, Richness estimates, Shannon index and Niches breadth were investigated. (E), Similarity of microbial communities visualized by NMDS. (F), A db-RDA showing microbial community similarity when environmental factors were used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


Percent of community abundance on Phylum level
0
0.2
0.4
0.6
0.8
1
S1 S10 S11 S12 S13 S14 S15 S16 S17 S18 S19 S2 S20 S21 S22 S23 S24 S25 S26 S3 S31 S32 S4 S5 S6 S7 S8 S9 R10_1 R10_2 R10_3 R2_1 R2_2 R2_3 R3_1 R3_2 R3_3 R4_1 R4_2 R4_3 R6_1 R6_2 R6_3 R7_1 R7_2 R7_3 R8_1 R8_2 R8_3 R9_1 R9_2 R9_3
Firmicutes
Proteobacteria
Actinobacteriota
Chloroflexi
Bacteroidota
Planctomycetota
Acidobacteriota
Verrucomicrobiota
Deinococcota
Cyanobacteria
Patescibacteria
Desulfobacterota
Myxococcota
unclassified Bacteria
Gemmatimonadota
Crenarchaeota
Halobacterota
Nitrospirota
Methylomirabilota
MBNT15
Sva0485
others

Percent of community abundance on Order level
0
0.2
0.4
0.6
0.8
1
S1 S10 S11 S12 S13 S14 S15 S16 S17 S18 S19 S2 S20 S21 S22 S23 S24 S25 S26 S3 S31 S32 S4 S5 S6 S7 S8 S9 R10_1 R10_2 R10_3 R2_1 R2_2 R2_3 R3_1 R3_2 R3_3 R4_1 R4_2 R4_3 R6_1 R6_2 R6_3 R7_1 R7_2 R7_3 R8_1 R8_2 R8_3 R9_1 R9_2 R9_3
Exiguobacterales
Burkholderiales
Micrococcales
Rhizobiales
Pseudomonadales
Bacillales
Sphingomonadales
Flavobacteriales
Rhodobacterales
Pirellulales
Verrucomicrobiales
Vicinamibacterales
Lactobacillales
Anaerolineales
Propionibacteriales
Blastocatellales
Deinococcales
Gemmatales
Chthoniobacterales
Caulobacterales
Peptostreptococcales-Tissierellales
Alicyclobacillales
others

839

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.

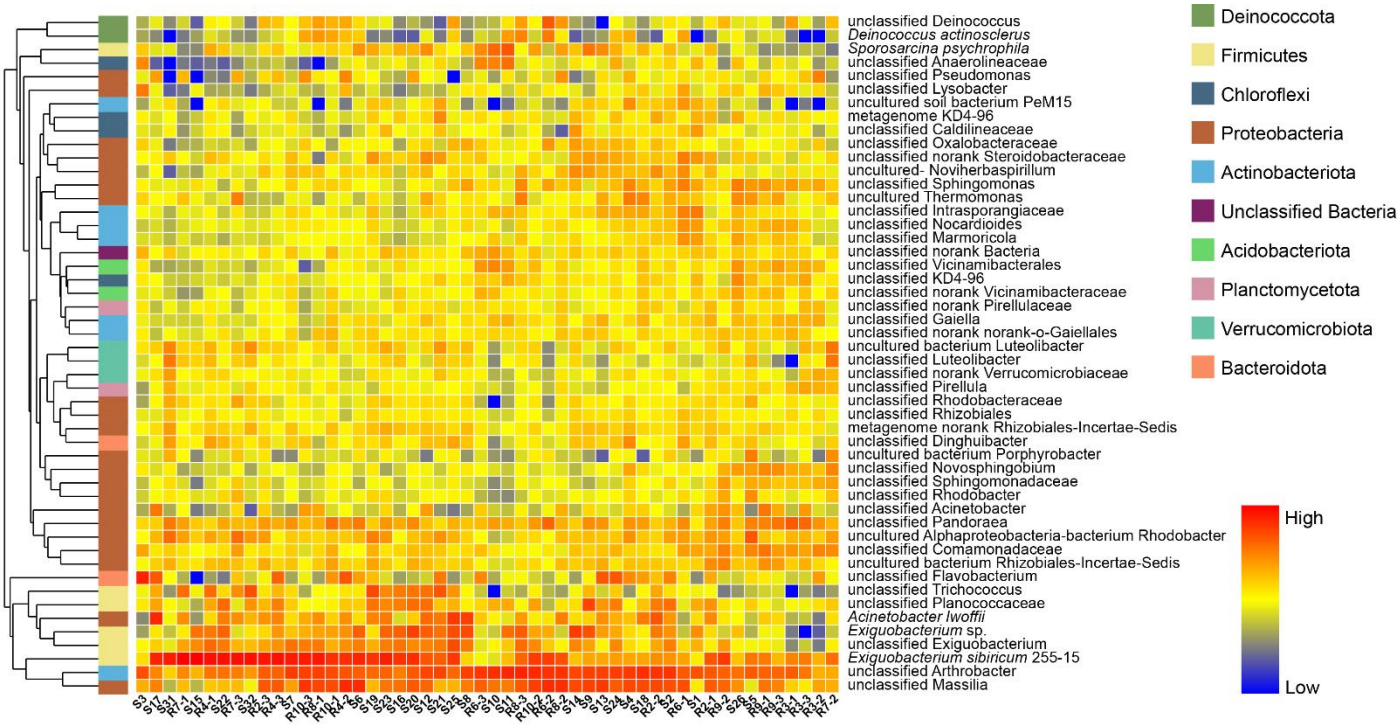


Figure 5. A heatmap was constructed for the 50 most dominant species.

