# Microbial diversity-ecosystem function in canals across a gradient of urban intensification in Suzhou, China

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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# Abstract

Urbanization is increasing worldwide and is happening at a rapid rate in China in line with rapid and continuing economic development. Although urbanization accelerates economic and social development, it can also lead to major changes to freshwater environments such as eutrophication, loss of biodiversity, and chemical and microbiological contaminations due to domestic, industrial and agricultural discharges. Suzhou is a good example for urbanization with its development in recent decades therefore, this project aimed at assessing the impact of urbanization on water quality and microbial diversity-ecosystem function using Suzhou canals as a model system. Nine sampling locations covering three urban intensity classes (High, Medium and Low) in Suzhou were selected and field studies were carried out in winter and summer over a two-year period (2015 and 2016). Three sampling locations in natural reserve mountains in Huangshan were added as control locations in summer 2016. Water samples were collected from each location for physico-chemical, microbiological and molecular analyses (microbial abundance and diversity) and leaf bag experiments were carried out to assess the organic matter (OM) breakdown and leaf associated microbial communities. The water quality results showed that pH, electrical conductivity (EC), total nitrogen (TN), total phosphorous (TP), ammonium nitrogen ($NH_4$-N), phosphate ($PO_4$-P), total viable count (TVC), total coliforms (TC) and fecal coliforms (FC) varied with urban intensification, whereas water temperature (WT), EC, TN, $PO_4$-P, nitrate-N ($NO_3$-N), nitrite-N ($NO_2$-N), chlorophyll *a* (Chl *a*) and FC showed seasonal variations. Higher levels of nutrients and microbial load were observed in the high urbanization locations as compared to medium and low urbanization, and the results correlated well with land use classification and anthropogenic activities. The OM breakdown rate was significantly affected by the seasons as compared to urbanization and high temperature was found to accelerate OM breakdown. The results of bacterial and fungal community diversity studied by next-generation sequencing of specific target genes (16S rRNA and ITS1, respectively) revealed that the water associated communities were distinct from leaf associated communities and obvious variations in the composition with seasons were also observed. The bacterial and fungal communities also varied among the sampling locations, but no clear trend was observed. However, the bacterial / fungal communities in water / leaf samples collected from Suzhou were very distinct from the samples collected at control locations in Huangshan. The phylum Proteobacteria was dominant (20-80%) in almost all the water and leaf samples tested and Burkholderiales was dominant (5-50%) in most samples at order level. Some of the bacterial genera (*Arcobacter*, *Massilia* and *Acinetobacter*) which are typically found in wastewater or associated with human / animal microbiomes were represented at high percentages at high and medium urban intensification areas. In the fungal community, the phylum Ascomycota was dominant (2-99%) in most samples and the order Pleosporales was dominant (1-99%) in most leaf samples. Fungal genera like *Trichothecium* associated with pathogens / microbiomes and human health were represented at high percentages in high urban intensification areas, whereas natural fungal flora (e.g. *Alternaria*) associated with decomposition / ecosystem function were represented at high percentages at low urban intensification areas and also at control locations. In microcosm studies, the influence of temperature, nutrients and heavy metals on OM breakdown rate and bacterial diversity were studied. The results revealed that the temperature was a key factor that affects the composition of bacterial community, whereas nutrients had fewer effects on the community but accelerated the OM breakdown rate in the short term. The heavy metals such as Cu had potential effects on the shifts in bacterial community, especially the high concentrations of Cu reduced the diversity and also OM breakdown rate. Selected fecal (total, human and avian-associated) markers and bacterial pathogens were quantified by qPCR by using the DNA extracted from water samples. The effect of urbanization was observed with total and human-associated fecal markers (BacUni and HF183) and these markers were observed in higher levels at high urbanization locations. Among the bacterial pathogens tested, *Enterococcus* spp. were the most frequently detected pathogens in water samples (100%), followed by *Arcobacter butzleri* (74%), Shiga toxin-producing *Escherichia coli* (STEC) (41%), *Shigella* sp. (36%), whereas

*Campylobacter jejuni* and *Salmonella* spp. were least frequently detected (10%). The overall results indicated that urbanization impacts the water quality with high level of nutrients, microbial contaminations including pathogens. The microbial community composition was affected by the season, and high levels of nutrients and temperature were found to be the key factors which affect both the microbial diversity and OM breakdown rate.

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## List of Abbreviations

| | |
|---|---|
| ACE | Abundance based Coverage Estimator |
| APHA | American Public Health Association |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pair |
| CA | Cluster Analysis |
| CFU | Colony Forming Units |
| Chl *a* | Chlorophyll *a* |
| Ct | Threshold Cycle |
| DNA | Deoxyribonucleic acid |
| DNQ | Detected but not quantifiable |
| dNTP | Deoxynucleotide triphosphate |
| EC | Electrical Conductivity |
| FC | Fecal Coliforms |
| FIB | Fecal Indicator Bacteria |
| LOD | Limit of Detection |
| LOQ | Limit of Quantification |
| MEP | Ministry of Environmental Protection |
| MST | Microbial Source Tracking |
| NBSC | National Bureau of Statistics of China |

| | |
|---|---|
| NCBI | National Centre for Biotechnology Information |
| NGS | Next Generation Sequencing |
| $NH_4$-N | Ammonia nitrogen |
| $NO_2$-N | Nitrite-N |
| NPS | Non-point sources |
| OTU | Operational Taxonomic Unit |
| PCR | Polymerase Chain Reaction |
| $PO_4$-P | Phosphate |
| QIIME | Quantitative Insights into Microbial Ecology |
| qPCR | Quantitative PCR |
| TC | Total Coliforms |
| TN | Total Nitrogen |
| TOC | Total Organic Carbon |
| TP | Total Phosphorous |
| TVC | Total Viable Count |
| UN | United Nations |
| USEPA | U.S. Environmental Protection Agency |
| WHO | World Health Organization |
| WT | Water temperature |

## List of Publications and Conference Presentations

### List of Publications in preparation:

**1. Tianma Yuan,** Kiran Kumar Vadde, Jonathan D. Tonkin, Jianjun Wang, Jing Lu, Zimeng Zhang, Yixin Zhang, Alan J. McCarthy and Raju Sekar. (2019) Urbanization impacts the physico-chemical characteristics and abundance of fecal markers and bacterial pathogens in surface water. *International Journal of Environmental Research and Public Health 16(10),1739.*

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**3. Tinama Yuan et al.** Bacterial diversity and ecosystem function in Suzhou canals with varying urban intensification. *Prepared for submission to FEMS Microbiology Ecology.*

**4. Tianma Yuan et al.** Changes in fungal diversity across a gradient of urbanization in Suzhou canals. *Prepared for submission to Fungal Ecology.*

### List of Conference Presentations:

**1.** R. Sekar, J. Tonkin, J. Wang, **T. Yuan**, Z. Zhang, K. K. Vadde, J. Zhang, F. Lim and A. J. McCarthy. Impact of urbanization on physico-chemical and microbiological characteristics of Canals in Suzhou, China. Oral presentation at Water Microbiology Conference, held in Chapel Hill, North Carolina, USA, during May 17-19, 2016.

**2. T. Yuan**, J. Tonkin, J. J. Wang, Y. Zhang, A. J. McCarthy and R. Sekar. Bacterial abundance, diversity and ecosystem function in Suzhou canals with varying urban intensification. Oral presentation at Cold Spring Harbor Asia Conference on 'Microbiology and Environment', held in Suzhou, China, during September 26-30, 2016.

# Chapter 1 - General Introduction

### 1.1. Urbanization Worldwide

Urbanization is happening globally and its rapid development was first observed in Europe and Northern America over the late 19th and 20th centuries with industrial revolution (United Nations, 2015). However, in recent decades, urbanization is increasing at an extremely rapid rate in most developing countries, particularly in Asia and Africa. At present, the most urbanized regions in the world include Northern America, Latin America and the Caribbean, Europe and Oceania. The levels of urbanization in Asia and Africa are approximately 50% and 43%, respectively (United Nations, 2018) (Figure 1.1).

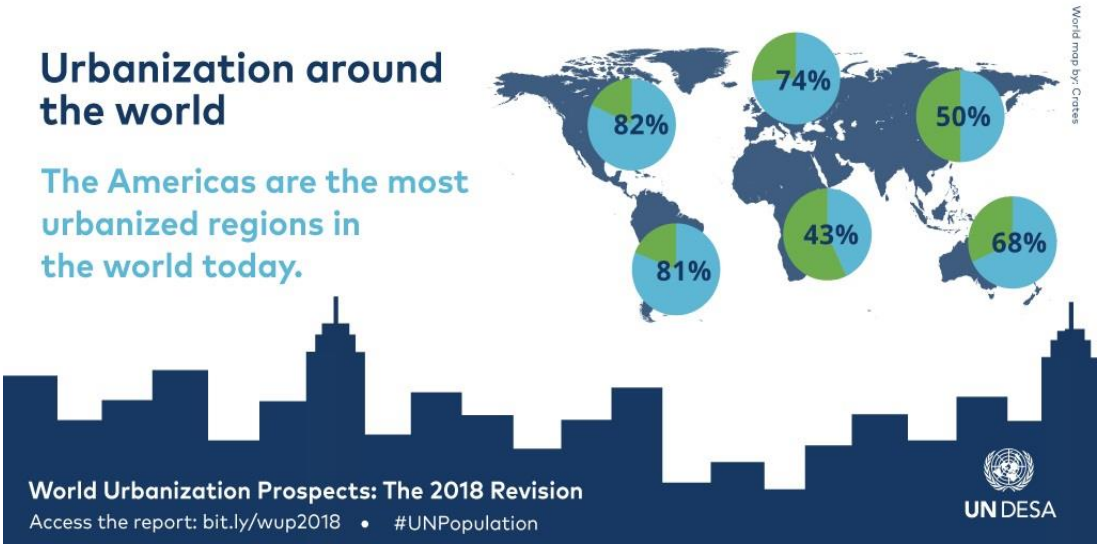



**Figure 1.1.** Urbanization in different regions of the world in 2018 (United Nations, 2018).

Currently, approximately 55% of the world population lives in urban areas, which is projected to increase to 68% by 2050. The Revised *World Urbanization Prospects* (2018) released by the United Nations Division of Economic and Social Affairs (UN DESA) shows that 2.5 billion people will migrate to urban areas by 2050, and nearly 90% of this increase will take place in Asia and Africa (United Nations, 2018). The increase in urbanization is expected to occur more in three countries: India, China and Nigeria, which will account for 35% of the increase in urban population (Figure 1.2) (United Nations, 2015).

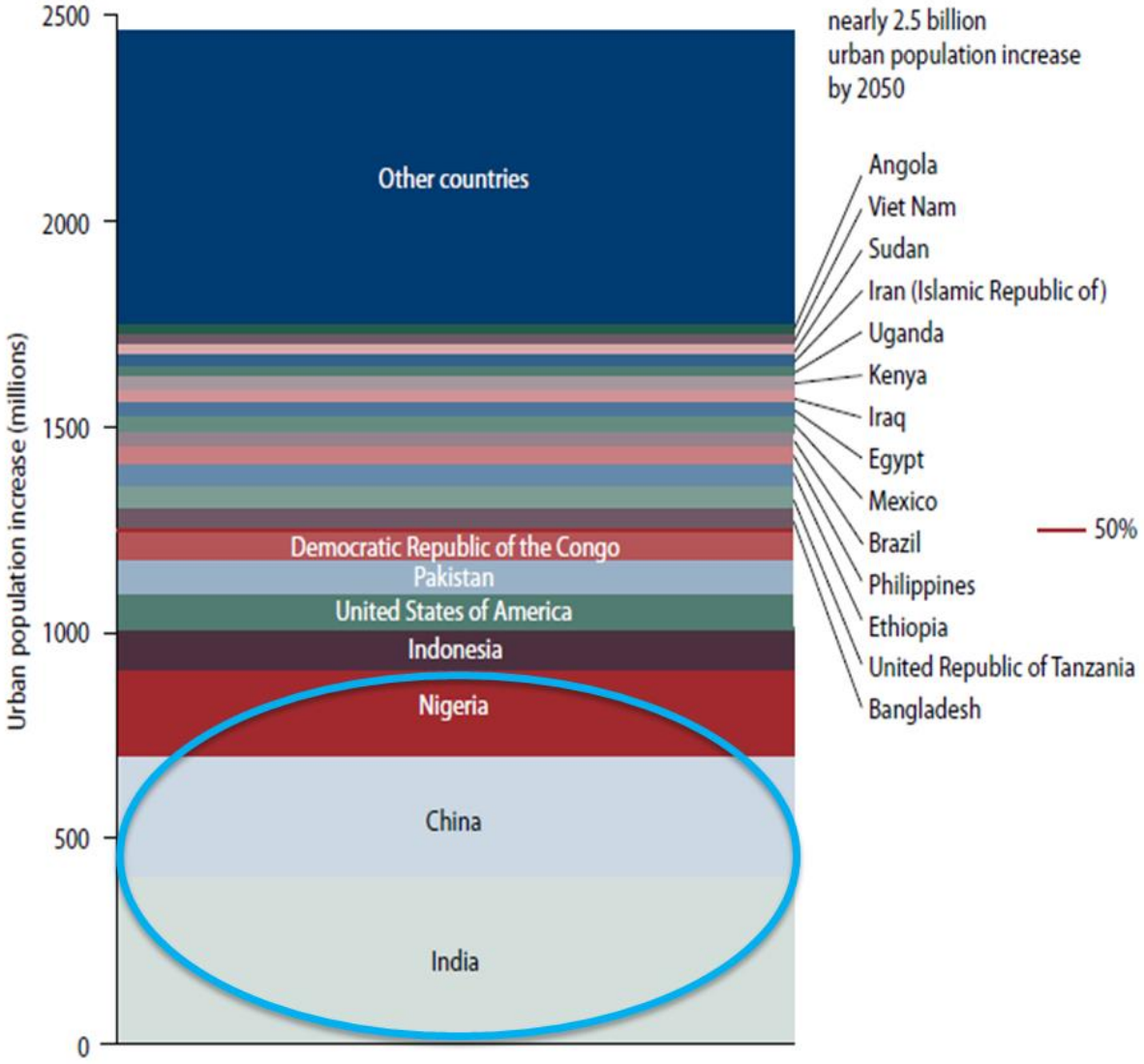



**Figure 1.2.** The contributions of different countries to increase in the global urban population from 2014 to 2050 (United Nations, 2015).

