*Accepted version of the manuscript: Yuan et al. Current Microbiology (2020): DOI: 10.1007/s00284-020-02138-5*

**Impact of temperature, nutrients and heavy metals on bacterial diversity and ecosystem functioning studied by freshwater microcosms and** **high throughput DNA sequencing**

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Running title: Impact of environmental factors on bacterial diversity and ecosystem functioning

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**Abstract**

Microbial communities are fundamental components in freshwater, and community shifts in ecosystem structure are indicative of changing environmental conditions. This study aimed at investigating the influence of key environmental parameters on bacterial diversity and ecosystem functioning (i.e. organic matter breakdown) in laboratory freshwater microcosms. The effects of varying temperatures (5, 20 and 35°C), nutrients (representing low, medium and high urbanization) and heavy metals Cuprum (Cu) and Zinc (Zn) on bacterial diversity and organic matter (OM) breakdown were studied by using leaf bags and capsules filled with polycaprolactonediol-2000 (PCP-2000), respectively. The leaf-associated bacterial diversity was determined by next-generation sequencing of SSU rRNA gene amplicons. The results showed that bacterial diversity increased at high temperature (35°C) with more operational taxonomic units (OTUs) as compared to medium (20°C) or low (5°C) temperatures, whereas nutrient variation had fewer effects on the bacterial community structure. In contrast, the presence of heavy metals, especially high concentrations (100μM) of Cu, reduced the number of OTUs in the leaf-associated bacterial community. The higher temperatures and nutrient levels accelerated PCP-2000 breakdown rate but this was impeded by a high concentration (100μM) of Cu in the short-term, though no effect of Zn on breakdown rate was observed. The overall results indicate that temperature and variated heavy metal effects are amongst the key factors that affect bacterial diversity and ecosystem functioning in freshwater systems.

**Keywords:** bacterial diversity, organic matter breakdown, laboratory microcosms, environmental factors, next-generation sequencing.

**Introduction**

Rapid economic development and high urbanization in China in the recent decades has positively impacted on various aspects of human society. However, urbanization has also been reported to have negative impacts, including issues with surface water quality [1]. Many pollutants associated with urbanization affect freshwater biodiversity, manifested as shifts in the diversity and activity of microbes [2]. Microbial communities can act as indicators of ecosystem structure as they play major roles in primary production, nutrient cycling, decomposition and degradation of pollutants [3, 4]. Advances in molecular techniques have enabled researchers to study microbial diversity at high resolution and to link diversity to metabolic activity or specific functions/processes [5, 6].

Combining eco-physiological studies with bio-molecular techniques in biodiversity-ecosystem functioning research reinforces the ability to link microbial diversity to ecosystem processes [7, 8]. One of the most regularly used measures for river ecosystem function is leaf litter (organic matter) breakdown rates - a key process that drives the flow of nutrients and energy in freshwater ecosystems [9, 10], which has been shown to respond to land-use change in varying ways [11]. As an alternative to leaf bag experiments, a new method using synthetic organic polymers has been applied to measure the ecosystem function [12]. Although this new method has not been widely used, a few recent studies indicate the potential of using polymer degradation to assess ecosystem function [13, 14].

Previous studies have shown that urban-influenced eutrophic waterways in an estuary harboured greater bacterial diversity than an oligotrophic lake [15], and due to land-use pressures, metals are major drivers of microbial communities and their functions in waterways as revealed by ecosystem genomics [16]. Identifying the genetic mechanisms that mediate responses of organisms to natural environments by using molecular methods enables fundamental ecological and evolutionary questions to be addressed [17]. However, the detailed effects of specific changes in environmental parameters caused by urbanization and land use shifts on microbial diversity–ecosystem function relationships have not been well studied. Here, the aim was to investigate the influence of temperature, nutrients and heavy metals as key environmental parameters on bacterial diversity and ecosystem functioning (i.e. organic matter breakdown) in the freshwater ecosystem through microcosm studies, designed on the basis of two years prior field experiments in Suzhou canals showing that high urbanization increased the levels of multiple nutrients, fecal markers and bacterial pathogens [18]. Microcosm studies complement fieldwork by enabling the controlled evaluation of multiple environmental factors on microbial diversity and ecosystem function.

