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1 *Article*

2 Effect of River Ecological Restoration on Biofilm
3 Microbial Community Composition

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16 **Abstract:** Across the world, there are increasing attempts to restore good ecological condition to
17 degraded rivers through habitat restoration. Microbial communities developing as biofilms play an
18 important role in river ecosystem functioning by driving organic matter decomposition and
19 ecosystem respiration. However, little is known about the structure and function of microbial
20 communities in riverine systems, and how these change when habitat restoration is implemented.
21 Here, we compared the biofilm bacterial community composition using 16S rRNA genes targeted
22 high-throughput Illumina Miseq sequencing in three river types, degraded urban rivers, urban
23 rivers undergoing habitat restoration and forested rivers (our reference conditions). We aimed to
24 determine: (i) the biofilm bacterial community composition affected by habitat restoration (ii) the
25 difference in bacterial diversity in restored rivers, and (iii) correlations between environmental
26 variables and bacterial community composition. The results showed that both water quality and
27 biofilm bacterial community structure were changed by habitat restoration. In rivers where habitat
28 has been restored, there has been an increase in dissolved oxygen, a reduction in organic pollutants,
29 a reduction in bacterial diversity and a related developing pattern of microbial communities, which
30 is moving towards that of the reference conditions (forested rivers). River habitat management
31 stimulated the processing of organic pollutants through the variation in microbial community
32 composition, however, a big difference in bacterial structure still existed between the restored rivers
33 and the reference forest rivers. Thus, habitat restoration is an efficient way of modifying the biofilm
34 microbial community composition for sustainable freshwater management. It will, however, take a
35 much longer time for degraded rivers to attain the similar ecosystem quality as the "pristine" forest
36 sites than the seven years of restoration studied here.

39

1. Introduction

One of the current aims in riverine ecology is to use ecological restoration techniques to improve the quality of river ecosystem health, especially in urban areas where rivers have often been degraded severely [1]. Degraded rivers are normally formed by water pollution, land reclamation, dredging, channelisation, altered hydrology and the clearing of riparian zones [2, 3]. Ecological restoration approach aims to recover river habitat quality by increasing river habitat complexity and heterogeneity; this is achieved by reconfiguring the river channel, increasing flood plain areas, adding in-stream islands, and aquatic vegetation [1]; all designed to enhance the hydraulic and substrate heterogeneity and macrophyte colonization. In combination, these treatments should increase food availability within the ecosystem [4, 5], and eventually, a complexity of aquatic habitats (e.g. riffle, run, pool, and debris dam classifications) will develop in these restored rivers [6].

Healthy river habitats not only allow the living micro-organisms, aquatic flora (e.g. algae, aquatic plants) and fauna (e.g. macro-invertebrates, fishes) to persist, but they can also provide important ecosystems services, for example by reducing pollutants, such as organic matter, nutrients and heavy metals [7]. Riverine habitats are known to influence the diversity and composition of aquatic biotas through river morphology, hydrology, sedimentation, and by changing environmental variables at the reach scale, the latter important for larger stream organisms such as fish and macro-invertebrates [8]. For example, the surface features of the stream may influence detritus accumulation [9], and hence form 'refuges' for predators [10, 11]. Moreover, the habitat complexity generated by surface irregularities exerts a significant impact on the abundance and diversity of benthic invertebrates in stream systems [6, 12, 13]. In a meta-analysis, in-stream habitat heterogeneity restoration (including wood, boulder additions and channel reconfigurations) enhanced macro-invertebrate richness [6]. Nuttle et al., (2017) also found that cutting gates, restoring substrates, and enhancing in-stream and riparian habitats, significantly enhanced (i) the taxon richness of macro-invertebrates, and (ii) the richness and abundance of fish in 18 mitigation sites [15]. In spite of this, very little is known about the effects of river habitat restoration on the composition of biofilm microbial communities.

Biofilms, are a complex assemblage of microbial communities composed of bacteria, archaea, fungi, algae, and exopolysaccharides produced by the microorganisms. They are important components of stream ecosystems, and are considered a good bio-indicator of environmental health [16], not only because of their high abundance in most natural environments, but also because of their sensitivity to environmental changes with short life-cycle. Biofilms are a basic component of freshwater food webs; they adhere to the surfaces of rock particles and aquatic plants, and are influenced by many environmental factors including temperature, light, shear forces, nutrients and contaminants [17-19]. They fix energy and carbon by photosynthesis and chemosynthesis and some can also fix nitrogen [20]. They also recycle organic nitrogen, impact on dissolved organic matter, and play key roles in nutrient cycling, organic compound degradation, water quality remediation and suspended sediment removal [21]. Effectively, altering any environmental factor can affect stream biofilm communities, and this may in turn alter their function of the whole stream ecosystem [22]. Bacteria are an indispensable part of the epilithic biofilm, usually occupying 1-5% of the epilithic biofilm, and playing key roles in nutrient cycling, metabolic processes and many other biogeochemical processes and ecosystem functions [23-25]. The rates of bacterial-mediated nitrification, denitrification, and heterotrophic nitrogen (N) uptake in small streams have been shown to affect downstream water quality [25-27]. However, the impact of habitat restoration on biofilm bacterial community composition is still unclear.

To address this lack of information about biofilms during riverine restoration, we compared microbial populations in three different river types along a disturbance gradient. The most disturbed sites in this study were in urban areas, and the least disturbed sites were in forested catchments. In between, were rivers in urban areas where the habitat had been restored within the last seven years

89   as part of an ecological restoration strategy. We measured a range of environmental factors and
90   assessed the microbial community using a standardized field procedure followed by 16S rRNA
91   Illumina MiSeq. Through comparing the relationship among habitat status, environmental
92   parameters and bacterial community composition, we aimed to determine: (i) the biofilm bacterial
93   community composition affected by habitat restoration (ii) the difference in bacterial diversity in
94   restored rivers and urban degraded rivers, and (iii) any correlations between bacterial community
95   composition and selected environmental variables. We hypothesized that habitat restoration would
96   alter the biofilm bacterial community composition in these restored rivers compared to the degraded
97   ones and that they would become similar to the reference forest rivers. The bacterial diversity would
98   be shifted toward near-natural state where habitat had been restored. The substrate composition and
99   physico-chemical variables like dissolved oxygen, nutrient and organic pollutant might be leading
100 factors affecting the bacterial community composition in river groups.