### 1.2. Urbanization in China

The recent data released by the National Bureau of Statistics of China (2018) indicate that at the end of 2017, 58.52% of the population in China lived in urban areas (NBSC, 2018), an increase of about 26% from 1990 (Figure 1.3). It has been estimated that by 2030, the middle class population, which will live in the cities, could reach up to one billion and correspond to 70% of China's projected total population (UN-HABITAT, 2016). This is the biggest driver of urbanization in China in the coming decades. With its continuing rapid

economic development since the late 1970s, China is facing the largest rural-to-urban human migration in its history (NBSC, 2010), driven by economic, social and environmental push or pull factors. Economic factors comprise prospects for high income and remittance, job opportunities, improved living conditions and health care; social factors include improved educational opportunities, marriage and family prospects (Gong et al., 2012).

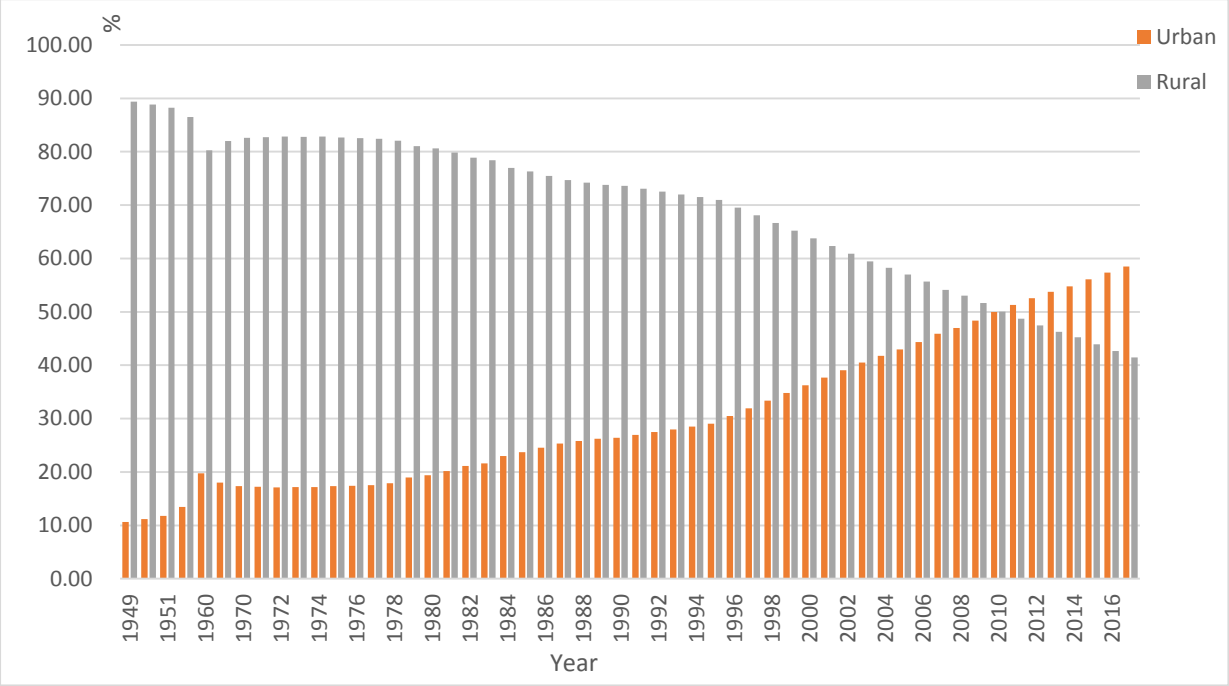



**Figure 1.3.** The urbanization rate (percent of total population) in China from 1949 to 2017. The data were obtained from the National Bureau of Statistics of China (2018).

### 1.3. Urbanization and Economic importance of Suzhou

Suzhou (formerly known as Soochow), is the second largest city in the South-Eastern region of Jiangsu Province of China and it is located 100km North-West of Shanghai (one of the largest cities in China). As a city in the Yangze River Delta Region, Suzhou is a prime example of urbanization with its recent expansion of Suzhou Industrial Park (SIP), Suzhou New District (SND), Wuzhong Taihu New Town and Wujiang Taihu New Town. Wharton Economic Research Institute in Shanghai recently issued 'Ranking list of China's best 100 cities in 2018' based on both economic (61.8%) and non-economic (38.2%) indices, in which

Suzhou was listed in 7th place after Beijing, Shanghai, Guangzhou, Shenzhen, Hangzhou and Chengdu (WERI, 2018). The report also indicated that the top ten cities on this list has not been changed in the last 4 years. Based on degree of business resource aggregation (25%), city pivotability (20%), urban activity (17%), diversity of lifestyle (18%) and future plasticity (20%), China Business Network (CBN) classified cities in China into 6 levels in 2018: first-tier (4), new first-tier (15), second-tier (30), third-tier (70), fourth-tier (90) and fifth-tier (129). In this list, Suzhou was fifth amongst the new first-tier cities in the last three years with huge potential for development (China Business Network, 2018). The Globalization and World Cities (GaWC) Research Network also classified cities in the world into 4 large levels in 2018: Alpha (++, +, none and -), Beta (+, none and -), Gamma (+, none and -) and Sufficiency (high and none). According to this standard, Suzhou belongs to the Beta group as the 11th ranked city in mainland China (GaWCRN, 2018). It should be noted that Suzhou belonged to the Gamma group on the 2016 list, indicating further and very recent rapid development of Suzhou. Established in 514 BC on the network of rivers in the Yangtze floodplain, Suzhou has over 2,500 years of history with its notable canals, and attracts many tourists due to its high cultural and rich historical significance (Mao et al., 2008, Wang et al., 2015). Suzhou is famous as a representative water city in China and is often referred to as "Venice of the East" by visitors from the western world. In 1980, Suzhou and Venice became designated as sister cities.

### 1.4. Positive and Negative impacts of Urbanization

Rapid urbanization has created wealth and improved social factors and human well-being (De Sherbinin et al., 2007). Urbanization is one of the main drivers of global economic growth in this era, and evidence of the positive link between economic development and urban areas is overwhelming both in China and worldwide. On a worldwide basis, in 2014,

with only 54% of the global total population, cities account for more than 80% of total GDP. In China, the top ten cities account for 20% of the national GDP (UN-HABITAT, 2016). However, urbanization has also created many problems, such as pressure on resources caused by industrialization, challenges of providing employment and city services, environmental pollution and public security issues. Environmental pollution has, via contamination of food, water and air, already seriously affected human health (Shao et al., 2006, Zhu et al., 2011). UN-HABITAT pointed out that cities were facing four main types of environmental challenges today: i) effective and equal access to resources and public services ii) managing environmental hazards iii) the effects of urbanization on land conditions/transformation and exchanges for access to water resources, markets and diversified livelihoods and iv) shifts in resource consumption for a low-carbon world and more sustainable societies (UN-HABITAT, 2016). In China, overcoming the negative environmental impacts of rapid urbanization is also becoming one of the major themes of environmental studies (Cui and Shi, 2012, Zhu, 2012), which is associated with many fields, such as climate change (urban heat island effects) (Zhang and Chen, 2014), public health (Zhu et al., 2011, Gong et al., 2012), water resource management (Shen et al., 2005) and pollutant emissions (Pal et al., 2014, Yu and Lu, 2018).

### 1.5. Impact of Urbanization on Freshwater Systems

Urbanization can affect the water quality of freshwater systems in many ways – eutrophication and increasing chemical and microbial contaminations through discharge of excessive nutrients, endocrine disrupting chemicals, heavy metals, antibiotics, steroid hormones, pharmaceuticals, personal care products (PCPs), fecal matter and pathogens (Paul and Meyer, 2001, Shen et al., 2005, Chang et al., 2009, Du et al., 2010, Oster et al., 2014, Prasad et al., 2014, Dai et al., 2016, Santiago-Rodriguez et al., 2016, Sun et al., 2016b, Xu et al., 2018). The major sources of contamination are households, hospitals, animal/dairy farms,

accidental leakage from sewer systems and agriculture run-off through heavy rainfall and flooding (Ongley, 2004, Liu and Raven, 2010, Qu and Fan, 2010). All of these have flow-on effects on the condition of the urban/freshwater systems, and can lead to loss of biodiversity caused by hydrological alterations, habitat degradation and loss, overexploitation, invasive species, toxicity and the multiple impacts of climate change (Worm et al., 2006, Cardinale et al., 2012, Hooper et al., 2012, Reich et al., 2012, Darwall et al., 2018).

In China, water pollution has been a serious environmental issue in the past 40 years from late 1970s with rapid economic development and urbanization, and remains a significant threat although improvements have been made in more recent years (Liu and Raven, 2010, Qu and Fan, 2010, Jiang, 2015, Hao et al., 2018). The quality of groundwater, rivers, lakes and reservoirs has deteriorated across the country, particularly in the developed regions and large cities with high population density and increased human activity (Ongley, 2004, Liu and Raven, 2010, Qu and Fan, 2010). It has been reported that industrial effluent, domestic sewage and agricultural pollution are the main causes for the pollution of various water resources in China (Liu and Raven, 2010, Qu and Fan, 2010, Zhu et al., 2018). Many urban rivers in China are facing issues, such as pollution from heavy metals, multiple nutrients and chemicals, which threatens the safety of drinking water supplies (Qu and Fan, 2010, Zhang et al., 2017). Urbanization has been reported to have significant impact on water quality indicators, and drinking water quality was found to be closely related to housing construction, population urbanization, energy consumption and industrialization during the urban development (Zhang et al., 2017). During the earlier urbanization stages in China, water quality in city rivers deteriorated rapidly, and rapid increases in the volume of domestic discharge was the major cause of water quality deterioration (Qin et al., 2014). As an example, Li reported that water quality deterioration in Shanghai was primarily caused by input of nutrients such as ammonia nitrogen (Li et al., 2018). With continued urbanization,

the wastewater and pollutants (total nitrogen and total phosphorus) from household consumption (including both direct pollution sources caused by economic activity and indirect pollution sources produced through interindustry exchanges from food manufacturing industries, textile and transportation) had a huge influence on emission loads (Liu and Huang, 2014).

Landscape characteristics have been significantly correlated with water quality in watersheds with high urban intensification (Shen et al., 2014). The industrial land use showed negative effect on water quality only at smaller scales, whereas the impact of urbanization turned to be more obvious as spatial scale increased (Zhao et al., 2015). As a result, rapid urbanization with resulting intense land use, land cover change and population growth have a great impact on water quality variables (dissolved oxygen, ammonium-nitrogen, chemical oxygen demand, permanganate index and biochemical oxygen demand) (Chen et al., 2016). Human activities with rapid urbanization and industrialization in developing countries were also the most important factor for increase in the pollution by heavy metals, which is a concern for human health and the environment (Lu and Yu, 2018). An overall increase in both indicator bacteria such as fecal coliform (FC) and nutrients from suburban headwaters to urbanized sections of the river has been detected (Glinska-Lewczuk et al., 2016). It has been reported that fecal indicators (*Enterococcus* spp., *Escherichia coli*, enterophages and coliphages) of microbial water quality positively correlated with urbanization in a tropical watershed in Puerto Rico and such conditions lead to increased human health risk (Santiago-Rodriguez et al., 2016).

### 1.6. Microbial diversity – Ecosystem function

Microorganisms are abundant in freshwater systems and they play vital roles in the biogeochemical processes, nutrient recycling and organic matter decomposition (Finlay and

Esteban, 2001, Hahn, 2006, Zhang et al., 2013). Ecosystem function refers to the biological, geochemical and physical processes that occurs within an ecosystem, and primary production and decomposition by microorganisms are important drivers (Bell et al., 2009). The advance in molecular techniques such as PCR, high throughput sequencing and functional metagenomics, enables researchers to study the structure and function of the microbial communities without cultivation of the indigenous microorganisms themselves (Smith and Osborn, 2009, Steffen et al., 2012, Llorens-Mares et al., 2015, Tang et al., 2015).