**Materials and Methods**

**Microcosm experimental design**

The microcosm experiments were constructed as shown in the schematic diagram (Fig. S1). Four water baths were set up at three different temperatures: one each at 5°C (minimum) and 35°C (maximum), and two at 20°C (average) to study the influence of varying temperature on bacterial diversity and ecosystem function. The temperature set for the experiment represents minimum, average and maximum water temperature observed in the field studies in Suzhou canals [18]. Water baths at 20°C were used for nutrient studies using water samples collected from Suzhou canals representing three different urban gradients (high, medium and low urbanization) based on data from a two year field study [18]. The urban intensity classification of sampling locations was based on population density/km2 for each category: >8000, 1700–2100 and 800–1100 persons/km2 for high, medium and low urbanization respectively. The dominant land use types in the high urbanization locations were high-density residential land and commercial land. In medium urbanized locations, the dominant land use types were research and education institutions as well as associated residential areas and industrial land. In contrast, the land use types in low urbanization locations were rivers and lakes, agricultural land and public green land. Significant variations in nutrients particularly total nitrogen, total phosphorus, phosphate and ammonia were observed between sampling locations in different urbanizations [18]. In the microcosm experiments, KH2PO4 and NH4Cl (1μM and 50μM, respectively) were added, to address the environmental effects of ammonia and phosphate [19].

In a second water bath set at 20 °C, three different concentrations (1μM, 10μM and 100μM) of CuCl2 and ZnCl2 were used to study the effect of heavy metals. Serious heavy metal pollution in watersheds within the Yangtze River Delta (which includes Suzhou) was reported earlier [20] and in our study, Cuprum (Cu) and Zinc (Zn) were selected as examples of heavy metal pollution impact in this microcosm study. The south section of the Grand Canal runs through the Yangtze River Delta and the concentrations of dissolved Cu in surface water in most urban areas within this section were below 9μg/L, except in one small city, in which the concentration of Cu was in the range 13.6 to 71.2μg/L. The concentrations of Zn in three locations in the south section of the Grand Canal in the Yangtze River Delta were over 120μg/L [21]. The heavy metal concentrations selected in the previous microcosm studies which focused on studying the leaf litter decompositions were 0.03mg/L, 0.98mg/L, 9.8mg/L for Zn and 2mg/L, 4mg/L and 8mg/L for Cu [22, 23]. Therefore, based on the water quality of the sampling locations in different urban gradients, local standards for monitoring surface water quality and references from previous microcosm studies three different concentrations (1μM, 10μM and 100μM) of Cu and Zn were selected here. All the microcosms were maintained under a light/dark regime of 12:12 hours, which simulated the urban freshwater environment, and the study was conducted for 42 days. Five hundred mL water samples were used in each microcosm and the water renewed every two weeks for all the microcosms until the end of the experiments. The water samples were also used to characterize various physico-chemical and microbiological parameters as described previously [18]. Three grams of dried Willow (*Salix* sp.) leaves were weighed and placed in ready-made nylon bags (15 x 10 cm dimension with 0.5-1.0 mm mesh) along with number tags. The leaf bags were deployed in each microcosm and the bags and PCP-2000 were retrieved after the same time (14and 42 days) for further analysis. Capsules filled with polycaprolactonediol-2000 (PCP-2000) (Sigma-Aldrich USA) (1g each) were deployed in the microcosms and collected on the 14th and 42nd day to assess the OM decomposition rate [19, 24] to represent short and long term measurements similar to our field leaf bag experiments in Suzhou canals [25].

**DNA extraction and bacterial community analysis**

Water samples (500mL each) were filtered through 0.22μm polycarbonate membrane filters (Millipore, UK) to collect microorganisms for DNA extraction. Leaf samples were stored at -20°C prior to DNA extraction. Genomic DNA was extracted from membrane filters and leaf samples using PowerSoil DNA isolation kit (Mo Bio, USA) according to the manufacturer’s instructions. The leaf samples were ground using liquid nitrogen and the extracted DNA was quantified using a Nanodrop, verified by gel electrophoresis and stored at -20°C until further processing.