2. Materials and Methods

2.1. Study Sites

103 This study compared three stream types in the winter of 2017: (i) degraded rivers in urban areas,
104 (ii) restored rivers, where an aquatic habitat restoration scheme had been implemented within the
105 last seven years for each river; and (iii) rivers in forested catchments as reference conditions. Nine
106 streams with similar-sized watersheds within the Anji City Region, Zhejiang Province PRC were
107 selected for this study (Figure 1, Table S1). There were three replicates of each stream type, all located
108 in different places in Anji City. The average day/night temperatures of the region were 12 ℃/5 ℃ in
109 winter, and an average precipitation of 50 mm.
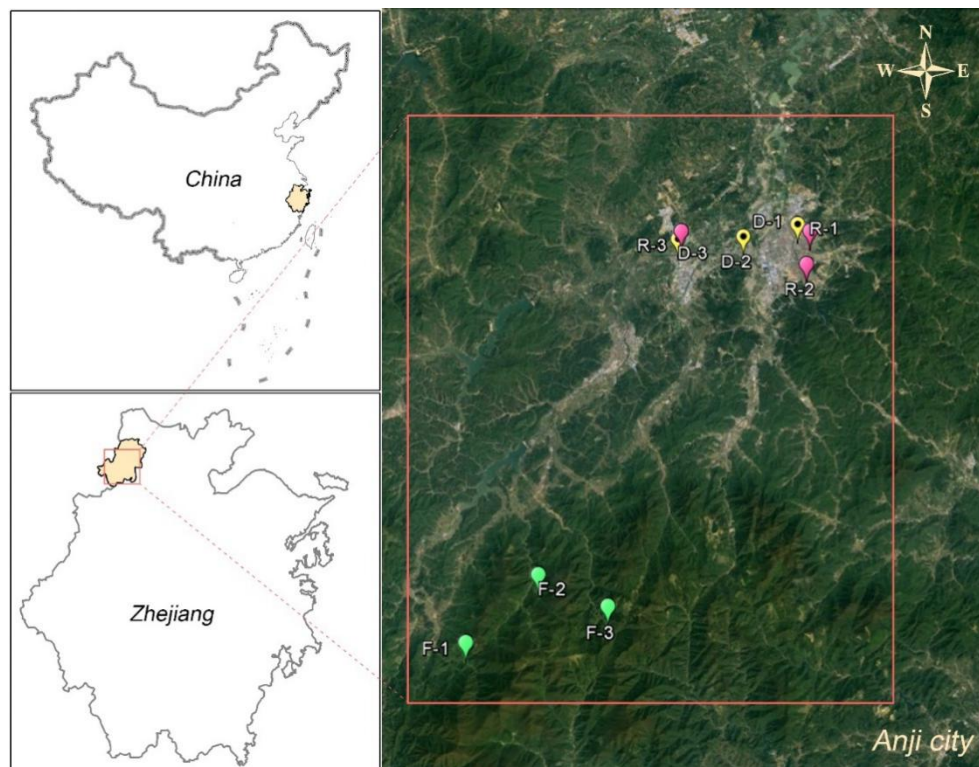
Figure 1. Location of the sampling sites within the Anji City Region, PRC; Containing three degraded urban rivers (D), three restored rivers (R) and three Forested rivers (F). The three forest streams (F) were upstream from Anji City; the three restored rivers (R) and the three degraded urban rivers (D) were downstream of the forest ones.

115   The three urban degraded sites (denoted D) were similar to the pre-restoration status of our
116 restored rivers, Tongxin River is located in the city center, and the other two are located in the
117 suburban districts. The three restored rivers (denoted R) have been restored for up to seven years
118 using a mixture of ecological restoration techniques to reconstruct a natural river form. The
119 techniques used included channel re-meandering, creation of riffles, pools and run areas,
120 construction of floating islands, aquatic plant re-introduction, and riparian zone afforestation. A
121 subsidiary aim was to provide ecosystems that could be used for ecological research, education and
122 entertainment. Three forest streams (denoted F) were in the Tianmu Mountains (maximum elevation
123 590 m), 40-km upstream from Anji City were set as our "reference" conditions, because pristine rivers
124 were not available in the city area. There has been relatively little human interference on these forest
125 streams, and they represent pre-urban landscape form where the urban rivers have derived [28].

2.2. Habitat Survey and Physico-chemical Parameters of Stream Water

127   Habitat surveys were performed in December 2017 and January 2018. Reach canopy cover was
128 estimated visually and presence of various mesohabitat counted (island, pool, riffle). To estimate the
129 variation of sediment grain size within each reach studied, 100 sediment particles were selected
130 randomly on the river bed and proportions of boulders (> 256 mm in diameter), cobbles (64-256 mm),
131 pebbles (4-64 mm) and sand grains (2-4 mm) were counted [29]. The substrate diversity was
132 calculated using the percentage cover of all substrate classes using the Shannon diversity index H'
133 [30] for each study site.

134   Thereafter, within each river, the river width was measured using a 100 m tape. Water velocity
135 and river depth were measured at five evenly-spaced points across the channel using Teledyne flow
136 meters (ISCO, Lincoln, Nebraska, USA) and a steel ruler. Water quality in each river was monitored
137 at three different points with 3 m interval at the maximum by *in situ* measurement of temperature,
138 pH, both using a HACH pH/temperature meter (HACH, LA-pH 10, USA), dissolved oxygen (DO),
139 using a YSI Professional Plus probe (YSI Propolus, USA), and turbidity, using a turbidity meter
140 (HACH, DR2100Q, USA). A 1 litre water sample was collected from each stream and filtered in the
141 field through 0.45 um Jingteng syringe tip filters and preserved at 4 ℃ before sending to the
142 laboratory. These water samples were analyzed within 48 hours for (i) total nitrogen (TN) and total
143 organic carbon (TOC), measured using a total organic carbon analyzer with a total nitrogen module
144 (Multi N/C3100, analytik-jena, German), (ii) ammonium nitrogen (NH_4^+), nitrate-nitrogen (NO_3^-), and
145 total phosphorus (TP), measured using a QuickChem® Flow Injection Analysis system (Lachat
146 Instrument, Hach, USA), and (iii) chemical oxygen demand (COD), measured using a DR1010 COD
147 analyzer (HACH, USA).

2.3. Biofilm Sampling Procedure

149   Biofilm was sampled by placing four 10 cm × 10 cm autoclaved unglazed tiles, at 0.3 m water
150 depth in each river for 39 days; thereafter the biofilms were collected by scraping the accumulated
151 materials from the tiles into 50 ml tubes covered with aluminum foil, and transported in a cool box
152 to the laboratory. The material in each 50 ml tube was then separated into two, one part was filtered
153 through 0.45 um membrane filter (Jingteng) to measure chlorophyll *a* (Chl-*a*) using a fluorimeter
154 (10AU, Turner Designs, Sunnyvale, California, USA) after acetone extraction [31], and the other part
155 was filtered on 0.22 μm pore size polycarbonate membrane filters (Millipore, USA) using a vacuum
156 pump; these filters were stored in sterile Petri dishes at -20 °C until DNA extraction.

2.4. DNA Extraction and Analysis of Bacterial Community Composition

158   The genomic DNA of all the biofilm samples was extracted using DNA extraction Kit (MO BIO
159 PowerBiofilm® DNA Isolation Kit, USA) based on a standard protocol. The DNA concentration was
160 quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the

161 ratio of absorbance at 260 nm and 280 nm checked to insure the quality of DNA obtained. All DNA
162 samples were then preserved at -80 ℃ before processing for bacterial community analysis.