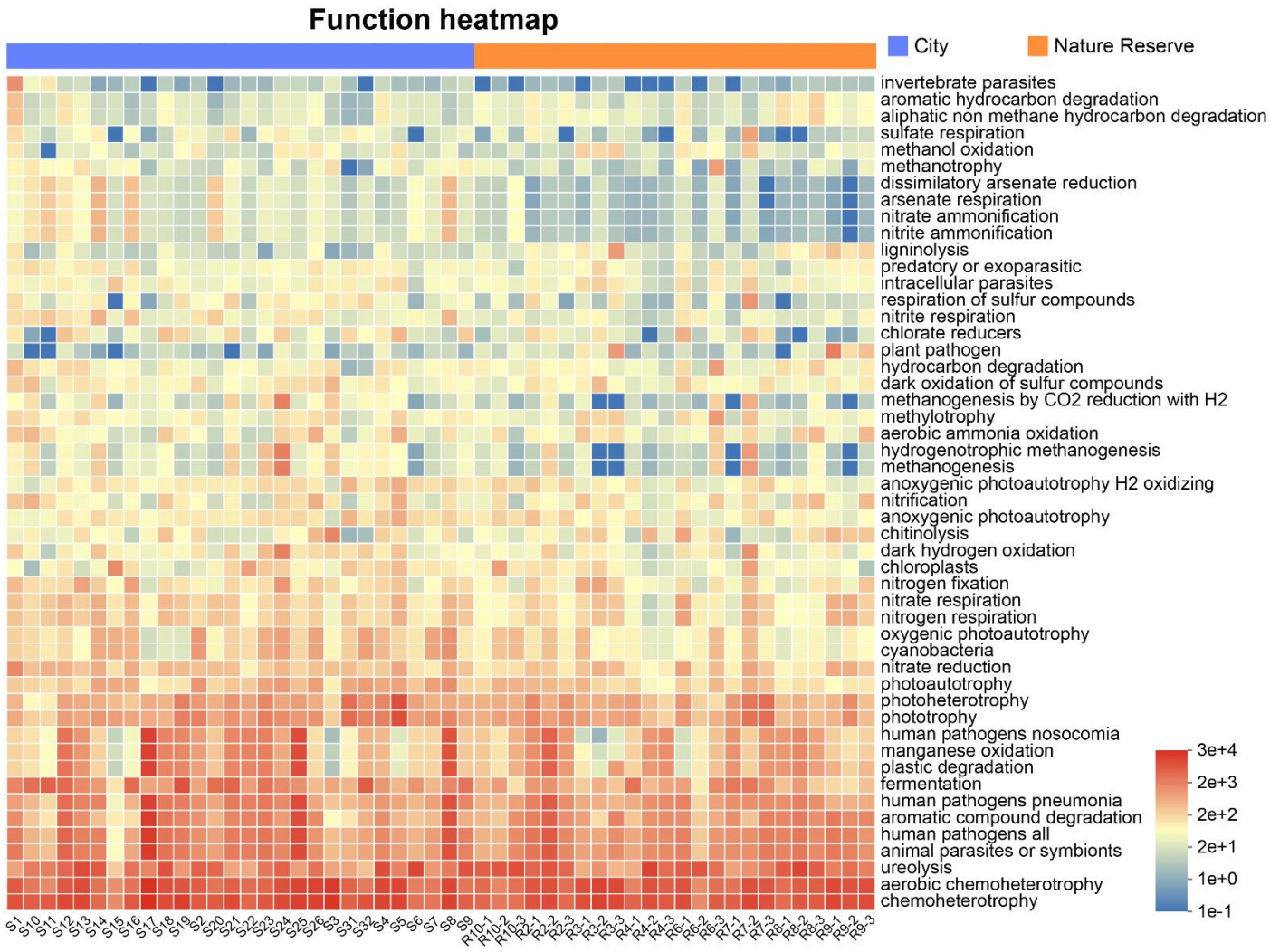


Figure 6. Prediction of the functional diversity and metabolic pathways between urban areas and nature reserve areas.