The bacterial communities in water and leaf samples were studied by next generation sequencing. Primer sequences that target V3 and V4 regions (CCTACGGRRBGCASCAGKVRVGAAT and GGACTACNVGGGTWTCTAATCC) [26] of the 16S rRNA gene were used to study bacterial diversity by using the MiSeq250 platform.

The PCR reactions were performed in triplicate. The PCR mixture (20µL) contained 2µL of 10× Taq Buffer, 2µL of 2.5mM dNTPs, 0.8µL of each primer (5µM), 0.2µL of Takara rTaq Polymerase, 0.2µL of BSA and 10ng of template DNA. The PCR cycling conditions used were: 95°C for 3min, followed by 27 cycles at 95°C for 30s, 55°C for 30s, and 72°C for 45s and a final extension at 72°C for 10min. The PCR amplified products were extracted and purified using AxyPrep DNA extraction kit and sequenced using Illumina MiSeq platform at GENEWIZ, Inc. Suzhou, China. The sequencing results were analyzed by using VSEARCH (1.9.6) [27] for sequence clustering and operational taxonomic units (OTUs) were clustered with 97% similarity cut-off. Silva 128 [28] was used as the reference database for 16S rRNA analysis and RDP (Ribosomal Database Program) [29] classifier Bayesian algorithm was used for OTU analysis. The sequence data were normalized and sequence reads of 51166 for each sample were used for comparison between samples. The sequences obtained from this study were submitted to the National Center for Biotechnological Information (NCBI) Short Read Archive (SRA) database under the accession numbers SAMN10721271 to SAMN10721312.

**Determination of organic matter breakdown rate**

The organic matter breakdown rate was calculated on the basis of PCP-2000 weight loss over the period when it was immersed in the microcosm. One gram of dry PCP-2000 samples was placed in a capsule (3.3cm height and 3.1cm diameter) along with a number tag. The capsules had 0.2mm-diameter openings on the top to ensure that the water could flow into the capsule smoothly. The PCP-2000 samples were collected after 14 and 42 days and dried to constant weight, and the weight loss calculated.

**Statistical analyses**

The bacterial diversity and richness within each sample (alpha diversity) was carried out by diversity indices (Shannon, Simpson, abundance-based coverage estimator and Chao1 richness estimator) by using QIIME (1.9.1). The variation in the diversity between samples (beta diversity) was studied by non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis distance matrix, and weighted unifrac was applied in heatmaps. OTU analysis was carried out by using QIIME (1.9.1) and VSEARCH (1.9.6), while bacterial relative abundance analysis and all the plotting were carried out using the R statistics software package. As shown in Table 1, the samples were grouped into different categories for further analyses.

All the data obtained for OM breakdown rate were analysed statistically using the IBM SPSS Statistics 20 software. The variation in OM breakdown rate with urban intensifications (high, medium and low), temperature, nutrients and heavy metals were analyzed by ANOVA.

**Results**

**Changes in environmental variables**

The physico-chemical and microbiological characteristics of water samples collected from three urban gradients in Suzhou (designated as H, M and L) for microcosm studies are shown in Table S1. Higher levels of nutrients particularly TN, TP and PO4-P in highly urbanized locations were observed as compared to medium and low urbanization locations. The results reflect the actual water quality gradient in Suzhou canals as observed in our two-year field studies.

**Changes in the alpha diversity**

The rarefaction curves displayed a saturated trend in the observed OTUs (Fig. S2) and high Good’s coverage (0.995-1) (Table 2) were obtained for all the water and leaf samples, which indicated that the sequencing depths for all the samples were sufficient. The number of sequencing reads ranged from 62309 to 102037 in the samples analyzed but the normalized sequences of 51166 were used for further analysis.

The alpha diversity measures revealed that bacterial communities in leaf samples were more diversified with higher indices (Shannon, Simpson, ACE and Chao1), at higher temperature than lower temperature (Table 2). Nutrients had less effects on bacterial diversity in leaf samples and heavy metals decreased the bacterial diversity with lower indices in leaf samples, especially at high concentration of Cu (labelled as 12L14 and 12L42 in the Table 2).