163 The bacterial diversity and community composition of all biofilm samples were measured using
164 the Illumina Miseq sequencing at Suzhou Genewiz Company. Using 30-50 ng DNA as the template,
165 the 16S rRNA genes covering the V3-V4 regions were first amplified from the DNA extracts using
166 the forward primer 347F "CCTACGGRRBGCASCAGKVRVGAAT", and the reverse primer 802R
167 "GGACTACNVGGGTWTCTAATCC". PCR amplification was conducted in triplicate for each
168 sample using 25 μl PCR reactions mixture containing 2.5 μl TransStart Buffer, 2 μl dNTPs, 2 μl of
169 each primer, 0.2 μl BSA, 0.4 μl FastPfu DNA polymerase, 20 ng DNA template and ddH$_2$O. PCR was
170 performed using the following conditions: initial denaturation at 95 ℃ for 3 min, 24 cycles of
171 denaturation at 94 ℃ for 30 s, annealing at 57 ℃ for 90 s, and extension at 72 ℃ for 10 s. The PCR
172 amplicons were checked by 2% agarose gel electrophoresis and purified using MagPure Gel Pure
173 DNA Mini Kit (Magen). The purified amplicons were pooled and paired-end sequenced on the
174 Illumina MiSeq platform (Illumina, USA) at a read length of 2 × 300 bp.

175 After 16S rRNA sequencing, the reads were sorted to the samples according to barcodes, and the
176 barcodes and primers were then removed. The low-quality reads were discarded, including the reads
177 which did not exactly match the primer, the reads containing ambiguous character (N), a sequence
178 length <200 bp, and reads with an average quality score <20. Then chimeric sequences were detected
179 and removed by comparing the sequences with the reference database (RDP Gold database) [32]
180 using UCHIME algorithm [33]. The high-quality sequences were clustered into operational
181 taxonomic units (OTUs) using the clustering program VSEARCH9 (1.9.6) against the Silva 128 16S
182 rRNA database with 97% sequence identity threshold. The Ribosomal Database Program (RDP)
183 classifier was used to assign taxonomic category to all OTUs at a confidence threshold of 0.8. The 16S
184 rRNA gene sequences were submitted to National Centre for Biotechnological Information (NCBI)
185 Sequence Read Archive database under the accession numbers MH889163 - MH890450.

186 *2.5. Statistical Analysis*

187 We evaluated differences in habitat characteristics, physico-chemical features, bacterial diversity
188 and richness in different stream types (forest, urban restored and degraded) using one-way analysis
189 of variance [34], followed by the Tukey's HSD post-hoc test for comparison of means. Pearson
190 correlation coefficients were used to explore relationships between environmental parameters and
191 all microbial variables. Differences were accepted as significant at p = 0.05 level. These statistical
192 analyses were performed in the R statistical Environment [35].

193 Based on the results of the operational taxonomic units (OTUs) analysis, α-diversity indices
194 (Shannon-Weiner index; Chao1 richness) were calculated in QIIME1.9.1 [36]. Non-metric Multi-
195 dimensional Scaling (NMDS) plot was performed to display β-diversity based on Euclidean
196 dissimilarities between each samples using the 'vegan' package [37] within the R statistical
197 Environment [35]. Analysis of similarities (ANOSIM) was then performed to evaluate the bacterial
198 community similarity among three river types using the vegan package. Venn diagrams were drawn
199 to analyze overlapped and unique OTUs of each sample based on cluster analysis of OTUs. Metastats
200 [38] was performed to detect the differentially abundant taxonomic groups at phylum and genus
201 levels between different river types. The relationships between the bacterial community and
202 environmental parameters (pH, turbidity, DO, TN, TP, TOC, NH$_4^+$-N, NO$_3$-N and COD) were
203 assessed using redundancy analysis (RDA) within Canoco 4.5 for windows [39].

204 **3. Results**

205 *3.1. Habitat Characteristics*

206   There was a significant difference in canopy cover among the different river types ($F_{2,6}$ = 13.435,
207 p = 0.006); canopy cover was significantly greater in forest rivers, intermediate in degraded rivers,
208 and lowest in restored rivers. Forest and restored rivers had greater diversity of river bed habitat
209 types than degraded rivers. In forest and restored rivers, riffles, pools, islands were commonly found
210 whereas in the degraded rivers only pools, and a few islands were observed. In terms of substrate
211 composition, the Shannon diversity (H') of substrate (ranging from 0 to 1.13) was significantly greater
212 in forest and restored rivers (p = 0.001) and lowest in degraded rivers. Only granules were found in
213 degraded rivers, whereas the restored and forest rivers had boulders (forest-only), cobbles, pebbles
214 and granules. Degraded sites had much smaller substrates, whereas restored and forest rivers had
215 bigger substrates.

216 *3.2. Effects of Habitat Restoration on Physico-chemical Properties of Stream Water*

217   Physico-chemical values (Table 1a) revealed no significant differences among river types for
218 river width ($F_{2,6}$ = 0.336) and mean depth ($F_{2,6}$ = 0.791), and no difference in the surface water for pH
219 ($F_{2,6}$ = 1.815), NH_4^+ ($F_{2,6}$ = 1.533), NO_3^- ($F_{2,6}$ = 0.374), TN ($F_{2,6}$ = 2.708), TP ($F_{2,6}$ = 0.042) and COD ($F_{2,6}$ =
220 5.069). However, significant differences were observed in surface water properties among the stream
221 types for DO ($F_{2,6}$ = 7.398, p = 0.024), turbidity ($F_{2,6}$ = 7.69, p = 0.022), TOC ($F_{2,6}$ = 17.86, p = 0.003) and
222 Chl-*a* ($F_{2,6}$ = 8.94, p = 0.016). The forest and restored rivers had similar concentrations of DO, and both
223 had significantly greater DO concentrations than the degraded rivers (p < 0.05) (Figure 2A). The
224 turbidity in degraded rivers was much greater than forest rivers (p = 0.018), while no differences were
225 observed between forest rivers and restored rivers, restored rivers and degraded rivers (p > 0.1)
226 (Figure 2B). Degraded rivers and restored rivers had greater TOC concentrations than forest rivers (p
227 = 0.002 and p = 0.027, respectively). Although no significant difference was detected when comparing
228 restored rivers with degraded rivers (p > 0.1), a reduction in TOC concentration was observed (Figure
229 2C). In terms of Chl-*a*, no differences were detected when comparing forest rivers with restored rivers
230 and degraded rivers (p > 0.1), whereas rivers under restoration had a much higher Chl-*a*
231 concentration than degraded rivers (p = 0.013) (Figure 2D).

232

233   **Table 1.** Mean values of (a) physico-chemical variables and (b) microbial diversity in different types
234   of rivers within the Anji City Region, PRC. The values represent the mean ±standard error of three
235   replicate samples.