Earlier studies have not only addressed physico-chemical contaminants, but also the ecological structure of freshwater systems (Meyer et al., 2005, Young et al., 2008, Huang et al., 2013a). Many pollutants that impact waterways have also had flow-on effects on freshwater biodiversity, shifts in activity and diversity of multiple biological communities, especially the microbial functional groups (Death et al., 2009, Sandin and Solimini, 2009, Zhang et al., 2015). Loss of biodiversity and ecosystem function have been identified (Qu and Fan, 2010, Englert et al., 2013, Ostfeld and Logiudice, 2013) as major issues resulting from declining water quality in China's waterways. Ecosystems have multiple functions, which require biodiversity (Worm et al., 2006). However, different ecosystem functions are influenced by significantly different sets of species (Hector and Bagchi, 2007, Gotelli et al., 2011). To investigate the relationship between microbial biodiversity and ecosystem function, the key point is to understand how differences in the structure and performance of microbial communities translate into differences in function (Naeem et al., 2009). The link between microbial diversity and ecosystem processes is a fundamental research goal for microbial ecology (Bernhard and Kelly, 2016). Microbial communities are the fundamental components of freshwater ecosystems and their composition can indicate shifts in ecosystem structure (Paerl et al., 2003). Ecosystems that are more species-rich are more efficient at removing nutrients than ecosystems with fewer species, so that conservation of biodiversity could be a

useful tool for controlling nutrient levels in watersheds (Cardinale et al., 2012). Therefore, we need to not only consider how urbanization impacts water quality, but also how these changes can in turn affect microbial diversity and thus the function of these ecological communities (Paul and Meyer, 2001).

In recent years, there has been a push to not only assess the structure of freshwater communities but also the function of these communities (Paul et al., 2006, Young et al., 2008, Duarte et al., 2010, Delgado-Baquerizo et al., 2016). Combining eco-physiological studies with contemporary bio-molecular techniques in biodiversity-ecosystem functioning (BEF) research reinforces the ability to link microbial diversity to ecosystem processes (Dudgeon and Gao, 2010, Krause et al., 2014). One of the more regularly used measures of river ecosystem function is leaf breakdown rates (Gessner and Chauvet, 2002, Clapcott et al., 2012, Collier et al., 2013, Thompson et al., 2016), which can be indicative of a number of important biological and ecosystem features. As functional and structural indicators of river ecosystem health, leaf breakdown rates have been shown to respond to land-use change in varying ways (Young and Collier, 2009, Gardeström et al., 2016). Leaf-litter breakdown rate was positively related to macroinvertebrate abundance, but was unrelated to nutrient concentrations in the urbanized North Branch of the Chicago River watershed (Cook and Hoellein, 2016). Since leaf breakdown rate is a key indicator for ecosystem function, leaf litter placed in bags were deployed within the water systems and collected following the retrieval schedule to measure the decay rate of leaf litter (Benfield, 2007). This is considered as leaf bag experiment, the traditional method to assess water ecosystem function (Young et al., 2008). In addition to leaf bag experiments, a new method was extended by using synthetic organic material – polymers filled in capsules instead of leaf bags, and polycaprolactonediol (PCP) was proved as a convenient polymer for river ecosystem assessment. Data on breakdown rates of PCP

complemented traditional leaf bag experiments in assessing water ecosystem function (Rivas et al., 2016).

The potential cosmopolitan nature of microbes in freshwater ecosystems has led questions about the relationship between species-richness and specific ecosystem functions (Finlay et al., 1997, Gessner and Chauvet, 2002), which is especially likely for canals of the Yangtze floodplain where there is an high level of interconnectivity across the landscape. Recent research showed that urban-influenced waterways in an urbanized estuary harbored significantly greater bacterial richness and diversity than Lake Michigan, an oligotrophic lake (Newton and McLellan, 2015), and land-use pressures and eco-genomics revealed that the presence of polluting metals such as copper and aluminum are major drivers of microbial communities compositions and their functions in the waterways of a megacity (Saxena et al., 2015). Microbial diversity increased with urbanization in vernal pools of the Cuyahoga River watershed (USA) (Carrino-Kyker et al., 2011). Watershed urbanization altered the composition and function of stream bacterial communities, receiving a high level of scouring flows containing nutrient, contaminant and thermal pollution (Wang et al., 2011b). Freshwater fungi are a diverse group of organisms and fulfil important functions in the food web dynamics of surface water ecosystems, and they play a key role in the breakdown of organic matter such as leaves, which accounts to 99% of the total input energy of surface water. Therefore, the colonization of organic matter by aquatic fungi and other microorganisms represents an essential component in the food web of running water (Ittner et al., 2018). The dynamics of aquatic fungal communities in a heavily-contaminated tropical river were found to be mainly driven by seasonality, geographical distance and physico-chemical parameters (e.g. pH, nitrate, total organic carbon) (Ortiz-Vera et al., 2018). As riparian land use changed from natural forest sites to urban sites with anthropogenically disturbed conditions, fungal richness and biomass were lower and leaf litter breakdown rates

became slower (Iniguez-Armijos et al., 2016). Following the initial leaching period of the leaf decomposition process, fungi and bacteria colonized the leaves for microbial conditioning. During this process, the leaves were broken down because the fungal community released enzymes capable of digesting structural materials (cellulose and lignin) of leaves (Suberkropp and Klug, 1976). Therefore, fungi play a key role for leaf degradation during microbial conditioning. In streams, urbanized watersheds were more contaminated with nutrients and xenobiotics than forested watersheds. Fungal biomass in decomposed *Alnus* leaves was lower in urbanized watersheds than in forested watersheds because of pesticide toxicity. However, leaf microbial decomposition rates were higher in urbanized watersheds, and compensatory effects of nutrients over xenobiotics were confirmed (Rossi et al., 2019). Urbanization also alters other watershed ecosystem functions, such as nutrient budgets and retention processes (Hale et al., 2014). Nutrient budgets and retention processes are indices for evaluation of whole-ecosystem nutrient-cycling function through quantification of inputs and outputs. Groffman et al. found that urban and suburban watersheds had much higher nitrogen (N) losses than the forested watershed, and retention of N in the suburban watershed was extremely high, 75% of inputs were dominated by home lawn fertilizer and atmospheric deposition (Groffman et al., 2004). Urbanization and precipitation have interacted in shaping aquatic ecosystem responses to environmental forcing mechanisms (stressors), which included physical variables (i.e. water temperature, flow rate) and variables associated with fluxes of dissolved solutes (i.e. conductivity, salinity, dissolved nutrients) (Vogt et al., 2016). It has been reported that urbanization had severe impacts on ecosystem function in Ampang River (Yule et al., 2015). In Taihu Basin, urban development caused net primary production (NPP, an indicator of vegetation functions) loss during 2000 to 2010, which had negative effects on the ecosystem: Low levels of urban development and land utilization could affect a broader area than a highly developed urban area, while the latter showed greater effects (Li et

al., 2016). However, the impact of urbanization on the relationship of microbial diversity-ecosystem function in canals in the Yangtze floodplain has not been studied.

### 1.7. Canals as a model system to study the impact of Urbanization

In China, canals dominate the landscape of the Yangtze River Delta (YRD), and major cities such as Hangzhou and Suzhou are made famous for their widespread occurrence. The Grand Canal of China is considered as the oldest and largest artificial river in the world, and it connects Hangzhou and Beijing through 1776 km of its length (Wang et al., 2010). Canals connect all the water systems in Suzhou, including Taihu Lake and the Grand Canal. With hundreds and thousands of year's developments of urban water systems in Suzhou, there are totally 21084 canals excavated until now (Urban Commerce, 2016). Canals play important roles on supplying water for irrigation of crops, urban plantations, transportation, recreational activities, flood control during rainy seasons, and receipt of municipal and industrial discharges and agricultural run-off (Wang et al., 2010) (Figure 1.4). Canals are subject to increasing stress from urbanization, industry, agriculture and aquaculture because of rapid economic development in recent years (Chen et al., 2004, Wang et al., 2010, Yu et al., 2012). Thus, alteration of hydrological regimes and deterioration of water quality in canals due to contaminants, such as heavy metals, excess organic matter and multiple nutrients, have been reported (Chen et al., 2004, Wang et al., 2010, Yu et al., 2012). These pollutants may further lead to contamination of the groundwater, which can have flow-on effects on the local human population by entering the food web through water and soil contamination (Weng and Chen, 2000, Chen et al., 2004, Wang et al., 2010, Yu et al., 2012). In past decades, significant deterioration of water quality in canals has caused serious ecological and sanitary problems (Wang et al., 2010, Zhuang et al., 2016a, Zhuang et al., 2016b, Cao et al., 2018). The level of urbanization has been significantly related to water quality parameters in the Grand Canal,

with a descending order of electrical conductivity, nutrients and metals (Yu et al., 2012, Zhuang et al., 2016a, Zhuang et al., 2016b, Cao et al., 2018). Canals are generally regarded as a resource for exploitation rather than an ecological resource that needs to be protected. Since the canal water is used for irrigation of crops and urban plantations, it is important that these canals do not harbour high concentrations of pollutants such as heavy metals and pesticides, which can freely enter in to the food chain. In recent years, a number of studies have focused on the environmental effects of urbanization on aquatic systems located in large cities in China, such as Beijing and Shanghai (Liu and Huang, 2014, Zhao et al., 2015, Chen et al., 2016, Dai et al., 2016, Li et al., 2018) or large lakes (e.g. Taihu Lake) or canals (e.g. Grand Canal) (Chen et al., 2004, Qin et al., 2007, Wang et al., 2010, Wilhelm et al., 2011, Huang et al., 2013b, Sun et al., 2016b, Zhuang et al., 2016a, Zhuang et al., 2016b, Cao et al., 2018, Xu et al., 2018). Much less focus has been given to ecological studies in canals located in Suzhou. The water quality of the Suzhou canals has been reported in recent years, mainly by the local Water Conservancy Bureau (Suzhou Water Conservancy Bureau, 2018), but such reports mainly focus on the concentration of nutrients such as total nitrogen and phosphorus. No detailed studies on the impact of urbanization on water quality or microbial diversity and ecosystem function with reference to Suzhou canals appear to have been performed. Therefore, this study aims to fill this knowledge gap by investigating the influence of urbanization on water quality and microbial diversity-ecosystem function relationships by sampling across a gradient of urban intensification (high, medium and low urbanization) in the canals of Suzhou. The study was carried out in nine sampling locations covering three urbanization gradients (High, Medium and Low urban intensity) in Suzhou and environs, with sampling locations in Huangshan as selected as controls as they are located in natural reserve forests. The urban intensity classification for locations in Suzhou was based on population density/km$^2$. Both Huangshan and Suzhou have a subtropical monsoon climate

and similar weather conditions were observed in summer 2016 (Huangshan Statistics Bureau, 2017, Suzhou Statistics Bureau, 2017), so that the control sites are meaningful. The details of the sampling locations including the geographic coordinates and land use patterns are shown in Chapter 2.

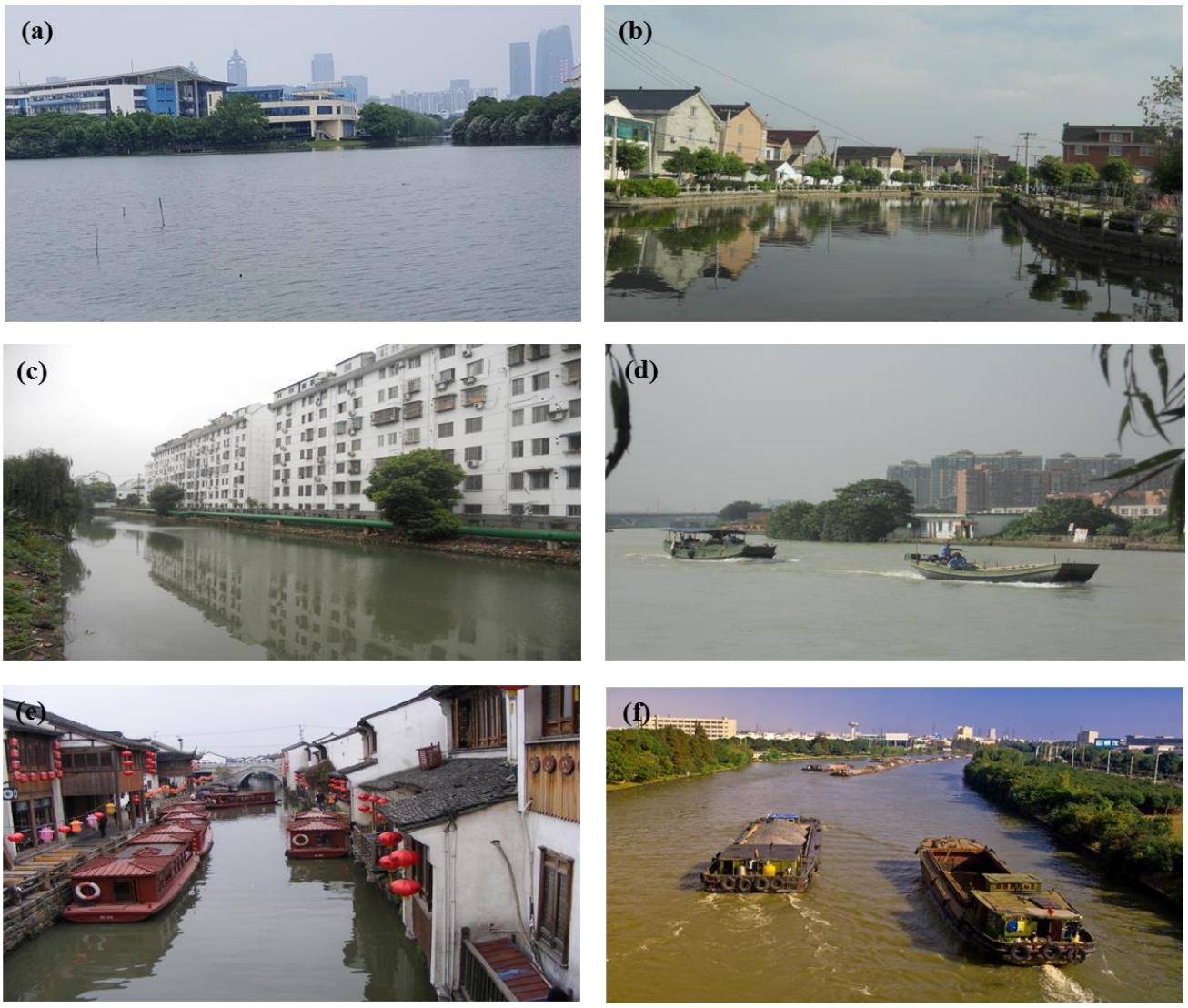



**Figure 1.4.** Canals in Suzhou serving multiple purposes. Pictures a to d – canals in urban and semi-urban areas where samples were collected for the present study. e and f – tourism and transportation activities in canals–pictures reproduced from wikimedia.org.