The OTU analysis results for bacterial community data obtained for water and leaf samples from microcosm experiments indicated that there were more unique OTUs in water samples (507) than in leaf samples (0-152) (Fig. 1). For leaf samples, more OTUs were observed on 42nd day (4-152) than on 14th day (0-19) and more OTUs were observed at high (35°C) temperature (19-152) than at medium and low (5 and 20°C) (≤ 20) temperatures. The heavy metals particularly Cu reduced OTU numbers in long term (42 day) but nutrients had fewer effects on OTUs (Fig. 1).

**Effect of various treatments on changes in bacterial community composition**

The relative abundance of the sequences obtained for each group of samples at the phylum level is shown in Fig. 2, and the relative abundance of the sequences in individual samples is shown in Fig. S3. The bacterial community in water samples was obviously different from the leaf samples. The water samples (Group: W) were dominated by Proteobacteria (37%) and Actinobacteria (35%) followed by Bacteroidetes (10%) and Cyanobacteria (9%) at the phylum level. At the order level, Burkholderiales (20%) and Frankiales (30%) were dominant in water samples (Fig. 3A and S4; Group: W).

The leaf samples were dominated by Proteobacteria (20-85%) followed by Bacteroidetes (10-70%) and Firmicutes (5-20%) at the phylum level. Proteobacteriawere represented at extremely high level (70-80%) in leaf samples with low temperature (5°C) (Group: A1 and A2), and Bacteroidetes were represented at high level (30-65%) in leaf samples with medium temperature (20°C) (Group: B1, D1, E1, F1 and B2, D2, E2, F2) as compared to other temperatures, which indicated the effect of temperature on shifts in bacterial community. In short-term (14 day), Proteobacteria wererepresented at a higher level (60%) in leaf samples treated with Cu (Group E1) as compared to untreated samples (20%; Group B1), whereas Bacteroidetes were represented at a lower level (30%) in leaf samples treated with Cu than in untreated samples (70%; Group B1), which indicated obvious effect of Cu on changing the composition of bacterial community in short-term. At the order level, Enterobacterialeswere represented at extremely high level (50%) in leaf samples with low temperature (5°C; Group: A1 and A2), and Bacteroidales were represented at high level (20-65%) in leaf samples with medium temperature (20°C; Group: B1, D1, E1, F1 and B2, D2, E2, F2), which was similar to the results at the phylum level and the results indicated the effect of temperature on changing the bacterial composition. In short-term (14 day), Enterobacteriales wererepresented at higher level (30%) in leaf samples treated with Cu (Group E1) as compared to untreated samples (15%; Group B1) at 20°C, whereas Bacteroidales were represented at lower level (20%) in leaf samples treated with Cu (Group E1) than in untreated samples (67%; Group B1), which was also indicated the obvious effect of Cu on changing composition of bacterial community in short-term. At the genus level, *Pseudomonas* represented at high level (15-25%) in leaf samples with low temperature (5°C) (Fig. 3B and S5; Group: A1 and A2); *Macellibacteroides* were represented at high level (10-25%) in leaf samples with medium temperature (20°C) (Group: B1, D1, E1, F1 and B2, D2, E2, F2) and *Prevotella\_9* were represented at extremely high level (45-50%) in leaf samples with medium temperature (20°C) on 14th day in control (B1) and nutrient (D1) groups.

In summary, bacterial community composition results indicated that i) the bacterial community varied between water and leaf samples (W vs. A-F); ii) the temperature affected the bacterial community in both short (A1 vs. B1 vs. C1) and long-term (A2 vs. B2 vs. C2) and iii) the Cu affected the bacterial community in short-term (B1 vs. E1). No obvious effect of nutrients on shifts in bacterial community was observed in leaf samples at both short- (B1 vs. D1) and long-term (B2 vs. D2), which is consistent with the results observed in our field studies [25].