236 **(a)**

River Type	Width (m)	Mean Depth (cm)	Dissolved Oxygen (mg/l)	pH	Turbidity	NH4-N (mg/l)	NO3-N (mg/l)	Total N (mg/l)	Total P (mg/l)	Chemical Oxygen Demand (mg/l)	Total Organic C (mg/l)	Chlorophyll *a* (mg/l)
Forest	8.83± 1.64	35.87± 7.97	14.16± 0.80	7.33± 0.11	0.62±0. 14	0.02± 0.01	1.06± 0.13	1.99± 0.21	0.18± 0.02	2.44±0 .15	0.48± 0.16	0.61±0.2 3
Restored	13.17± 3.09	28.13± 7.22	13.14± 0.65	7.64± 0.14	3.52±0. 85	0.08± 0.02	1.13± 0.40	2.74± 0.77	0.17± 0.02	3.35±0 .76	2.81± 0.32	1.22±0.1 9

| Degra ded | 11.57± 5.72 | 22.87± 3.86 | 7.91±1. 52 | 7.38± 0.11 | 22.81±1 4.93 | 1.37± 1.19 | 0.79± 0.40 | 4.01± 0.76 | 0.18± 0.05 | 8.82±3 .40 | 6.70± 2.21 | 0.20±0.0 9 |

(b)

River Type	Observed OTUs	Unique OTUs	Diversity Indices	
			Chao 1 Value	Shannon-Weiner Index
Forest	604.11 ±38.87	14.67 ±0.88	715.45 ±36.27	6.42 ±0.12
Restored	585.00 ±19.86	5.67 ±3.18	708.84 ±21.18	5.89 ±0.15
Degraded	666.89 ±69.17	30.00 ±14.80	769.73 ±72.81	6.98 ±0.17

3.3. Effects of Habitat Restoration on Bacterial Community Composition

A total of 3,300,566 reads were obtained from the 27 samples. After filtering, denoising, and chimera removal, 1650283 high-quality 16S rRNA gene-reads were obtained, ranging from 48,473 to 69,662 reads per sample. Mean OTUs and α-diversity values (Table 1b) showed that bacterial diversity measured by Shannon diversity index (H') was different between the river types ($F_{2,6}$ = 14.067, p = 0.005), being significantly greater in degraded rivers ($F_{2,6}$ = 6.98, p = 0.004) than restored rivers, whereas no distinct difference was found between restored rivers and forest rivers with respect to bacterial diversity (Figure 2F). Bacterial richness (Chao 1 Index) varied from 629 to 874, however, no significant differences were detected among river types for bacterial richness (Figure 2E).
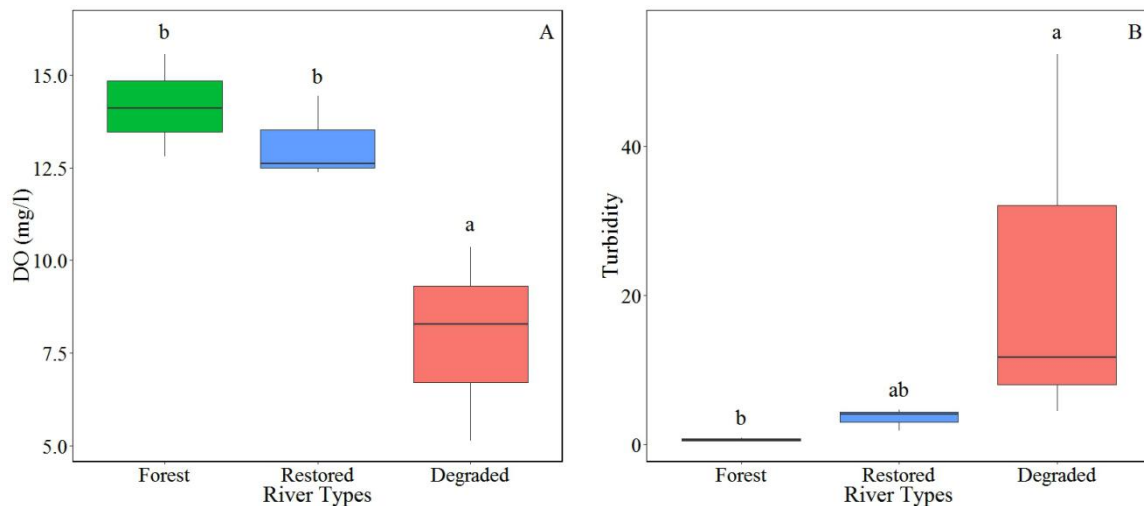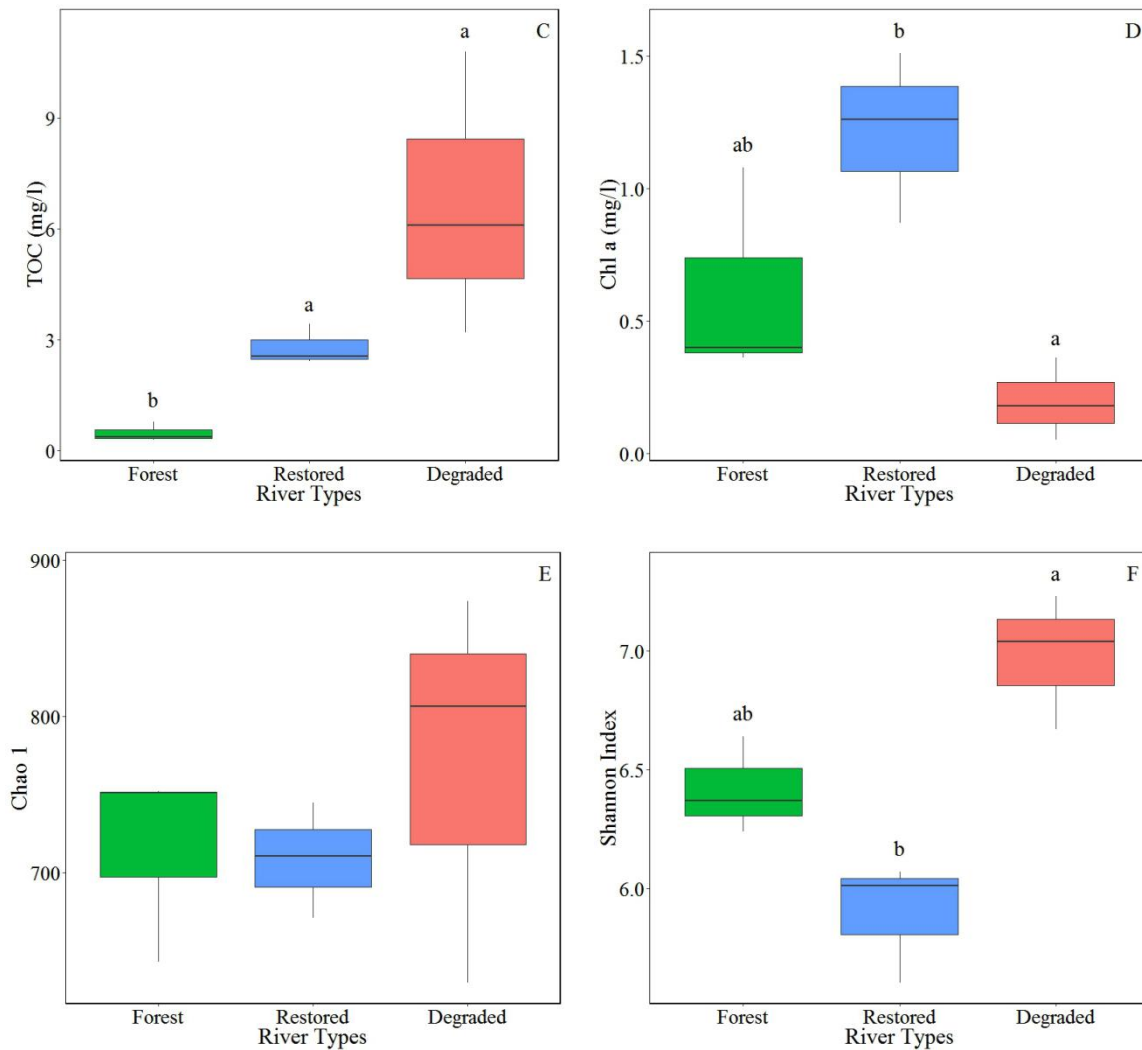
Figure 2. Boxplots representing the variance of physico-chemical parameters (A) dissolved oxygen (DO), (B) turbidity, (C) total organic carbon (TOC), (D) Chl-*a* and bacterial α-diversity (E) bacterial richness (Chao 1 Index), (F) bacterial diversity (Shannon Index) in forested, restored and degraded rivers within the Anji City Region, PRC. Black line: median value; box: quartile interval; whiskers: minimum and maximum value. Different lowercase letters indicate the significant difference observed at p = 0.05 level.