## 1.8. Aims of the Project and Research objectives

The main scope of this research is to investigate the influence of urbanization on water quality, organic matter breakdown rate, changes in microbial abundance and microbial diversity-ecosystem function relationships in Suzhou canals. We hypothesized that urbanization impacts the water quality with high level of nutrients and microbial contaminations (including fecal markers and pathogens) in Suzhou canals and urbanization also reduces microbial diversity and ecosystem function (organic matter breakdown rate).

The specific objectives are as follows:

- To study the water quality (nutrients and microbial contaminations) in Suzhou canals across a gradient of urban intensification (High, Medium and Low);
- To assess the pattern of microbial (bacterial and fungal) diversity in water and leaf samples and organic matter (leaf-litter) breakdown rate in Suzhou canals across a gradient of urban intensification;
- To evaluate the influence of key environmental parameters (temperature, nutrients and heavy metals) on the microbial diversity and ecosystem function by using laboratory microcosms;
- To quantify the abundance of selected (general and human host-associated) fecal markers and pathogens in Suzhou canals.

# Chapter 2 - Impact of Urbanization on Physico-chemical and Microbiological Characteristics of Water in Suzhou Canals

***Abstract***

The study reported in this chapter was aimed at assessing the impact of urbanization on physico-chemical and microbiological characteristics of water across a gradient of urban intensification in Suzhou, using canals as a model system. Nine locations covering three urban intensities (High, Medium and Low) were sampled at four time points (winter and summer 2015 and 2016) over a two-year period. In summer 2016, three sampling locations in Huangshan were included in the study as control sites as they are located in natural reserve mountains. Water samples were collected from each location and physico-chemical (e.g. multiple nutrients) and microbiological parameters (e.g. total and fecal coliforms) were analysed. The parameters such as pH, electrical conductivity (EC), total nitrogen (TN), total phosphorus (TP), phosphate ($PO_4$-P), ammonium nitrogen ($NH_4$-N), total viable count (TVC), total coliform (TC) and fecal coliform (FC) counts were significantly varied with respect to urbanization. The differences were obvious between high vs. medium and high vs. low urban intensity. However, the differences were less apparent between medium vs. low urban intensity. Significant seasonal variations were observed for several parameters, particularly nutrients, chlorophyll *a* (Chl *a*) and total coliforms (TC). High levels of pollution with nutrients and high microbial load were mainly observed in locations with high urban intensification, which correlated with land use types of these locations and anthropogenic activities. The levels of nutrients and microorganisms observed in water samples from Huangshan were extremely low compared to Suzhou canals, indicative of good water quality in the absence of any influence from urbanization and human activities. There was a positive correlation between the nutrients and total / fecal coliform levels. The overall results indicate that urbanization had an impact on water quality, particularly nutrients and microbiological parameters. Further studies on the impact of urbanization on organic matter breakdown and microbial diversity patterns are discussed in the following chapter.

## 2.1. Introduction

Freshwater ecosystems such as lakes, rivers and canals are affected by varying levels of pollution from multiple sources. Decline in water quality has been a major problem in China in the past few decades although significant progress has been made to protect the surface water quality (Liu and Raven, 2010, Qu and Fan, 2010, Jiang, 2015, Hao et al., 2018). The freshwater systems in China are affected by both non-point and point source pollution, which leads to eutrophication, excessive algal growth and chemical and microbial contamination (Hu et al., 2011, Wilhelm et al., 2011, Huang et al., 2013b, Li et al., 2019, Zhang et al., 2019). In addition, factors such as urbanization and land use can increase the pollution of water bodies. Although urbanization has accelerated economic and social development (De Sherbinin et al., 2007), it has created many environmental problems such as water pollution (Chen et al., 2011, Zhu et al., 2011, Cui and Shi, 2012). Urbanization has been reported to affect the water quality of city rivers with excess nutrients, endocrine disrupting chemicals, heavy metals, antibiotics and steroid hormones (Paul and Meyer, 2001, Shen et al., 2005, Chang et al., 2009, Du et al., 2010, Prasad et al., 2014) particularly in the developed regions and large cities with increased human activity (Ongley, 2004, Liu and Raven, 2010, Qu and Fan, 2010, Goel et al., 2018) and this further threatens the safety of drinking water supplies and sustainability in the region (Wu et al., 2008, Zhang et al., 2017). Industrial effluent, domestic sewage and agricultural pollution were reported to be the main causes of water pollution (Liu and Raven, 2010, Qu and Fan, 2010, Pires et al., 2015, Zhu et al., 2018). Urbanization density has potential for predicting water quality (Yu et al., 2013). Urbanization was positively related to multiple nutrients (TN and TP) and indicator bacteria (fecal coliforms) in the watershed, therefore development density of the city correlated with decreased water quality (Carle et al., 2005). Previous studies showed that bottomland and forestland had a negative correlation to nutrient parameters and non-point source (NPS)

pollutants, whereas residential and industrial land had a positive correlation (Yang et al., 2007, Yang et al., 2008) with those parameters. The landscape characteristics were significantly correlated with water quality in a watershed with high urban intensification (Shen et al., 2014). Industrial land use showed a negative effect on water quality only at smaller scales, whereas the impact of urbanization tended to be more obvious as spatial scale increased (Zhao et al., 2015). The impact of shifts in land use on water quality was more substantial in less-urbanized suburban areas as compared to highly-urbanized central cities (Tu, 2011). As a result, rapid urbanization with intense land use, land cover change and population growth have a great impact on physico-chemical variables (Chen et al., 2016). Qin et al. reported that at the early stage of urbanization, water quality in rivers of Shenzhen deteriorated rapidly, and increase in domestic discharge was reported to be the major cause for deterioration of water quality (Qin et al., 2014). Li et al. reported that water quality deterioration in Shanghai (one of the largest cities in China), was primarily caused by input of ammonia nitrogen (Li et al., 2018) and with continued urbanization, the wastewater and pollutants (total nitrogen and total phosphorus) from household consumption had a huge influence on emission loads (Liu and Huang, 2014).

Climate change is also a key factor that affects water quality parameters, such as water temperature, total suspended solids (TSS) and dissolved oxygen (DO) (Putro et al., 2016, Sun et al., 2016a). Combining climate change with urbanisation led to deterioration in water quality in rivers (Astaraie-Imani et al., 2012). Besides nutrient parameters, indicator bacteria (heterotrophic plate count and fecal coliform) were also reported to increase from suburban headwaters to urbanized sections of the river in Poland (Glinska-Lewczuk et al., 2016) and fecal indicators (*Enterococcus* spp., *Escherichia coli*, enterophages and coliphages), as microbiological parameters of water quality, were positively correlated with urbanization in a

tropical watershed in Puerto Rico, which led to increased human health risks (Santiago-Rodriguez et al., 2016).

Suzhou is a water city with many canals, and these canals connect the water systems of the city, including Taihu Lake and the Grand Canal, which serves for multiple purposes including transportation and supply water for irrigation. However, urbanization has been reported to cause contamination of hydrological regimes in these canals with heavy metals, excess organic matter and nutrients (nitrates and phosphates) (Chen et al., 2004, Wang et al., 2010, Yu et al., 2012). These pollutants may further lead to contamination of the groundwater, which can have flow-on effects on the local human population by entering the food web through both water and soil contamination (Weng and Chen, 2000, Chen et al., 2004, Wang et al., 2010, Yu et al., 2012). Canals are subject to increasing stress from urbanization, industry, agriculture and aquaculture with rapid economic development. In past decades, significant deterioration of water quality in canals has induced serious ecological and sanitary problems (Wang et al., 2010, Zhuang et al., 2016a, Zhuang et al., 2016b, Cao et al., 2018).

The aim of this study was to assess the water quality characteristics (physico-chemical and microbiological parameters) in Suzhou canals across a gradient of urban intensifications. Land use pattern analysis and multiple data analysis were conducted to research the relationships between water quality parameters, land use pattern and urbanization. As far as we are aware, this is the first detailed study to assess the impact of urbanization in Suzhou and Suzhou Industrial Park (one of the special economic zones in China) using canals as a model system. Thus far, only a few reports are available on water quality measurements of Suzhou canals in which only a limited number of parameters were analysed. For example, an earlier study reported a high concentration of phosphorus (P) in suspended sediments from a heavy eutrophic canal system in Suzhou (Li and Huang, 2013). The water quality of the Suzhou canals has been reported in recent years mainly by the local Water Conservancy

Bureau (Suzhou Water Conservancy Bureau, 2018), but these reports only address the overall hydrology in Suzhou, such as precipitation, water-carrying capacity and a few water quality parameters such as total nitrogen and total phosphorus. In the present study, comprehensive physico-chemical and microbiological analyses were carried out to assess the impact of urbanization on the water quality of Suzhou canals.

## 2.2. Materials and Methods

### 2.2.1. Study sites

All sampling was conducted in or around the outskirts of Suzhou; the control locations were located in the Huangshan area. Nine sampling locations in Suzhou were selected representing three urbanization gradients (High, Medium and Low urban intensity) (Figure 2.1). The urban intensity classification was based on population density/$km^2$. The population densities in the urban classifications High, Medium and Low were >8000, 1700-2100 and 800-1100 persons/$km^2$, respectively. The sampling locations were identified as 1-1, 1-2, 1-3 for High, 2-1, 2-2, 2-3 for Medium and 3-1, 3-2 and 3-3 for Low urban intensifications. In summer 2016, three sampling locations in Huangshan area were selected as control locations for this project and identified as H-1, H-2 and H-3. Huangshan is located at the natural reserve in mountain areas (approximately 500 km away from Suzhou), where population density, urban intensification and human activity are low as compared to Suzhou, and the waterways in Huangshan area are protected by the local government. Both Huangshan and Suzhou have subtropical monsoon climate and similar weather conditions were observed in summer 2016 (Huangshan Statistics Bureau, 2017, Suzhou Statistics Bureau, 2017), which maintained the credibility of the control sites. Climatic parameters, including temperature and precipitation in Suzhou for 2015 and 2016 and in Huangshan for 2016 (Supplementary Figures S2.1, S2.2 and S2.3), were gathered from published reports by the local governments

(Suzhou Statistics Bureau, 2016, Suzhou Statistics Bureau, 2017, Huangshan Statistics Bureau, 2017) to explore the relationship with other parameters observed in this study. The details of the sampling locations including the geographic coordinates and land use patterns are included in Table 2.1 and Supplementary Figures S2.4-2.12. The sampling locations are shown in Figure 2.2.