Heatmaps of clustered bacterial community at phylum, order and genus levels are shown in Fig. S6. At phylum level, water samples (Group W) clustered individually, which indicated distinct bacterial community between water and leaf samples. Among leaf samples, Group A1 and A2 (5°C) clustered together, which indicated the effect of temperature on bacterial community in leaf samples. Group B1 and D1 (20°C, 14th day: control and nutrients group) were clustered together, Group B2 and D2 (20°C, 42nd day: control and nutrients group) were also clustered together, which indicted that nutrients did not have significant effect on bacterial community both at short and long-term. Group E2 and F2 (20°C, 42nd day: Cu and Zn) were clustered together, but they were not clustered together with Group B2 (control), which indicated that the effect of heavy metals on bacterial community in leaf samples. The clustering observed at order level were the same as at phylum level. However, at genus level some changes were observed: Group E1 and E2 (20°C, Cu: 14 and 42nd day) were clustered together and similarly, Group F1 and F2 (20°C, Zn: 14 and 42 day) were also clustered together. Another observation made was that column clustering usually grouped samples into 2 large groups: leaf samples deployed at 20°C on 14th day and 42nd day. At the phylum level, only a few bacterial communities with high dominance were observed in leaf samples on 14th day while on 42nd day, bacterial communities became more diversified in leaf samples, with low dominance in leaf samples as compared to the samples collected on 14th day. This finding indicated that in the short-term, only a few bacterial species play roles in leaf litter breakdown, while in the long-term, more bacterial species contribute to leaf litter breakdown by secondary colonization on the leaf.

**Variations in bacterial community observed through beta diversity analysis**

NMDS (non-metric multidimensional scaling) was used to show the variation in bacterial communities between different samples on a two-axis matrix. The NMDS diagram (Fig. 4) in this study indicates high differences in bacterial communities between water (W) and leaf samples (A-F). The NMDS1 value indicated obvious variation between water and leaf samples. In leaf samples, they formed four large groups: Group 1 (A1 and A2), Group 2 (B1, D1, E1 and F1), Group 3 (C1, B2, D2, E2 and F2) and Group 4 (C2). The NMDS2 values for these four groups ranged from small to large with higher temperature and time, indicating the effect of temperature and incubation period on bacterial composition in leaf samples. However, the nutrients (B vs. D) and heavy metals (B vs. E and F) did not show any obvious effects on shifts in bacterial community in leaf samples.

**Organic matter (OM) breakdown rate influenced by temperature and heavy metals**

PCP-2000 (OM) breakdown rate was measured for each group of the experiments tested i.e. the influence of temperature, nutrients and heavy metals. The degradation rate varied significantly (P < 0.001) between the temperatures tested; the higher temperature (35ºC) accelerated OM breakdown rate, in both short-term and long-term (Fig. 5A; Table S2A and S2B). The OM breakdown rate in the nutrient group was compared with control group. Higher concentrations of nutrients accelerated OM breakdown rate, particularly in the short-term (14 day) (Fig. 5B). However, no significant variation between control and nutrient group was observed (Table S3).

The influence of heavy metals, Cu and Zn at varying concentrations on the OM breakdown rate was compared with the control group. PCP-2000 breakdown rate indicated that high concentration (100μM) of Cu reduced OM breakdown rate in short-term (14 day) however, no effect of Zn on breakdown rate was observed (Fig. 5C and Table S4A and S4B). In short-term, significant variation in the OM breakdown rate between High vs. Control, High vs. Low and High vs. Medium was observed.