The NMDS analysis produced a stress value <0.094, indicating that the ordination produced a good summary of the observed distances between samples with obvious clustering (Figure 3). The bacterial community structures among all three river types were distinct from each other (R = 0.508, p = 0.001) as shown by analysis of similarities (ANOSIM) (Table 2). Although there was some overlap between restored and degraded rivers, the bacterial community composition was significantly different (R = 0.256, p = 0.008) and there was a clear shift in bacterial community composition along the first axes from degraded to restored rivers, and from restored to forest rivers.

Table 2. Analysis of similarities (ANOSIM) of biofilm bacterial communities in contrasting river types within the Anji City Region, PRC.

River-type Comparison	ANOSIM	
	R	*p*

Forest vs. Degraded	0.645	0.001
Forest vs. Restored	0.733	0.001
Restored vs. Degraded	0.256	0.008

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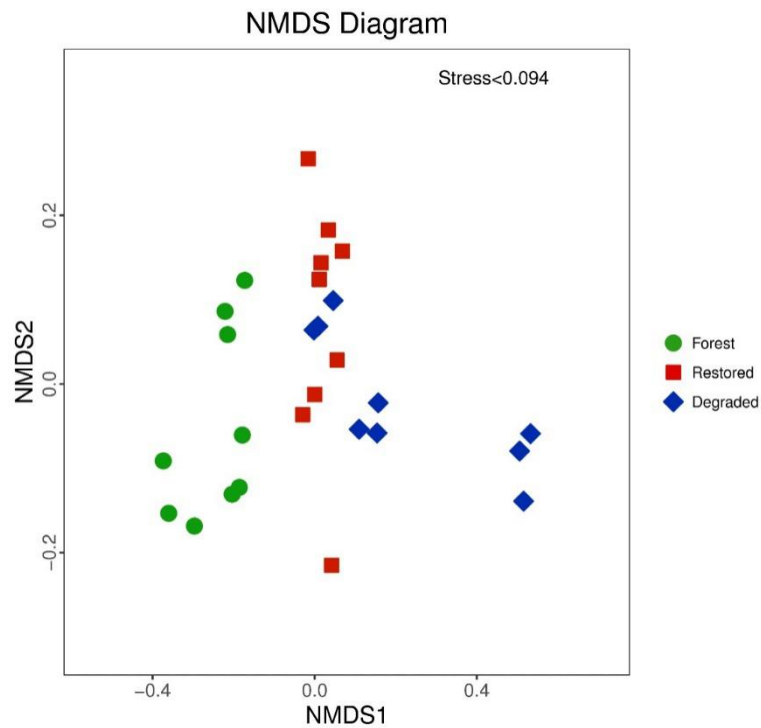
Figure 3. Non-metric Multi-dimensional Scaling (NMDS, stress<0.094) ordination of biofilm bacterial communities in forested, restored and degraded rivers within the Anji City Region, PRC within the Anji City Region, PRC.

In total, 383 OTUs were detected, 232 OTUs (61%) of which were universally present from biofilms in all rivers, and the three types of rivers contained 11.5% (forested), 4% (restored) and 23% (degraded) unique OTUs, respectively (Figure 4). The degraded rivers had greater percentage of unique OTUs, including genera Rhodocyclales, Cytophagales, Sphingobacteriales, however, no statistical differences were detected among river types for unique OTUs ($F_{2,6}$ = 2.81).
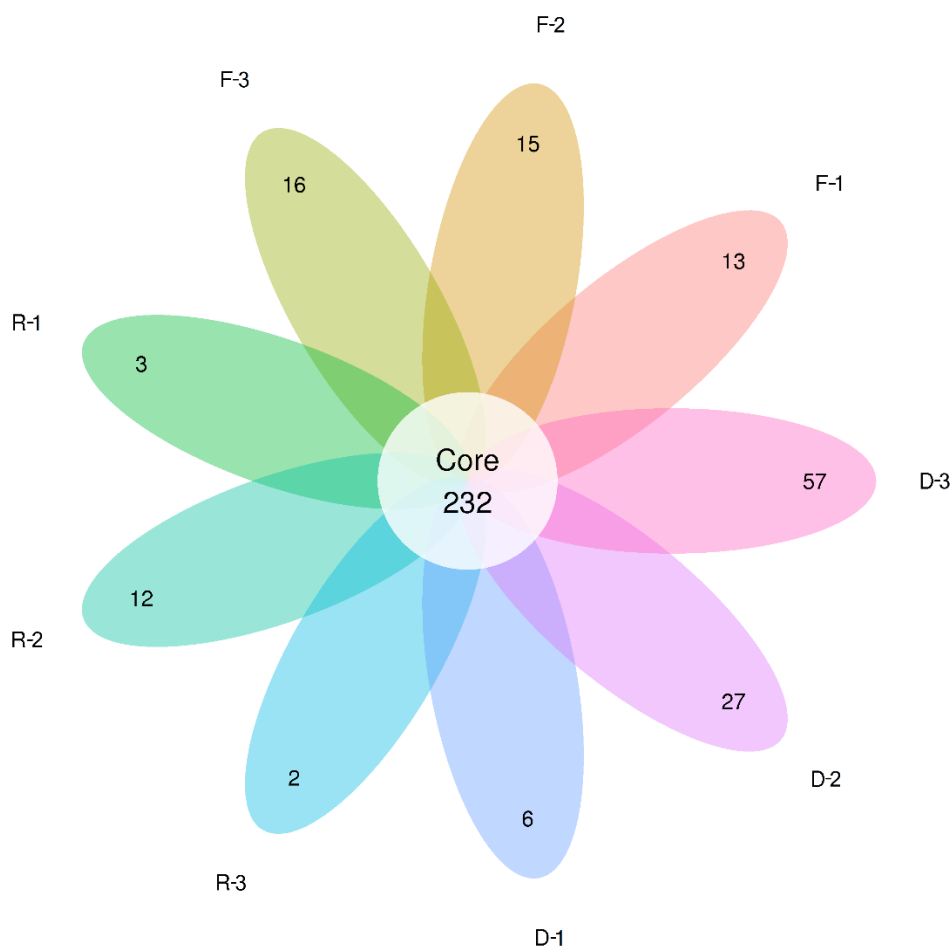
Figure 4. Venn diagram showing the number of unique and shared Operational Taxonomic Units (OTUs) among biofilms in forested (F), restored (R) and degraded (D) rivers within the Anji City Region, PRC.