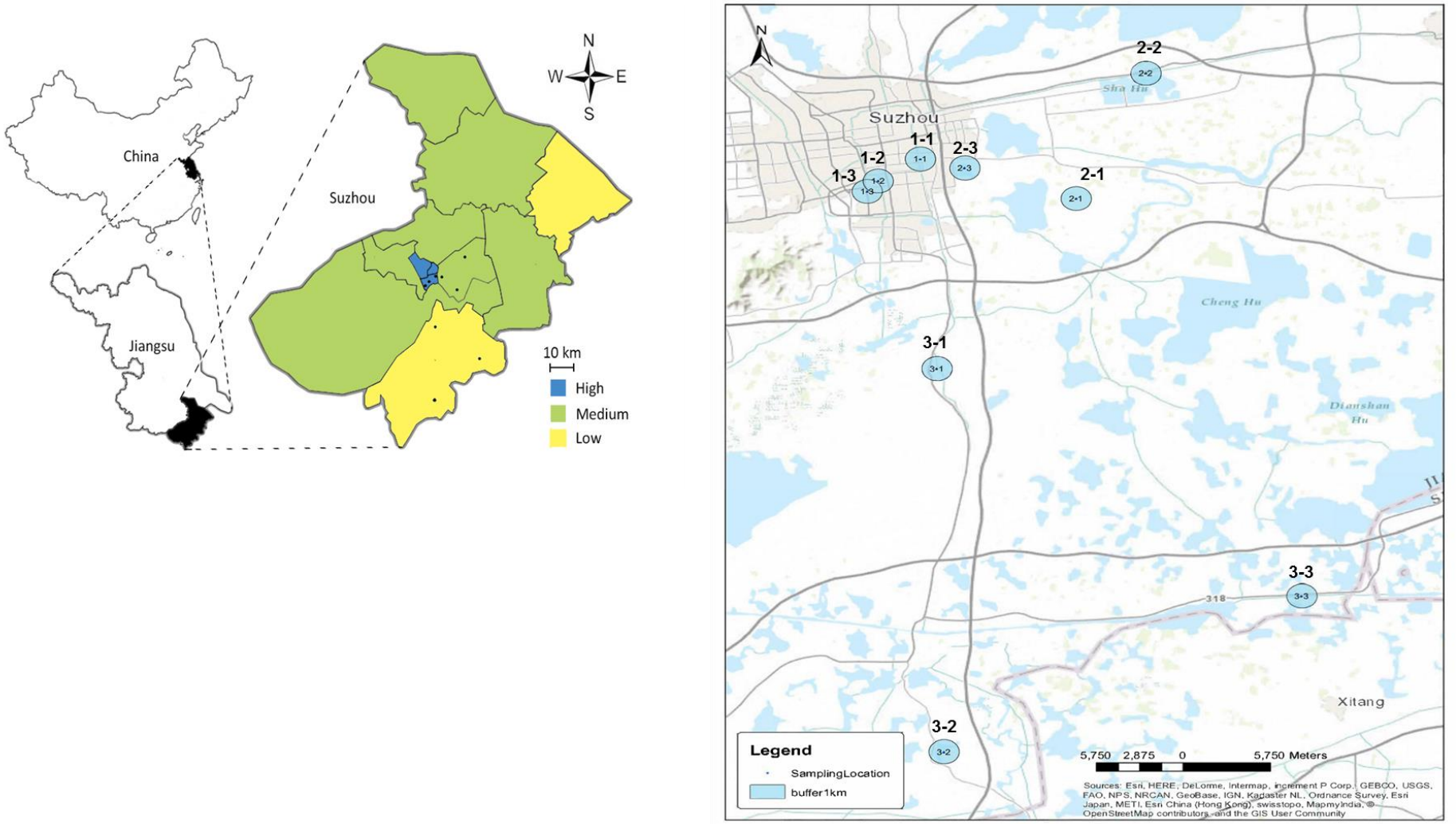



**Figure 2.1.** Sampling locations in Suzhou representing high, medium and low urban gradients (3 each) selected for this study.

**Table 2.1.** Description of sampling locations along with coordinates and corresponding land use types*.

| Sampling location | Description of location and land use types | Coordinates | |
|---|---|---|---|
| | | **Latitude** | **Longitude** |
| 1-1 | Suzhou old town; High Density Residential Land (47%), Education & Research (17%), River and lake (11%), Commercial Land (8%), Public Greenland (8%), Road (6%), Reserved Land (3%). | N31°17′49″ | E120°38′42″ |
| 1-2 | Suzhou old town; High Density Residential Land (36%), Commercial Land (18%), Road (12%), Public Greenland (10%), River and Lake (8%), Education Research (4%), Others (12%). | N31°16′58″ | E120°37′13″ |
| 1-3 | Suzhou old town; High Density Residential Land (57%), Road (13%), Commercial Land (9%), Education and Research (4%), River and Lake (3%), Public Greenland (3%), Others (11%). | N31°16′32″ | E120°36′32″ |
| 2-1 | Suzhou Industrial Park; Education & Research (63%), Road (11%), Reserved Land (9%), Public Greenland (7%), River and Lake (4%), High Density Residential Land (3%), Others (3%). | N31°16′19″ | E120°44′17″ |
| 2-2 | Suzhou Industrial Park; Industrial Land (49%), High Density Residential Land (19%), Road (9%), Public Greenland (7%), Protective Greenbelt (7%), Others (9%). | N31°21′9″ | E120°46′51″ |
| 2-3 | Suzhou Industrial Park; High Density Residential Land (24%), Road (14%), River and Lake (12%), Municipal Utilities (11%), Education and Research (10%), Industrial Land (7%), Commercial Land (7%), Low Density Residential Land (7%), Public Greenland (6%), Others (2%). | N31°17′26″ | E120°40′21″ |
| 3-1 | Suzhou outskirt; High Density Residential Land (21%), Commercial Land (12%), River and Lake (11%), Reserved Land (11%), Road (11%), Public Greenland (10%), Education and Research (4%), Residential Land (3%), Industrial Land (3%), Hospital (3%), Others (11%). | N31°09′46″ | E120°39′18″ |
| 3-2 | Suzhou outskirt; High Density Residential Land (22%), River and Lake (34%), Agriculture land (16%), Public Greenland (6%), Commercial Land (5%), Low Density Residential Land (5%), Road (5%), Others (7%). | N30°54′53″ | E120°39′40″ |
| 3-3 | Suzhou outskirt; Public Greenland (29%), River and Lake (24%), Agricultural Land (20%), Industrial Land (14%), High Density Residential Land (7%), Road (6%). | N31°0′55″ | E120°52′19″ |
| H-1 | Close to village at the foot of the mountain; Farmland, Family farming activities (e.g. poultry). | N30°16′13″ | E118°4′45″ |
| H-2 | Close to the roads to the mountain; Protected nature area. | N30°10′15″ | E118°3′32″ |
| H-3 | In the mount mountain; Protected nature area. | N30°6′28″ | E118°1′40″ |

* Land use in 1 km buffer zone


**Figure 2.2.** A view of sampling locations in Suzhou (high, medium and low are degrees of urbanization) and Huangshan selected for this study.

### 2.2.2. Field sampling

One 5-liter water sample was collected from each sampling location. The samples were collected in sterile polypropylene containers. Parameters including air temperature (AT), water temperature (WT) and electrical conductivity (EC) were measured in the field using a thermometer and an EC/TDS/TEMP WATERPROOF COMBO METER (COM-100) (HM Digital Inc. Culver City, CA, USA). The samples were transported to the lab at ambient temperature for nutrients and microbiological analyses and processed within 8 h of sample collection. Samples which were used for nutrients and microbiological analyses were kept on ice until they were brought to the laboratory. The sampling was carried out at four time points (winter and summer 2015 and 2016) to assess the impact of urbanization and seasonal variations on these parameters. In summer 2016, the sampling was conducted in the Huangshan area, to provide a control dataset. For each season, field sampling was conducted twice to ensure the credibility of water quality characteristics, and the samples were conducted 6 weeks apart in a season.

### 2.2.3. Physico-chemical analysis of water samples

Air temperature (AT), water temperature (WT), pH, electrical conductivity (EC), total nitrogen (TN), total phosphorous (TP), nitrate-N ($NO_3$-N), nitrite-N ($NO_2$-N), ammonium nitrogen ($NH_4$-N), phosphate ($PO_4$-P), total organic carbon (TOC) and chlorophyll *a* (Chl *a*) were measured.

The pH was measured using a Eutech pH 700 instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA). TN and TP were measured by peroxodisulphate oxidation and spectrophotometric methods. Nitrate nitrogen ($NO_3$-N), nitrite nitrogen ($NO_2$-N), ammonium nitrogen ($NH_4$-N) and phosphate ($PO_4$-P) were determined using a continuous flow analyzer (Skalar SA 1000, Breda, The Netherlands) (Wang et al., 2011a). TOC was measured with a

Shimadzu analyzer (model 5000; Tokyo, Japan) by high-temperature oxidation. Chlorophyll *a* was measured based on the procedures of water and wastewater analysis used by the American Public Health Association (APHA, 2005).

### 2.2.4. Microbiological analyses of water samples

The total viable count (TVC) was carried out by using plate count agar (PCA) (Reasoner and Geldreich, 1985). Serial dilutions were made for collected water samples and 50 µL samples were plated on PCA plates in triplicate and incubated at 30 °C for 48 h. The colonies were counted to determine the average number of colony forming units (CFU) per mL.

Total coliforms (TC) in water samples were determined by the pour plate method (Lange et al., 2013). For this, serial dilutions were made and 100 or 200 µL samples were plated on Harlequin$^{TM}$ *E. coli* / Coliform Medium (LabM, Heywood, UK) plates in triplicate. Plates were incubated at 37 °C for 24 h and then the number of *Escherichia coli* (*E. coli*) (blue-green colonies) and coliform (rose-pink colonies) were counted to determine the average number of total colony forming units (CFU) per mL.

Fecal coliform counts (FC) in water samples were carried out by membrane filtration and pour plate methods. Serial dilutions were made and 2 mL samples filtered through 0.22 µm isopore$^{TM}$ membrane filters (Merck Millipore Ltd. Tullagreen, Carrigtwohill Co. Cork, Ireland), which were then placed on MFC agar medium (containing 1% Rosalic acid) (Difco, Sparks, MD, USA) plates in triplicate. Plates were incubated at 44.5 °C for 24 h and the colonies that exhibited blue shades were counted to determine the number of colony forming units (cfu) per mL. Because of the limitations of time and reagents, all the microbiological parameters were only analyzed for one time point in each season (42$^{nd}$ day).

### 2.2.5. Statistical analyses

All the data collected in a two-year period, including physico-chemical and microbiological parameters, were analysed statistically using the software "IBM SPSS Statistics 20" and "GraphPad Prism 5". Seasonal variations (Winter and Summer) and variations with urban intensifications (High, Medium, Low and Huangshan) were analyzed by Tukey two-way ANOVA (Fujikoshi, 1993, Taylor, 2012, Zaiontz, 2018). The correlations between microbiological parameters (TVC, TC and FC) and physico-chemical parameters (temperature, pH, conductivity and nutrients) of water samples were determined by two-tailed Spearman's non-parametric rank correlation test.

The land use maps were prepared by using the geographical information system software of ArcGIS 10.2 (Environmental Systems Research Institute, Inc. (ESRI), Redlands, CA, USA). On the basis of the Open Street Map of Suzhou city, two layers of buffer zones were created with radii of 500 m and 1,000 m respectively around all sample points. By referencing the official land use maps of Gusu district, Suzhou Industrial Park district and Wujiang district of Suzhou as well as Google maps covering the sample areas, the detailed land use types were digitalized within these buffer zones in accordance with the national code for classification of urban land use and planning standards of development land (GB50137-200), and the land use composition within the areas of buffer zones calculated. The specific explanation of each land use classification can be seen in Table 2.2.

**Table 2.2.** The specific explanation of each land use classification.

| No. | Land Use Classification | Explanation |
|---|---|---|
| 1 | Administrative Land | Government agencies, non-profit organizations and other facility land |
| 2 | Commercial and Residential Mixed Land | Land used for both commercial and residential land |
| 3 | Commercial Land | All sorts of commercial, business and entertainment use facility |
| 4 | Cultural Entertainment Land | Libraries, exposition etc. cultural facility land |
| 5 | Education & Research Land | Higher-education institution, secondary technical education institution, middle schools, primary schools, research institutions, as well as affiliated dormitories |
| 6 | High Density Residential Land | Middle to High rise residence with relatively complete amenity |
| 7 | Hospital | Hospital, health care, habitation related land |
| 8 | Industrial Land | Industry and mining factories |
| 9 | Low Density Residential Land | Low-rise residence |
| 10 | Protective Greenbelt | Greenland functions as sanitation and safety buffers |
| 11 | Municipal Utilities | Land for facilities providing services, environment and safety |
| 12 | Public Greenland | Open for public primarily for recreational purpose |
| 13 | Reserved Land | Land reserved for future use |
| 14 | River & Lake | Non-development land, all sorts of water bodies |
| 15 | Road | Urban road and traffic facility land |
| 16 | Sports | Sports facility and training facility land |

## 2.3. Results

### 2.3.1. Variations in physico-chemical parameters

The results of physico-chemical parameters of water samples collected from various sampling locations in Suzhou and Huangshan are shown in Table 2.3 and Figures 2.3 to 2.5.