**Discussion**

Microcosms are artificial and simplified ecosystems that are used to mimic and predict the reaction of natural ecosystems under controlled conditions, which provides an experimental approach for ecologists to study natural ecological processes [30]. Thus, in this study, the effects of temperature, nutrients and heavy metals on bacterial diversity and OM breakdown rate were studied by microcosms. A comprehensive bacterial community analysis was carried out in this study. The results showed that besides the observed variation in the bacterial diversity between water and leaf samples, temperature was found to be a key factor that affected the bacterial community composition. Bacterial diversity increased at high temperature with more OTUs as compared to medium or low temperatures. The bacterial community results observed in this study were consistent with recent research at Kalamas River [31], which runs through an agricultural, nature protected and urban sewage polluted areas in Northwest Greece and the results showed that temporal variations in bacterial taxonomic and functional diversity were more pronounced than spatial variations [31]. Another study reported that variation in the bacterial community was related to the temperature and sampling locations in a pond ecosystem [32]. The effect of seasonal variation on specific functional bacterial groups [33, 34] or biodegradation genes [35] in aquatic ecosystems was also confirmed through multiple diversity analysis. All these findings further demonstrate the profound effect of temperature on shifts in composition of specific bacterial communities, which have flow-on effects on ecosystem function. The field and lab microcosm studies by Martinez et al. (2014) confirmed the positive relationship between temperature and leaf breakdown rate [36] and in their study focused on the effect of temperature on headwater stream functioning by using leaf bag experiments and the laboratory microcosm. Earlier studies reported that temperature stimulated the microbial leaf decomposition and also by changing the diversity and activity of aquatic microbes [37, 38]. The results from our studies showed that the PCP-2000 (OM) breakdown rate was highly influenced by temperature, and higher temperature accelerated the breakdown of OM, which is consistent with the results from a leaf litter decomposition study carried out previously [25].

In our study, the nutrients had less effect on the composition of bacterial community than temperature, and this finding is somewhat different to results that have been reported previously. For example, urban-influenced waterways harbored significantly greater bacterial abundance and diversity in Lake Michigan, which is an oligotrophic lake [15]. Urbanization was reported to have severe impacts on bacterial diversity related leaf-litter decomposition in Ampang River, a tropical stream in Malaysia [39]. A few earlier studies also showed that nutrient enrichment contributed to microbial-mediated leaf litter breakdown [11, 40]. Among them, the study by Gardeström et al. (2016) showed that pesticides interacted with nutrients to impact microbial diversity and ecosystem processes [11], and Tant et al. (2015) reported that nutrient enrichment accelerated decomposer (fungi and bacteria) contributions to litter breakdown. In our study, the water samples from low, medium and highly urbanized areas (which varied in nutrient levels) with amendments of phosphate and ammonium (1μM of KH2PO4 and 50μM of NH4Cl, respectively) were used for the microcosms and no significant variation in OM breakdown between control and nutrient groups was observed. Water samples were amended with only one concentration of nutrients without any further nutrient enrichment, which could be one of the reasons for observing a lesser effect of nutrients on the bacterial community composition. The microbial communities in high and medium urbanized locations were already exposed to high concentrations of multiple nutrients therefore showed less effect in the microcosm study, which could also be one of the reasons for not observing high variations in bacterial community with nutrients. Due to nutrient kinetics within natural microbial communities, many microbial taxa with contrasting nutrient affinities could also be functioning under suboptimal conditions [41, 42], which is another possible reason for the lesser effect of nutrients on the bacterial community composition observed in this study.

In our study, a high concentration of Cu shifted the bacterial composition in leaf samples with reduced OTUs, and also reduced the PCP-2000 breakdown rate. These results are supported by the findings from the previous studies. Zn concentration was reported to cause changes in microbial functional genes and abundance [43] and high concentration of Cu was also found to change the composition of bacterial community[44], as Cu is a natural antimicrobial agent [45]. Although Zn also showed an antimicrobial effect, this heavy metal is an essential cofactor to support cellular growth for all living organisms. The levels are tightly regulated in microorganisms and are maintained well below the suitable range, and extremely high concentrations of Zn have been found to be cytotoxic [46]. It is likely that the concentrations of Zn used in this study were not sufficiently high to cause toxicity, and therefore minimal effects on bacterial community and breakdown rate were observed.