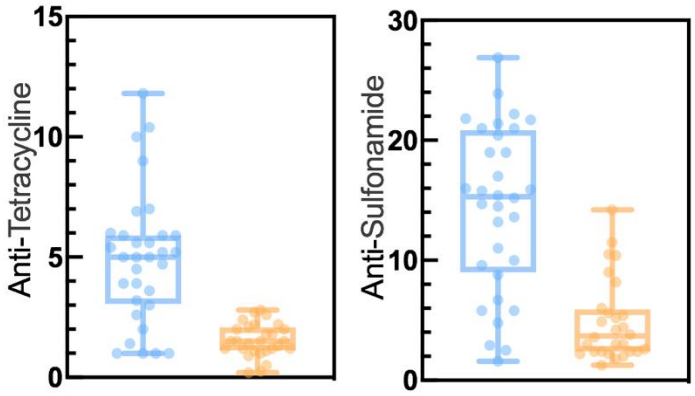


Figure 7. Antibiotic resistance rates. The blue circles indicate urban sites and orange circles indicate nature reserves sites, respectively. While no significant differences were observed among the nature reserve samples, the antibiotic-resistant rates exhibited variability among the samples of urban areas.

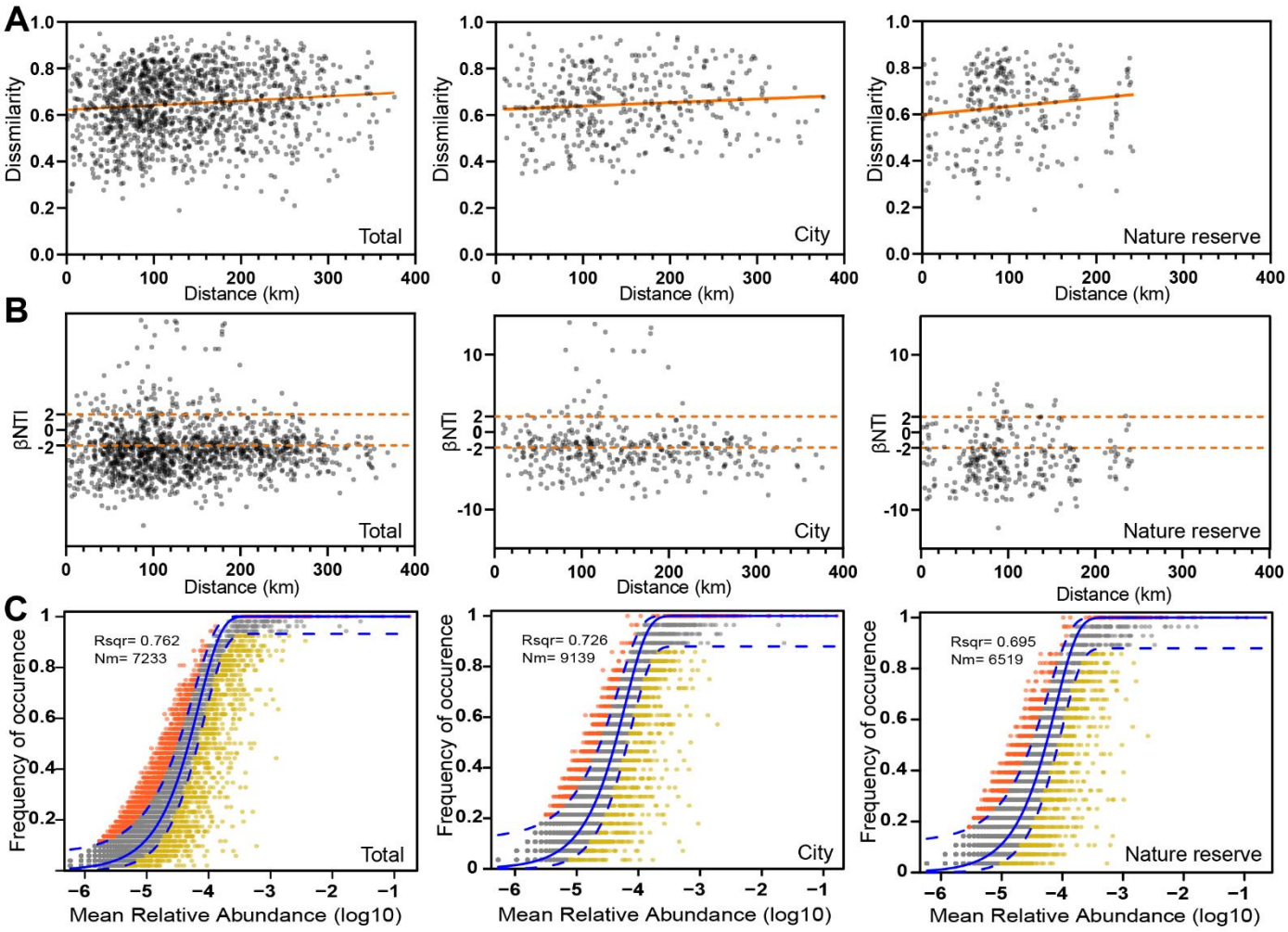


Figure 8. General patterns of microbial diversity in the river network system. (A) Distance-decay curves showing Bray–Curtis dissimilarity against geographic distances between sampling sites. Solid lines denote the ordinary least-squares linear regressions. (B) Scatter plot of βNTI values grouped by 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.