Both air and water temperature showed significant variation between seasons and also across the sampling locations (urbanization) (Figure 2.3, Tables 2.3 and 2.4). In winter, the air temperature ranged from -1 °C to 11 °C in Suzhou; In summer, the values ranged from 26-37.6 °C. The same pattern was observed with the water temperature. The air and water temperatures were obviously different between High vs. Medium and Medium vs. Low however, no significant variation between High vs. Low was observed. The pH values of the sampling locations ranged from 7-8 (with few exceptions – pH values were 8.71 and 8.16 for samples collected at 3-3 and H-2 collected in summer 2016, respectively) (Figure 2.3 and Table 2.3) and most of the values were within acceptable range (6.5-8.5) as reported in "Environmental quality standards for surface water in China" by the Ministry of Environment Protection (MEP, 2002). The pH values recorded during field sampling showed significant difference among the sampling locations (urban intensifications). However, no significant differences between seasons were observed (Table 2.4). Electrical conductivity values significantly varied between sampling locations and also seasons. The values were significantly high between high (393-832 µS/cm) and low (186-573 µS/cm) urbanization locations, and these values were extremely high as compared to values recorded in samples collected from the natural reserve mountain in Huangshan (45.6-146 µS/cm) (Figure 2.3 and Tables 2.3 and 2.4).

**Table 2.3.** Results of physico-chemical and microbiological characteristics of water samples collected from nine sampling locations across three urban intensifications in winter and summer 2015 and 2016. Samples from the control locations (Huangshan) were collected in summer 2016. The results of the statistical analysis (two-way ANOVA) is also shown in this table.

| **Parameters** | **Winter 2015 and 2016 Range (Min-Max)** | | | **Summer 2015 and 2016 Range (Min-Max)** | | | **Control location (Huangshan)** | **P values** | |
|---|---|---|---|---|---|---|---|---|---|
| | **High** | **Medium** | **Low** | **High** | **Medium** | **Low** | | **Urbanization** | **Season** |
| Air Temperature (°C) | -3-11 | -4-10 | -1-13 | 27-37.6 | 26-35.6 | 28.5-36.8 | 28.1-33.4 | 0.001** | 0.000*** |
| Water Temperature (°C) | 6-11 | 5.1-9.8 | 5.9-10 | 28-34.1 | 26-33.4 | 28.8-34.4 | 24.3-28.2 | 0.001** | 0.000*** |
| pH | 7.1-7.9 | 7.51-7.9 | 7.58-7.9 | 7.3-7.7 | 7.39-7.86 | 7.3-8.71 | 7.12-8.16 | 0.006** | 0.178 |
| Electrical Conductivity (µS/cm) | 422-832 | 474-615 | 395-573 | 393-534 | 389-544 | 186-563 | 45.6-146 | 0.013* | 0.000*** |
| TN (mg/L) | 2.85-16.5 | 2.14-4.48 | 1.57-4.21 | 2.11-4.67 | 1.92-10.58 | 0.96-4.63 | 0.29-1.17 | 0.001** | 0.038* |
| TP (mg/L) | 0.13-2.10 | 0.07-0.21 | 0.04-0.26 | 0.23-0.53 | 0.12-1.04 | 0.06-0.21 | 0.02-0.06 | 0.000*** | 0.102 |
| $NO_3$-N (mg/L) | 1.01-3.42 | 1.13-2.56 | 0.83-2.83 | 0.17-1.01 | 0.05-1.25 | 0.24-1.96 | 0.21-0.92 | 0.816 | 0.000*** |
| $NO_2$-N (mg/L) | 0.01-0.14 | 0.02-0.08 | 0.01-0.12 | 0.05-0.20 | 0.06-0.35 | 0.03-0.27 | 0.00-0.02 | 0.422 | 0.000*** |
| $PO_4$-P (µg/L) | 48-497 | 14.37-121.51 | 11.71-46.45 | 92.78-315 | 30.44-117 | 16.11-88.9 | 3.44-28.93 | 0.000*** | 0.002** |
| $NH_4$-N (mg/L) | 1.01-7.84 | 0.18-1.44 | 0.41-1.47 | 0.52-2.40 | 0.23-1.48 | 0.03-2.05 | 0.01-0.10 | 0.000*** | 0.148 |
| TOC (mg/L) | 1.99-42.3 | 3.17-13.23 | 3.55-13.8 | 3.72-20.8 | 3.75-23 | 3.67-15.6 | 1.31-3.35 | 0.936 | 0.745 |
| Chlorophyll *a* (µg/L) | 2.33-21.4 | 1.37-15.41 | 1.47-16.56 | 3.39-68.86 | 2.7-50.177 | 1.95-54.42 | 0.95-3.17 | 0.329 | 0.000*** |
| Total viable count (x $10^3$ cfu/mL) | 7-57.4 | 0.4-43.9 | 0.6-33.4 | 32-48.7 | 13.4-53.1 | 2-73.7 | 9.5-30.7 | 0.040* | 0.055 |
| Total coliform count (x $10^3$ cfu/mL) | 3.733-10 | 2.067-8.267 | 0.067-0.867 | 3.2-22.1 | 0.2-2.4 | 0-3.9 | 0.098-0.933 | 0.006** | 0.696 |
| Fecal coliform count (cfu/mL) | 90-120 | 55-85 | 0-23 | 218-480 | 18-253 | 0-233 | 0.5-17 | 0.036* | 0.032* |

*Statistically significant difference at $p<0.05$; **Statistically significant difference at $p<0.01$; *** Statistically significant difference at $p<0.001$.

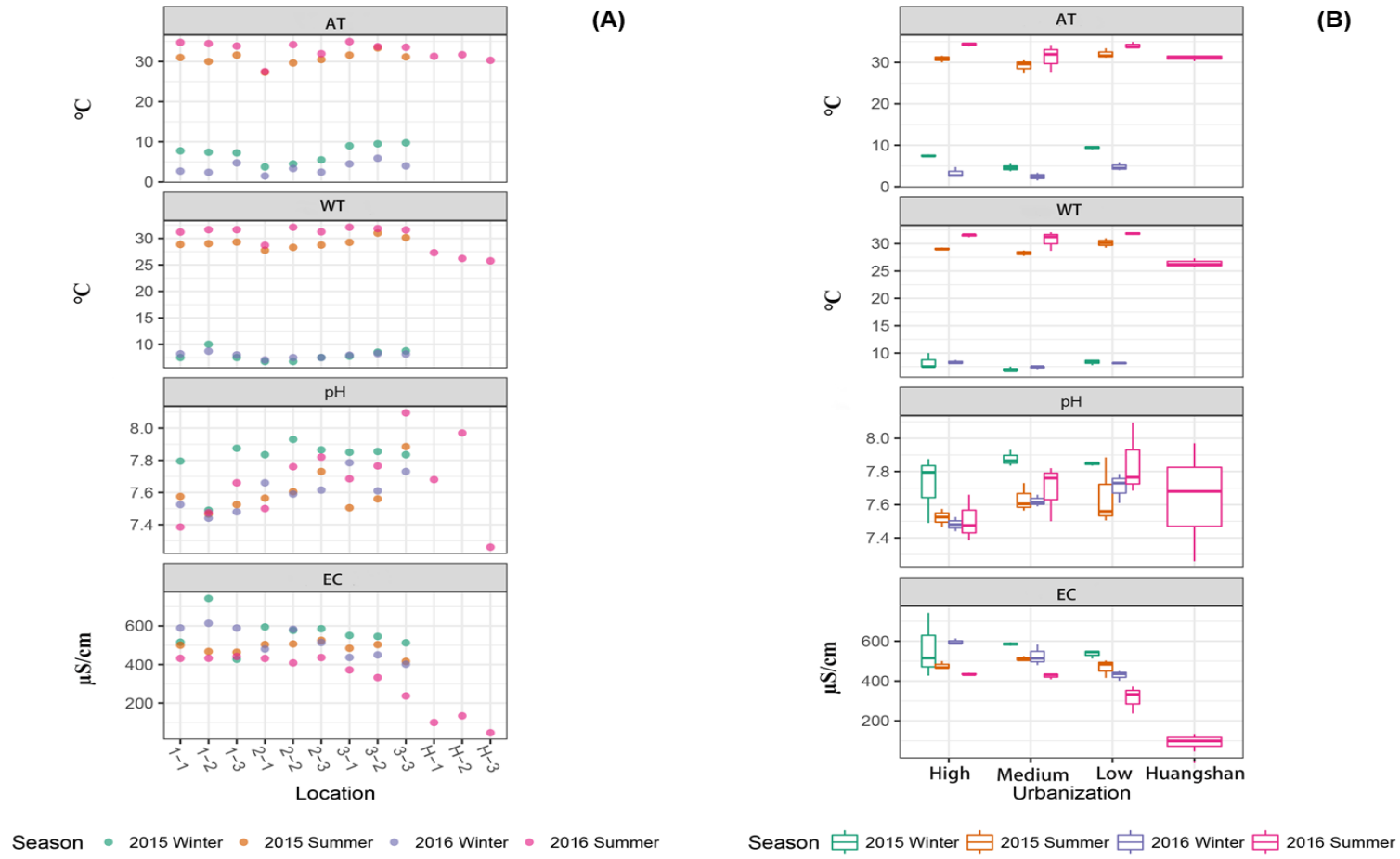



**Figure 2.3.** The variation in air and water temperatures, pH and electrical conductivity values observed in different sampling locations and seasons. The individual values for each parameter (A) and boxplots (B) with median value (line within each box), quartile interval (box) and the minimum and maximum value (whiskers) are shown.

**Table 2.4.** Two-way ANOVA for variations of physico-chemical and microbiological parameters of water samples.

| Source | df | SS | MS | F | P |
|---|---|---|---|---|---|
| **Air Temperature (AT)** | | | | | |
| A. Urbanization† | 2 | 0.391 | 0.195 | 9.852 | **0.001** |
| B. Season‡ | 1 | 8.847 | 8.847 | 445.955 | **0.000** |
| A×B | 2 | 0.249 | 0.124 | 6.270 | **0.007** |
| **Water Temperature (WT)** | | | | | |
| A. Urbanization | 2 | 0.026 | 0.013 | 9.099 | **0.001** |
| B. Season | 1 | 6.290 | 6.290 | 4406.812 | **0.000** |
| A×B | 2 | 0.009 | 0.005 | 3.170 | 0.056 |
| **pH** | | | | | |
| A. Urbanization | 2 | 0.002 | 0.001 | 6.074 | **0.006** |
| B. Season | 1 | 0.000 | 0.000 | 1.907 | 0.178 |
| A×B | 2 | 3.33E-05 | 1.67E-05 | 0.117 | 0.890 |
| **Electrical Conductivity (EC)** | | | | | |
| A. Urbanization | 2 | 0.097 | 0.049 | 5.050 | **0.013** |
| B. Season | 1 | 0.155 | 0.155 | 16.104 | **0.000** |
| A×B | 2 | 0.003 | 0.001 | 0.151 | 0.860 |
| **Total Nitrogen (TN)** | | | | | |
| A. Urbanization | 2 | 0.729 | 0.364 | 9.671 | **0.001** |
| B. Season | 1 | 0.177 | 0.177 | 4.691 | **0.038** |
| A×B | 2 | 0.123 | 0.061 | 1.632 | 0.212 |
| **Total Phosphorus (TP)** | | | | | |
| A. Urbanization | 2 | 3.216 | 1.608 | 19.592 | **0.000** |
| B. Season | 1 | 0.234 | 0.234 | 2.852 | 0.102 |
| A×B | 2 | 0.093 | 0.047 | 0.569 | 0.572 |
| **Nitrate ($NO_3$)-N** | | | | | |
| A. Urbanization | 2 | 0.024 | 0.012 | 0.205 | 0.816 |
| B. Season | 1 | 3.461 | 3.461 | 58.892 | **0.000** |
| A×B | 2 | 0.583 | 0.292 | 4.964 | 0.014 |
| **Nitrite ($NO_2$)-N** | | | | | |
| A. Urbanization | 2 | 0.156 | 0.078 | 0.887 | 0.422 |
| B. Season | 1 | 2.419 | 2.419 | 27.512 | **0.000** |
| A×B | 2 | 0.203 | 0.101 | 1.154 | 0.329 |
| **Phosphate ($PO_4$)-P** | | | | | |
| A. Urbanization | 2 | 7.207 | 3.604 | 69.510 | **0.000** |
| B. Season | 1 | 0.601 | 0.601 | 11.599 | **0.002** |
| A×B | 2 | 0.012 | 0.006 | 0.117 | 0.890 |
| **Ammonium ($NH_4$)-N** | | | | | |
| A. Urbanization | 2 | 3.917 | 1.958 | 12.379 | **0.000** |
| B. Season | 1 | 0.348 | 0.348 | 2.200 | 0.148 |
| A×B | 2 | 0.638 | 0.319 | 2.017 | 0.151 |
| **Total Organic Carbon (TOC)** | | | | | |
| A. Urbanization | 2 | 0.017 | 0.009 | 0.067 | 0.936 |
| B. Season | 1 | 0.014 | 0.014 | 0.107 | 0.745 |

| | | | | | |
|---|---|---|---|---|---|
| A×B | 2 | 0.037 | 0.018 | 0.144 | 0.867 |
| **Chlorophyll *a* (Chl *a*)** | | | | | |
| A. Urbanization | 2 | 0.427 | 0.213 | 1.156 | 0.329 |
| B. Season | 1 | 3.097 | 3.097 | 16.775 | **0.000** |
| A×B | 2 | 0.293 | 0.147 | 0.795 | 0.461 |
| **Total Viable Count (TVC)** | | | | | |
| A. Urbanization | 2 | 2.553 | 1.276 | 3.612 | **0.040** |
| B. Season | 1 | 1.418 | 1.418 | 4.013 | 0.055 |
| A×B | 2 | 0.302 | 0.151 | 0.427 | 0.656 |
| **Total Coliforms (TC)** | | | | | |
| A. Urbanization | 2 | 3.938 | 1.969 | 8.519 | **0.006** |
| B. Season | 1 | 0.037 | 0.037 | 0.160 | 0.696 |
| A×B | 2 | 1.912 | 0.956 | 4.136 | 0.046 |
| **Fecal Coliforms (FC)** | | | | | |
| A. Urbanization | 2 | 80422.33 | 40211.16 | 4.444 | **0.036** |
| B. Season | 1 | 53355.55 | 53355.55 | 5.897 | **0.032** |
| A×B | 2 | 22770.11 | 11385.05 | 1.258 | 0.319 |

† Urbanization: High vs. Medium vs. Low; ‡ Season: Winter vs. Summer;

df: degree of freedom; SS: Sum Square; MS: Mean Square.