The relative abundance of the bacterial community was calculated respectively both at phylum and genus level. At phylum level (Figure 5A), Proteobacteria was the most abundant phylum in all rivers, followed by Bacteroidetes, Firmicutes, Cyanobacteria, Verrucomicrobia, Acidobacteria and Actinobacteria. Rivers in forest and after restoration had a greater Proteobacteria abundance than degraded rivers ($p = 0.050$, $p = 0.049$, respectively), while no difference was detected between forest and restored rivers ($P > 0.05$). The relative abundance of bacteria in the phylum Bacteroidetes, a genera commonly assumed to be specialized in degrading high molecular weight (HMW) compounds [40], was slightly greater in degraded rivers than forest rivers ($p = 0.064$), while, no differences of Bacteroidetes were observed when comparing forest rivers with restored rivers, and restored rivers with degraded rivers ($p > 0.01$).

In terms of relative abundance at genus level, *Flavobacterium*, *Duganella*, *Pseudomonas*, *Undibacterium* and *Arenimonas* were commonly distributed in all studied rivers (Figure 5B). Degraded rivers showed significant numbers of reads allocated to *Flavobacterium* ($p = 0.001$), *Arenimonas* ($p = 0.026$) and *Acinetobacter* ($p = 0.001$). Forest rivers had a higher relative abundance of *Duganella* ($p = 0.022$), *Indobacter* ($p = 0.010$), *Clostridium_sensu_stricto_13* ($p = 0.006$), *Methylotenera* ($p = 0.001$) and *Rhodoferax* ($p = 0.007$) than degraded rivers. Among restored rivers, a greater relative abundance of *Flavobacterium*, *Pseudomonas*, *Acinetobacter* and a lower relative abundance of *Indobacter*, *Clostridium_sensu_stricto_13*, *Methylotenera* and *Rhodoferax* ($p < 0.05$) was found when comparing restored rivers with forest rivers. Restored rivers had a greater relative abundance of *Duganella* ($p =$

298 0.023) than degraded rivers. No difference in genus abundance was found between restored and
299 degraded rivers for other taxa.

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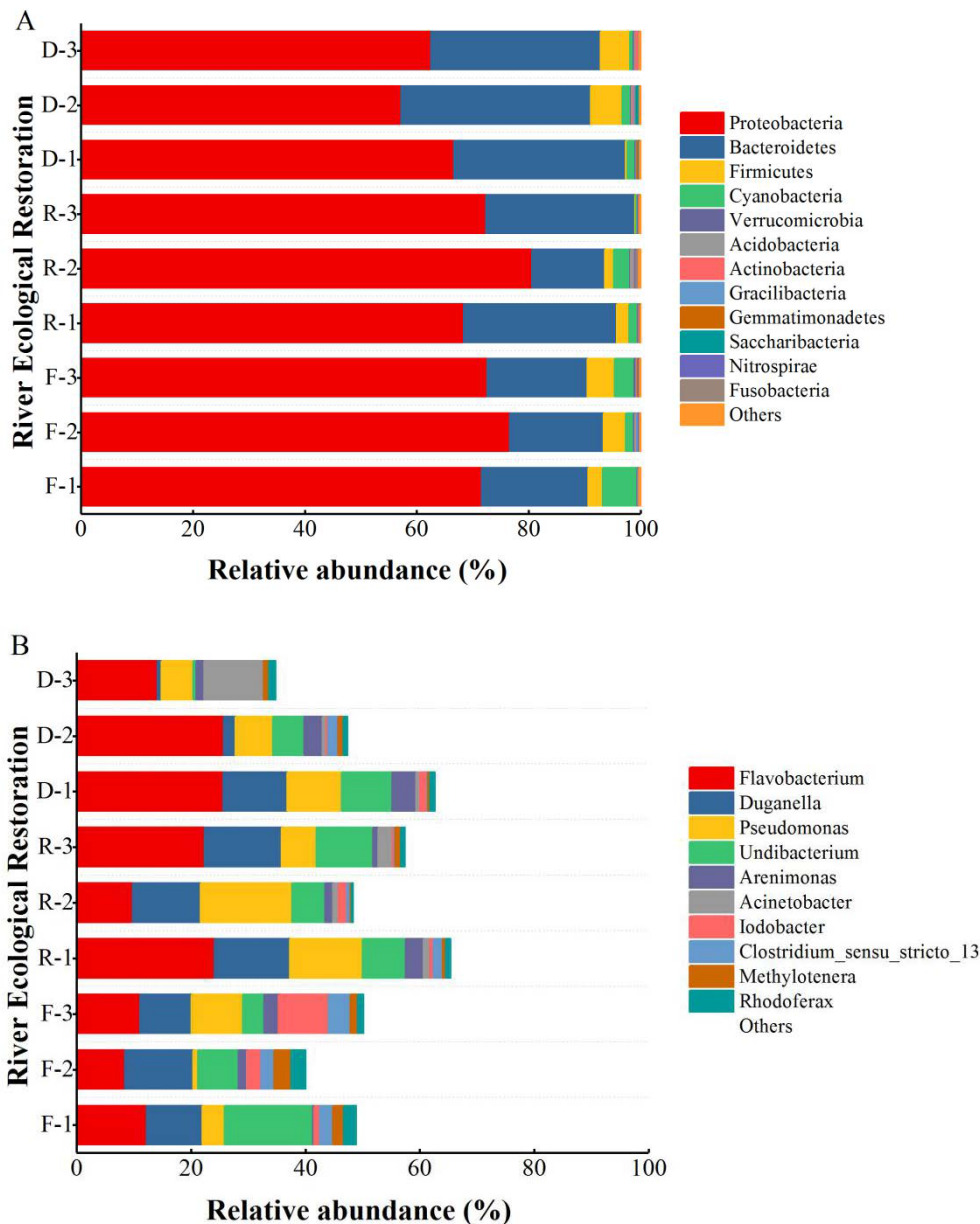
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303 **Figure 5.** Relative abundance of bacterial community at Phylum (A) and Genus level (B) in forested
304 (F), restored (R) and degraded (D) rivers within the Anji City Region.