Martinez et al. (2016) observed through a microcosm study that Cu pollution could negatively affect OM breakdown in a soil ecosystem [23] and the results were similar to our study in that a high concentration (0.1mM) of Cu reduced PCP-2000 breakdown rate. Many studies have proved that heavy metal contamination significantly inhibits litter decomposition, and the effect was usually stronger for laboratory microcosms than for field studies [47-49]. Comparing these reports to the results obtained in our study, it can be interpreted that low concentrations (≤0.1mM) of Zn have no effect on OM breakdown rate. Although the concentration of heavy metals in Suzhou canals was not measured in this specific study, we refer to the earlier studies that reported the levels of heavy metals in water and sediment samples collected from Grand Canal [21, 50] and the Yangtze River [20]. Cu in water samples collected from most urban sections of the Grand Canal met the Criterion Continuous Concentration (CCC) [51] of 9μg/L, but Zn in water samples collected from three towns and a large size city in the Yangtze River Delta was not within the acceptable limit of 120μg/L [21]. The background concentrations (defined as the concentration before industrialization) of Cu and Zn in sediment of lakes in Jiangsu Province were 22.3mg/kg and 62.6mg/kg respectively. Whereas, the concentrations of Cu in the lakes in and around Suzhou (e.g. Yangcheng Lake, Jinji Lake, Dushu Lake and Taihu Lake) were 38mg/kg, 370mg/kg, 45mg/kg and 26mg/kg respectively, and the concentrations of Zn in these lakes were 138mg/kg, 431mg/kg, 208mg/kg and 85mg/kg, respectively [20]. Pollution through multiple sources could have caused high levels of heavy metals in these lakes [20]. All these reports highlight the issues of heavy metal pollution in the freshwater ecosystem, and the concentration levels used in this microcosm study were representative of typical pollution levels observed in Chinese urban waterways.

Besides nutrients and heavy metals, various chemicals (e.g. endocrine disrupting chemicals), antibiotics, steroid hormones, pharmaceuticals, personal care products and pesticides are also important pollutants as multiple stressors to impact urban freshwater ecosystems [52]. The effects of these pollution factors on microbial diversity and ecosystem functioning are worth determining by microcosm studies. In addition, the leaf associated fungal community, and the functional genomics of microbial communities associated with OM breakdown could also be addressed. All these experiments and follow-up analyses will provide comprehensive information on the effects of multiple pollutants on microbial diversity and ecosystem function, and constructive suggestions for future management of urban freshwater ecosystems.

**Conclusions**

In this study, the influence of key environmental parameters - temperature, nutrients and heavy metals, on the bacterial diversity and ecosystem functioning (OM breakdown) in freshwater were studied in laboratory microcosms. Importantly, these microcosms were set up with water samples representative of an urbanization gradient previously characterized by field studies. The temperature was found to be a key determinant significantly affecting the diversity and composition of the bacterial community and OM breakdown rates. Bacterial diversity increased at high temperature with more OTUs compared to medium or low temperatures, and OM breakdown rate was also accelerated at higher temperature. In contrast, nutrient levels in the water samples had minimal effect on the composition of the bacterial community; the higher nutrient level accelerated the OM breakdown rate slightly in the short term, but the increase was insignificant. The heavy metals tested had potential effects on bacterial community; a reduced number of OTUs was observed in the presence of Cu in high concentration and OM breakdown rate was also reduced, however, the effect of Zn on breakdown rate was not observed. Overall, the temperature and variated effects of heavy metals were found to be the key factors that affect the bacterial diversity and ecosystem functioning in freshwater systems. Future microcosm studies will focus on studying the effect of factors such as pharmaceuticals, personal care products and pesticides on microbial diversity-ecosystem function and the abundance of selected functional genes.

**Acknowledgements**

The authors would like to acknowledge the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (Jiangsu University Natural Science Programme, Grant No. 13KJB180022), the Natural Science Foundation of Jiangsu Province (Grant No. BK20171238), and Key Program Special Fund in Xi’an Jiaotong-Liverpool University (XJTLU; Grant No. KSF-E-20) for funding. Financial support to Tianma Yuan was provided through Postgraduate Research Scholarship (PGRS-13-03-09) awarded by XJTLU. Thanks to Qiaoli Feng for her support with DNA extraction from leaf samples.

**Author Contributions:** Tianma Yuan,Raju Sekar, Alan J. McCarthy and Yixin Zhang conceived and designed the experiments; Tianma Yuan carried out the laboratory microcosm experiments and prepared the paper with the direction of his supervisors Raju Sekar, Alan J. McCarthy and Yixin Zhang; All the authors contributed to the revision of the paper.

**Compliance with Ethical Standards:** No ethical issues with the study reported in this manuscript.

**Conflict of interest:** All authors certify that there is no conflict of interest.

**Supplementary Materials:** Figures S1-6, Tables S1-4 can be found in the supplementary materials.

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