**Table 2.5.** Urbanization variation of physico-chemical and microbiological parameters of water samples.

| Urbanization | Mean Difference | Std. Error | Sig. (P) | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower Bound | Upper Bound |
| **AT** | | | | | |
| High vs. Medium | 0.157 | 0.048 | **0.003** | 0.059 | 0.256 |
| High vs. Low | -0.047 | 0.045 | 0.308 | -0.141 | 0.047 |
| Medium vs. Low | -0.205 | 0.048 | **0.000** | -0.303 | -0.106 |
| **WT** | | | | | |
| High vs. Medium | 0.038 | 0.011 | **0.002** | 0.016 | 0.060 |
| High vs. Low | -0.004 | 0.011 | 0.704 | -0.026 | 0.018 |
| Medium vs. Low | -0.042 | 0.011 | **0.001** | -0.064 | -0.020 |
| **pH** | | | | | |
| High vs. Medium | -0.009 | 0.003 | **0.019** | -0.016 | -0.001 |
| High vs. Low | -0.012 | 0.003 | **0.002** | -0.019 | -0.005 |
| Medium vs. Low | -0.003 | 0.003 | 0.380 | -0.010 | 0.004 |
| **EC** | | | | | |
| High vs. Medium | 0.001 | 0.028 | 0.974 | -0.057 | 0.059 |
| High vs. Low | 0.078 | 0.028 | **0.010** | 0.021 | 0.136 |
| Medium vs. Low | 0.078 | 0.028 | **0.010** | 0.020 | 0.135 |
| **TN** | | | | | |
| High vs. Medium | 0.144 | 0.056 | **0.016** | 0.029 | 0.258 |
| High vs. Low | 0.245 | 0.056 | **0.000** | 0.131 | 0.360 |
| Medium vs. Low | 0.101 | 0.056 | 0.080 | -0.013 | 0.216 |
| **TP** | | | | | |
| High vs. Medium | 0.349 | 0.083 | **0.000** | 0.180 | 0.518 |
| High vs. Low | 0.506 | 0.083 | **0.000** | 0.337 | 0.675 |
| Medium vs. Low | 0.157 | 0.083 | 0.067 | -0.012 | 0.326 |
| **$NO_3$-N** | | | | | |
| High vs. Medium | -0.032 | 0.070 | 0.651 | -0.175 | 0.111 |
| High vs. Low | 0.011 | 0.070 | 0.874 | -0.132 | 0.154 |
| Medium vs. Low | 0.043 | 0.070 | 0.542 | -0.100 | 0.186 |
| **$NO_2$-N** | | | | | |
| High vs. Medium | 0.037 | 0.086 | 0.673 | -0.138 | 0.211 |
| High vs. Low | 0.112 | 0.086 | 0.201 | -0.063 | 0.287 |
| Medium vs. Low | 0.075 | 0.086 | 0.386 | -0.100 | 0.250 |
| **$PO_4$-P** | | | | | |
| High vs. Medium | 0.484 | 0.066 | **0.000** | 0.350 | 0.619 |
| High vs. Low | 0.766 | 0.066 | **0.000** | 0.632 | 0.900 |
| Medium vs. Low | 0.282 | 0.066 | **0.000** | 0.147 | 0.416 |
| **$NH_4$-N** | | | | | |
| High vs. Medium | 0.375 | 0.115 | **0.003** | 0.140 | 0.609 |
| High vs. Low | 0.561 | 0.115 | **0.000** | 0.326 | 0.795 |
| Medium vs. Low | 0.186 | 0.115 | 0.116 | -0.049 | 0.420 |
| **TOC** | | | | | |
| High vs. Medium | -0.023 | 0.104 | 0.823 | -0.235 | 0.188 |
| High vs. Low | -0.037 | 0.104 | 0.720 | -0.249 | 0.174 |
| Medium vs. Low | -0.014 | 0.104 | 0.893 | -0.225 | 0.197 |
| **Chl *a*** | | | | | |
| High vs. Medium | 0.168 | 0.127 | 0.197 | -0.092 | 0.428 |
| High vs. Low | 0.162 | 0.124 | 0.203 | -0.092 | 0.415 |
| Medium vs. Low | -0.006 | 0.127 | 0.962 | -0.266 | 0.254 |

| | | | | | |
|---|---|---|---|---|---|
| **TVC** | | | | | |
| High vs. Medium | 0.497 | 0.249 | 0.055 | -0.011 | 1.006 |
| High vs. Low | 0.617 | 0.243 | **0.017** | 0.121 | 1.114 |
| Medium vs. Low | 0.120 | 0.249 | 0.633 | -0.388 | 0.629 |
| **TC** | | | | | |
| High vs. Medium | 0.571 | 0.278 | 0.064 | -0.039 | 1.182 |
| High vs. Low | 1.215 | 0.294 | **0.002** | 0.567 | 1.863 |
| Medium vs. Low | 0.643 | 0.294 | 0.051 | -0.005 | 1.291 |
| **FC** | | | | | |
| High vs. Medium | 110.667 | 54.919 | 0.067 | -8.991 | 230.324 |
| High vs. Low | 159.833 | 54.919 | **0.013** | 40.176 | 279.491 |
| Medium vs. Low | 49.167 | 54.919 | 0.388 | -70.491 | 168.824 |

The changes in the nutrients such as TN, TP, $NO_3$-N, $NO_2$-N, $PO_4$-P and $NH_4$-N in different locations and seasons are shown in Figures 2.4 and 2.5. The surface water quality in China is classified into six grades (MEP, 2002): Grade I-III are applicable to the water from sources or protected areas for centralized sources for drinking and such grades could be considered as good quality; Grade IV and V are applicable to water bodies for industrial and agricultural use, and such grades could be considered as moderately polluted; Grade V+ means seriously polluted. The results obtained in this study showed that TN concentrations were high in almost every location in Suzhou (highest in high and medium urbanized locations) as compared to the MEP standards (Grade V+: TN > 2mg/L) which indicates that these locations were seriously polluted with multiple sources. TN concentration was low and within the limit in control locations in Huangshan (0.29-1.17 mg/L) to the water in Suzhou canals. TP and ammonium concentrations were beyond the standards (Grade V+: TP > 0.4mg/L, $NH_4$-N > 2mg/L), especially in all the locations with high urbanization (0.13-2.10 mg/L of TP and 1.01-7.84 of $NH_4$-N). Particularly, all of the above three parameters (TN, TP and $NH_4$-N) were extremely high in the second location (1-2) in high urbanization during winter 2015. Besides these parameters, some other key water quality parameters such as $PO_4$-P and TOC, were also very high in the location 1-2 during winter 2015. The statistical analysis (two-way ANOVA) showed that significant variation between locations (urbanization) was observed for the parameters TN, TP, $NH_4$-N and $PO_4$-P (Table 2.4); among the three urban intensifications (High, Medium and Low) in Suzhou, the variation in the nutrient values were observed mainly between High vs. Medium and High vs. Low and almost no significant variations in the parameters (except for $PO_4$-P) were observed between Medium vs. Low (Table 2.5). In addition to the variation between the sampling locations, TN and $PO_4$-P values showed significant variation between seasons as well. However, the parameters such as $NO_3$-N, $NO_2$-N and chlorophyll *a* showed significant variation with

respect to only season rather than the degree of urbanization (Table 2.4). Chlorophyll *a*, as an indicator of algal growth in water bodies, was extremely high in location 2-2 in summer 2016 (750 µg/L, this was not shown in tables and figures to avoid influence of this value to the whole dataset). The parameter TOC did not show significant variation either between sampling locations or seasons (Table 2.4). As shown in Table 2.3 and Figures 2.4 and 2.5, the nutrient values observed in samples from Huangshan were extremely low as compared to Suzhou canals, indicating good water quality in the absence of any influence from urbanization and other anthropogenic activities.

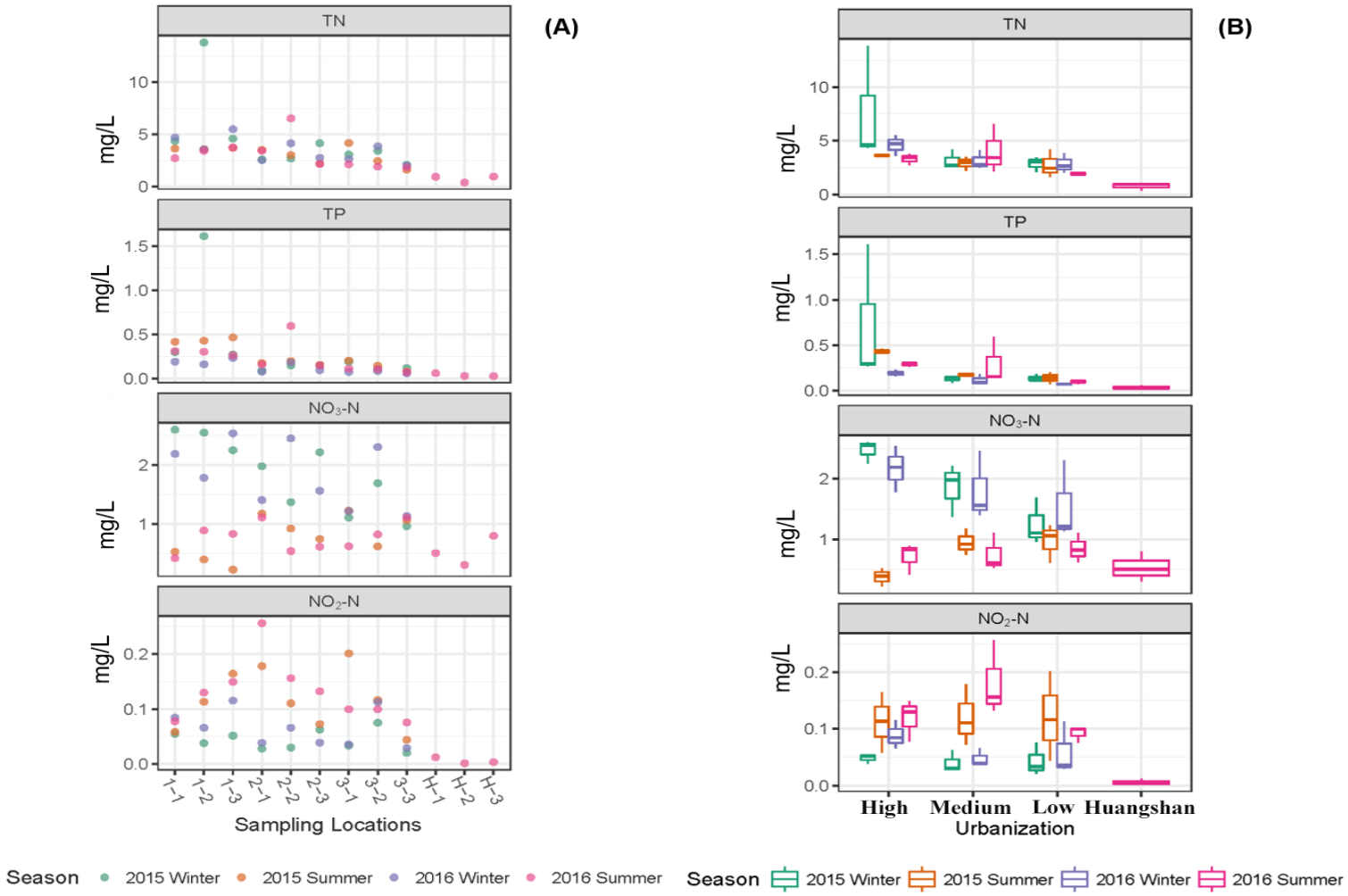



**Figure 2.4.** The variation in total nitrogen (TN), total phosphorus (TP), nitrate-N and nitrite-N values observed in different sampling locations and seasons. The individual values for each parameters (A) and boxplots (B) with median value (line within each box), quartile interval (box) and the minimum and maximum value (whiskers) are shown.