305 *3.4. Correlation between Bacterial Community Composition and Environmental Variables*

306 Bacterial richness (OTUs) showed a positive correlation with water turbidity and a negative
307 correlation with TP concentration (p = 0.049, p = 0.032, respectively). Bacterial diversity showed a
308 strong positive correlation with water turbidity (p = 0.006), COD (p = 0.023), and TOC concentration
309 (p = 0.019), and was negatively correlated with substrate diversity (p = 0.033). The relationship
310 between environmental parameters and the total bacterial community composition was further
311 evaluated by constrained redundancy analysis (RDA), which produced eigenvalues for the first two
312 axes of 0.322 and 0.159, respectively (Figure 6). The environmental variables explained 48.1% of
313 bacterial community structure variance. The biofilm bacterial assemblages in forest rivers were

314 positively correlated with substrate diversity (r = 0.156) , and Chl-*a* concentrations (r = 0.828), and
315 were negatively affected by NH_4^+ (r = - 0.621) and COD (r = - 0.629) of surface water. The reverse
316 pattern was found for biofilms in the degraded rivers, COD (r = 0.999), TOC (r = 0.984), NH_4^+ (r =
317 0.738) and TN (r = 0.635) in the surface water presented as major factors linking to the bacterial
318 structure in degraded rivers. For the restored rivers, the bacterial samples showed positive
319 correlations with DO (r = 0.571) and substrate diversity (r = 0.652), and was affected negatively by
320 COD (r = - 0.522) and NH_4^+ (r = - 0.526), though the correlations were not as strong as the forest rivers.
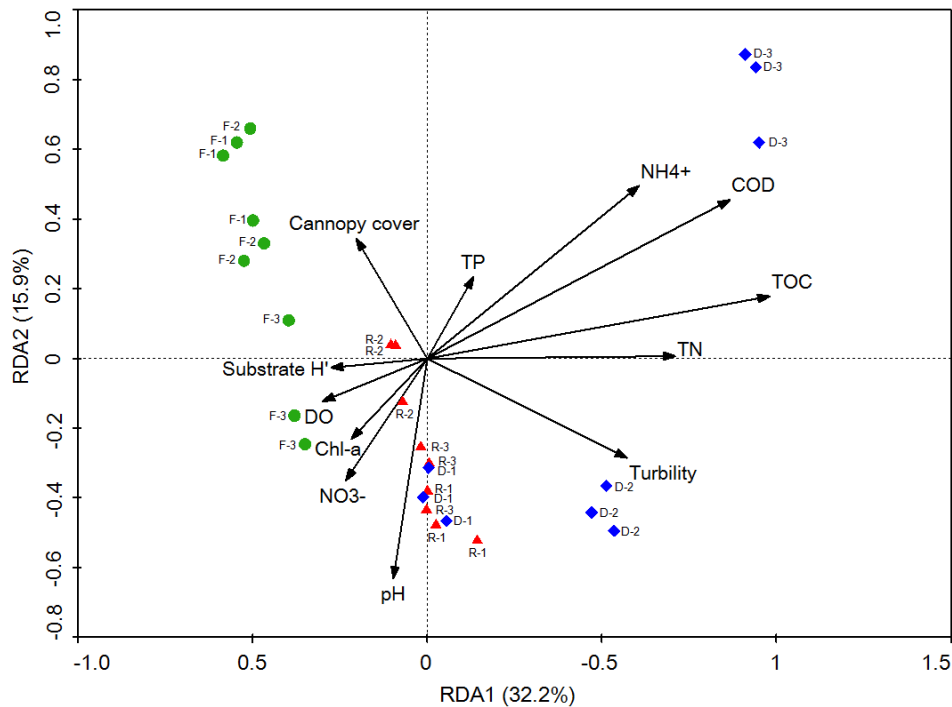
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322 **Figure 6.** Relationship between the biofilm bacterial community and environmental variables in
323 forested (F, circles), restored (R, triangles) and degraded (D, diamonds) rivers within the Anji City
324 Region, PRC.

4. Discussion

326 Rehabilitation of aquatic biota, through habitat restoration, is now being implemented around
327 the world to prevent further damage and mitigate existing freshwater degradation [41].
328 Accumulating evidence has linked aquatic rehabilitation to reducing nitrogen, phosphorus and
329 organic matter concentrations, and thereafter to improved conditions for macro-invertebrate and fish
330 populations [6, 15, 42]. Microbial communities are often ignored in stream restoration studies yet they
331 are crucial for supporting aquatic ecosystem processes and functions with key roles in driving
332 organic matter and nutrient cycling [43]. It is, therefore, imperative that we obtain a better
333 understanding of the underlying mechanisms of microbe-mediated processes. In this study,
334 therefore, we described the bacterial community composition including those involved in important
335 ecological functions in restored rivers, and compared them with both degraded urban sites and
336 "pristine" reference forest sites; to do this we used high-throughput 16S rRNA gene amplicon
337 sequencing methods. The results showed clear differences in the structure of biofilm microbial
338 communities among these three main river ecosystems, and these differences were strongly
339 correlated to the changes in habitat and physico-chemical characteristics in these river groups. This
340 finding is consistent with the results of surveys in New Zealand and USA, showing that local
341 environmental conditions, rather than spatial factors, such as latitude or elevation, best predicted the
342 variance of community composition and diversity [44, 45]. Suggesting that the differences in
343 microbial community here were mainly led by the variance in habitat and environmental

344   characteristic in the rivers, the longitudinal natural changes in rivers may account for some of the
345   environmental and biological variation observed [46].

346   *4.1. Habitat Restoration Impact on Physico-chemical Properties of Stream Water*

347   The consistent input of pollutants from both point and diffuse sources in the urban (pre-
348   restored) rivers caused high enrichment of TOC. Habitat restoration led to a reduction in TOC, and a
349   significant increase in DO in the surface water of the restored rivers. These results are consistent with
350   habitat restoration experiments in the Zenne River in Belgium [47]. Essentially, habitat restoration
351   improved conditions by reducing TOC and increasing DO, suggesting that organic pollutants
352   entering the degraded river were removed through habitat restoration. There was no difference in
353   DO concentration between restored and reference forest rivers, suggesting that habitat restoration
354   improved the physico-chemical environment of restored rivers.

355   *4.2. Impact of Habitat Restoration on the Bacterial Community*

356   The diversity and composition of bacterial communities change according to habitat
357   characteristics [48], hence, rehabilitation methods and the intensity of application should affect both
358   the composition and diversity of microbial communities. Here, no differences were detected among
359   river types for bacterial richness, and a significant decline in bacterial diversity was detected in
360   restored rivers compared to degraded rivers. This is consistent with studies in wastewater treatment
361   plant (WWTP) effluent in both urban and rural areas where a reduced diversity of biofilm bacteria
362   has been detected [49, 50]. The difference in bacterial diversity might reflect the physico-chemical
363   variables of surface water in the different river types. Dissolved inorganic nitrogen, dissolved organic
364   carbon and hydrological variability have been demonstrated to be the most important environmental
365   factors affecting biofilm responses [51]. In this study, the increase of DO concentration caused by
366   habitat restoration might lead to the development of aerobic microbial community and higher
367   efficiencies of chemical oxygen demand removal through oxidative decomposition [52]. The decline
368   in organic carbon quality could also influence the abundance of biofilm bacteria [51, 53], which might
369   have led to the decrease in heterotrophic anaerobic microorganism that rely on organic resources,
370   which lead to the decline of bacterial diversity in rivers after habitat restoration. Epilithic bacterial
371   populations can also be affected indirectly by inorganic nutrients via the influence of nutrients on
372   algal biomass [54, 55].

373   Distinct bacterial communities were detected in each of the river types, a dissimilar composition
374   was found between (i) forest rivers and degraded rivers, (ii) forest rivers and restored rivers, and (iii)
375   restored rivers and degraded rivers. These differences were strongly correlated with the changes in
376   habitat substrate diversity, and physico-chemical characteristics (DO, TOC and COD) of these river
377   types. The results from this study suggest that the differences in bacterial community compositions
378   were mainly caused by the variations in habitat and habitat-specific physico-chemical characteristics
379   [48, 56]. Rivers with diverse substrates may provide more dynamic surface and higher degree of
380   resource heterogeneity within the microhabitats for biofilms, shaping distinct bacterial communities
381   in forest and restored rivers from microbiome in degraded rivers. The variations in physico-chemical
382   attributes (e.g. TOC) in forest and restored rivers might led to the difference in bacterial community
383   composition between these two river types. Moreover, the bacteria clustered in the restored rivers
384   were distributed between the bacteria in the degraded and forest rivers, indicating that they were
385   moving in the correct direction, i.e. towards the reference forest rivers. There was, however, some
386   overlap between the restored and degraded rivers, indicating that there was still a legacy effect of the
387   previous degraded state. Overall, the degraded rivers possessed significantly greater bacterial
388   diversity than the restored rivers. Hence, restoration to "pristine" conditions will take longer than
389   seven years, and further studies are needed to determine exactly how long.

390   Compared with forest rivers, degraded rivers had a slightly greater abundance of Bacteroidetes,
391   a member of phylum specialized in degrading high molecular weight (HMW) compounds, and

392 possessed significantly higher relative abundance of *Flavobacterium*, *Arenimonas* and *Acinetobacter*,
393 which are capable of metabolizing/mineralizing organic compounds [57-59], and a remarkably low
394 abundance of *Duganella*, *Indobacter*, *Methylotenera*, *Rhodoferax* and *Clostridium_sensu_stricto_13*; these
395 genera are major players in cycling of carbon compounds in the environment [60, 61], and organic
396 matter utilization [62]. This suggests that the degraded rivers with a high TOC load and limited DO
397 have a distinct impact on the microbial community, shaping the microbiome with a greater ability to
398 degrade/mineralize high molecular weight (HMW) compounds in degraded rivers; this ability
399 differentiates these degraded rivers from the forest ones.

400 The restored rivers, however, had a greater relative Proteobacteria abundance than degraded
401 rivers; this phylum is often found in nutrient-poor conditions with a low TOC [47]. Moreover,
402 *Duganella* genus which utilized organic compounds, but required oxygen to survive [63] was greater
403 in restored rivers compared to the degraded ones. This may imply that along with the establishment
404 of more diverse substrates and aerobic and sub-aerobic system in the restored rivers, habitat
405 restoration shifted the dominant components of the bacterial community that mineralize and degrade
406 organic matter to bacteria that utilize organic matter for growth. At the same time, there is also a shift
407 from species that occur in predominantly anaerobic conditions to aerobic conditions. This is
408 consistent with the RDA results, where the bacterial community in the degraded rivers was strongly
409 correlated to organic pollutants TOC and COD, whereas, for restored rivers, the bacterial community
410 only showed weak positive correlations with substrate diversity and DO in the surface water.

411 In terms of the relationship between restored rivers and forest rivers, no significant differences
412 in bacterial diversity, bacterial richness, and relative abundance of the Proteobacteria and
413 Bacteroidetes were found. However, restored rivers possessed a lower abundance of *Indobacter*,
414 *Methylotenera*, *Rhodoferax* and *Clostridium_sensu_stricto_13* than forest rivers. Moreover, the
415 *Flavobacterium*, *Pseudomonas* and *Acinetobacter* were found in greater abundance in degraded rivers
416 were much greater in restored rivers compared to forest rivers. This suggests that restored rivers still
417 possess species that degrade/mineralize the high concentrations of organic compounds that persist
418 even after restoration. In summary, our results highlight effective dissolved oxygen enhancement,
419 organic pollutants reduction trends, and alongside changes in the microbial community during river
420 habitat restoration. However, restored rivers still have a long way to go to recover the natural status
421 of pristine rivers, and continued monitoring is needed to measure the time scale required for the
422 restored sites to attain the reference standards.

5. Conclusions

424 We examined the effect of habitat restoration on microbial community composition in biofilms using
425 high-throughput 16S rRNA gene amplicon sequencing. The results showed that habitat restoration
426 altered the bacterial community structure in a positive manner in the degraded rivers. Habitat
427 restoration induced a lower bacterial diversity, but greater abundance of genera that degrade organic
428 pollutants; these changes might be attributed to the status of dissolved oxygen and total organic
429 carbon variables in the surface water. These results suggest that applying habitat restoration
430 approaches to restore urban rivers by enhancing habitat heterogeneity, which can in turn alter the
431 physico-chemical characteristics and stimulate the processing of organic pollutants through the
432 variation of microbial community composition, which was moving in the right direction. Habitat
433 restoration is, therefore, an efficient way for the switching of microbial community composition for
434 sustainable freshwater restoration and management. It will take longer than seven years for degraded
435 rivers to attain the similar ecosystem quality as the reference sites, and continued studies are needed
436 to measure the time scale required for the recovery.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Materials

Table S1. Detailed location data and habitat information for the nine study sites within the Anji City Region, PRC; Habitat information include canopy cover, habitat types, substrate composition and substrate Shannon index (H′). F = forest streams; R = restored streams; D = degraded streams.

Site code	River name	Location (Longitude & Latitude)	Canopy cover (%)	Habitat types present			Substrate composition (%)				Substrate Shannon Index(H′)
				Island	Pool	Riffle	Boulders	Cobbles	Pebbles	Granules	
F-1	Longwang Mountain	30°25'3.93"N 119°24'30.52"E	70	✓	✓	✓	20.7	72	7	0.3	0.77
F-2	Yangjiao Mountain	30°26'59.18"N 119°27'55.03"E	90	✓	✓	✓	22.4	68.3	8.1	1.2	0.85
F-3	Zhebei Valley	30°25'24.05"N 119°30'33.60"E	85	✓	✓	✓	13.3	45.3	36.9	4.5	1.13
R-1	Shima Port	30°37'52.98"N 119°41'57.03"E	1	✓	✓	✓	0	13.3	38.7	48	0.99
R-2	Depu Port	30°36'22.34"N 119°41'39.80"E	2	✓	✓	✓	0	14.9	59.5	25.6	0.94
R-3	Wuxiangba	30°38'43.04"N 119°36'32.29"E	10	✓	✓	✓	0	68.5	29.7	1.8	0.69
D-1	Tongxin	30°38'13.96"N 119°41'28.86"E	20	-	✓	-	0	0	0	100	0
D-2	Wuzhuang	30°38'7.99"N 119°39'2.36"E	0.2	✓	✓	-	0	0	0	100	0
D-3	Chiyi	30°38'28.69"N 119°36'12.85"E	60	-	✓	-	0	0	0	100	0

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