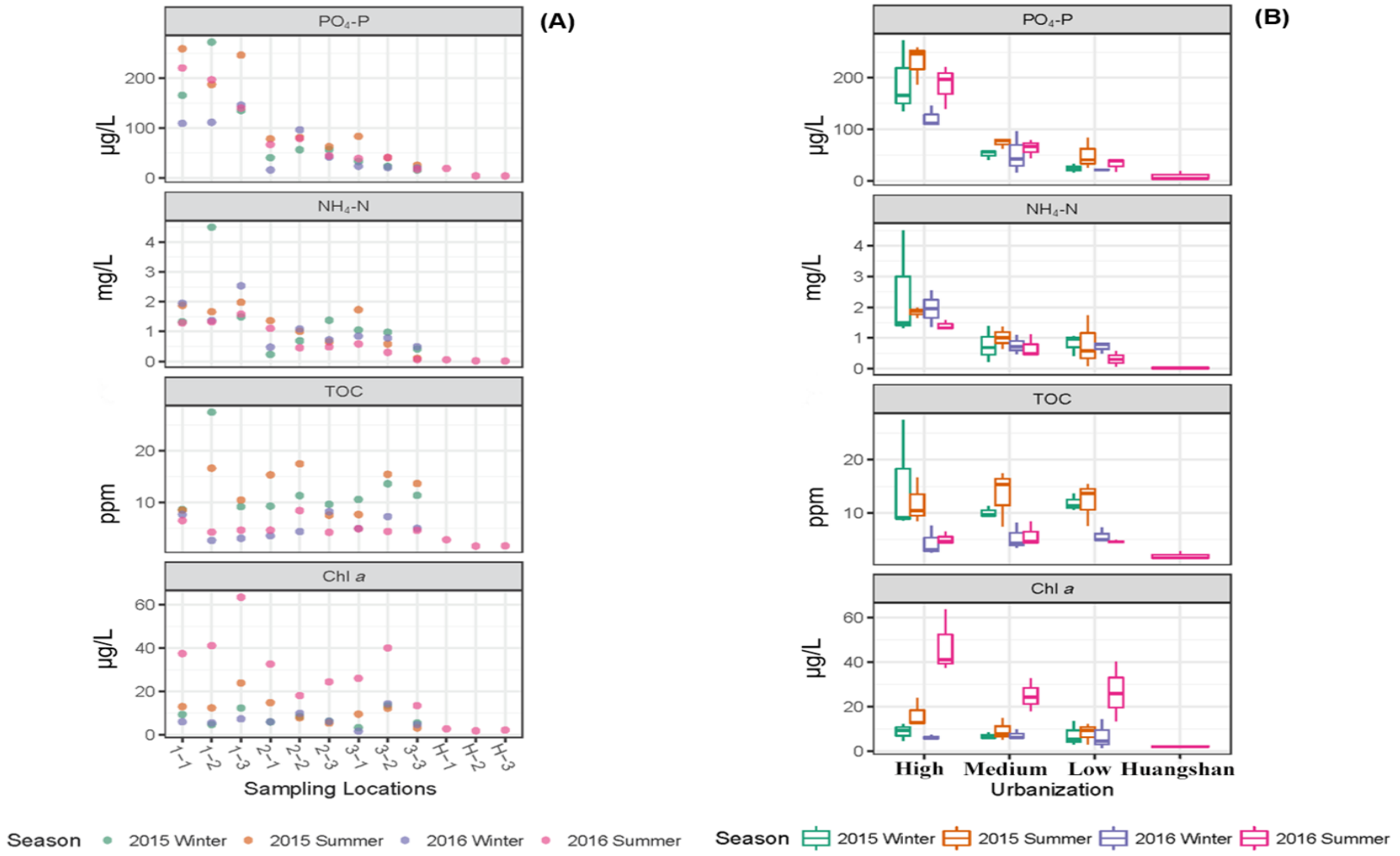



**Figure 2.5.** The variation in phosphate-P, ammonium-N, total organic carbon (TOC) and Chlorophyll *a* values observed in different sampling locations and seasons. The individual values for each parameters (A) and boxplots (B) with median value (line within each box), quartile interval (box) and the minimum and maximum value (whiskers) are shown.

### 2.3.2. Changes in microbiological parameters

As one of the culture dependent microbiological parameters, total viable count (TVC) was measured to quantitatively assess the microbial load in water samples collected from sampling locations in Suzhou and Huangshan (Figure 2.6). TVC showed significant variation with respect to urbanization (Table 2.4), and high TVC values were observed in locations with high urbanization, as compared to locations with low urbanization and Huangshan (Table 2.5).

Total coliform (TC) numbers were measured to assess the sanitary quality of the water. As the coliform group of bacteria normally originate from the digestive tracts of warm-blooded animals, their presence in water samples indicates bacteria of fecal origin although coliform themselves do not typically cause serious illnesses or diseases (Farrell-Poe, 2005). TC showed significant variation with respect to sampling locations in Suzhou (Table 2.4) and the TC were higher in locations in highly urbanized regions compared to low urbanized regions (Table 2.5). Extremely low levels of TC counts were observed in water samples collected from Huangshan.

Fecal coliform (FC) or thermotolerant coliform is a group of anaerobic facultative bacteria whose presence in high numbers indicates fecal contamination and increased health risk (Haller et al., 2009) however, their presence is not always an absolute indicator of fecal contamination or the presence of harmful bacteria in water samples (Doyle and Erickson, 2006) (Figure 2.6). The FC count showed significant variation with respect to both urbanization and season. Higher FC counts were observed in locations with high urbanization as compared to locations with low urbanization (Table 2.5). In water samples collected from Huangshan, extremely low FC counts were observed.

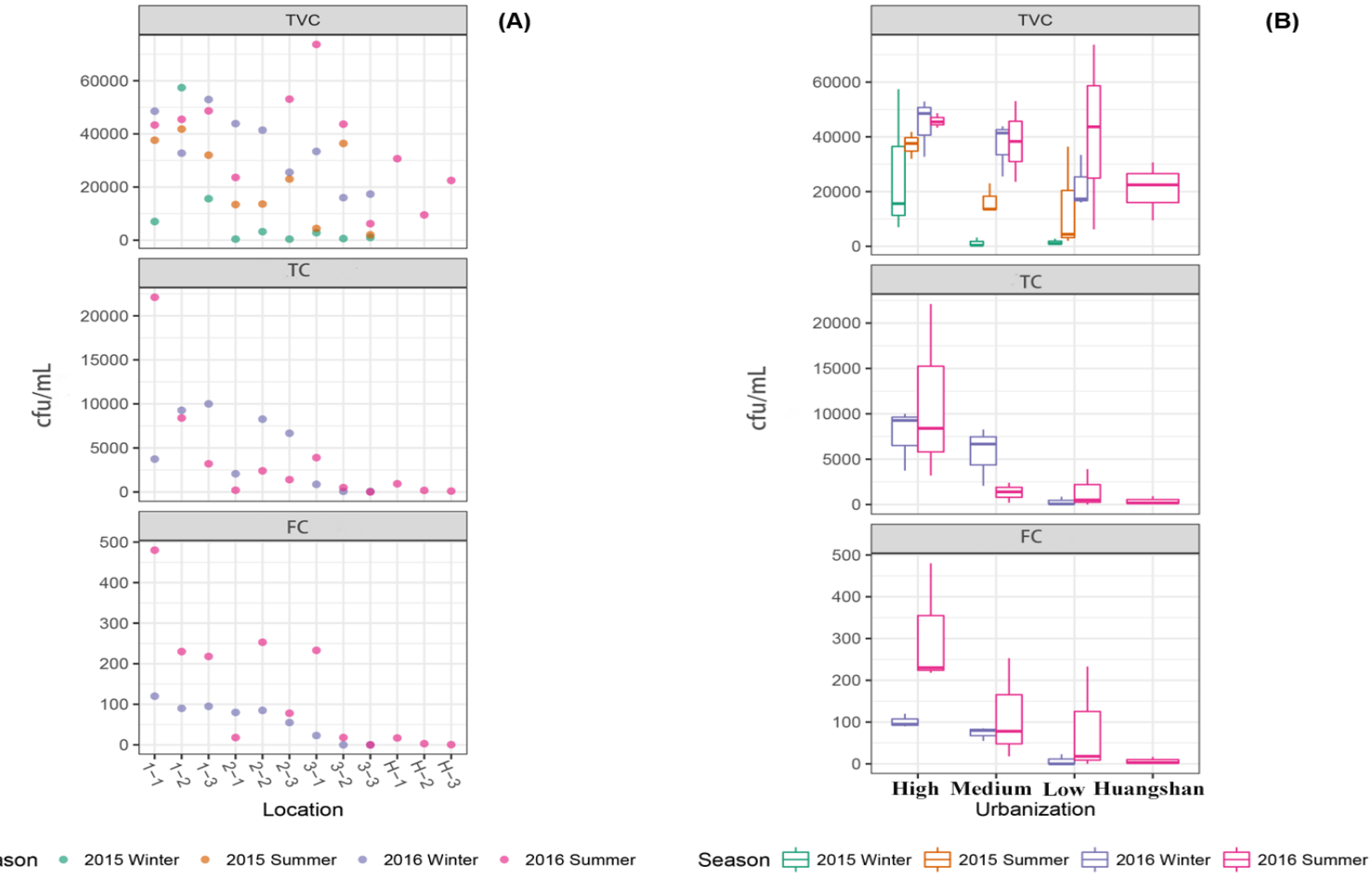



**Figure 2.6.** The variation in total viable count (TVC), total coliform (TC) and fecal coliform (TC) values observed in different sampling locations and seasons. The individual values for each parameters (A) and boxplots (B) with median value (line within each box), quartile interval (box) and the minimum and maximum value (whiskers) are shown.

### 2.3.3. Correlation between variables

Results of correlation analysis between the tested variables are shown in Table 2.6 and Table S2.1 (correlation coefficient [*r*] values and *P* values of correlation coefficient, respectively). Water temperature showed strong positive correlation with $NO_2$-N and chlorophyll *a*, and strong negative correlation with EC and $NO_3$-N. The pH did not show positive correlation with any of the water quality parameters studied. However, it had a moderate negative correlation with TVC and a strong negative correlation with total coliform (TC). Conductivity had strong positive correlation with parameters such as TN, $NH_4$-N and $NO_3$-N, and a moderate positive correlation with TC. Notably, TN had a strong positive correlation with many parameters such as $NO_3$-N, $NO_2$-N, $NH_4$-N, TP and $PO_4$-P (Table 2.6) and negative correlation with none of the parameters. Similarly, TP had strong positive correlation with $PO_4$-P, $NO_2$-N, $NH_4$-N and fecal coliform (FC) and no negative correlations with other parameters studied. The $NH_4$-N had strong positive correlation with $PO_4$-P and $NO_2$-N and the parameter, $PO_4$-P had strong positive correlation with $NO_2$-N, TC and FC. As an indicator of algal growth, chlorophyll *a* showed positive correlation with water temperature and nutrients such as TP, $NO_2$-N and $PO_4$-P. Interestingly, multiple nutrients (TN, TP, $NH_4$-N and $PO_4$-P) showed positive correlation with fecal indicator bacteria (total and fecal coliforms).

**Table 2.6.** Spearman's correlation coefficient values observed between different water quality parameters.

| | WT | pH | EC | TN | TP | $NO_3$-N | $NO_2$-N | $PO_4$-P | $NH_4$-N | TOC | Chl *a* | TVC | TC | FC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT | 1 | | | | | | | | | | | | | |
| pH | -0.156 | 1 | | | | | | | | | | | | |
| EC | **-0.508**[d] | -0.0534 | 1 | | | | | | | | | | | |
| TN | **-0.285**[a] | **-0.226**[a] | **0.526**[d] | 1 | | | | | | | | | | |
| TP | 0.172 | **-0.238**[a] | **0.377**[c] | **0.727**[d] | 1 | | | | | | | | | |
| $NO_3$-N | **-0.682**[d] | 0.0422 | **0.540**[d] | **0.560**[d] | 0.0264 | 1 | | | | | | | | |
| $NO_2$-N | **0.511**[d] | **-0.315**[b] | -0.0056 | **0.464**[d] | **0.528**[d] | -0.044 | 1 | | | | | | | |
| $PO_4$-P | 0.134 | **-0.279**[a] | **0.340**[b] | **0.663**[d] | **0.891**[d] | 0.0592 | **0.523**[d] | 1 | | | | | | |
| $NH_4$-N | -0.172 | **-0.307**[b] | **0.486**[d] | **0.822**[d] | **0.771**[d] | **0.282**[a] | **0.468**[d] | **0.799**[d] | 1 | | | | | |
| TOC | 0.0986 | 0.202 | **0.334**[b] | 0.209 | **0.360**[b] | 0.0007 | 0.185 | 0.189 | 0.220 | 1 | | | | |
| Chl *a* | **0.445**[d] | -0.0298 | **-0.164** | 0.212 | **0.406**[c] | -0.178 | **0.575**[d] | **0.359**[b] | 0.185 | 0.130 | 1 | | | |
| TVC | 0.234 | **-0.499**[b] | -0.137 | 0.046 | 0.292 | -0.284 | 0.3098 | **0.371**[a] | 0.061 | -0.1997 | **0.429**[b] | 1 | | |
| TC | -0.149 | **-0.683**[c] | **0.592**[b] | 0.362 | **0.655**[b] | 0.208 | 0.320 | **0.823**[d] | **0.547**[a] | 0.146 | 0.256 | **0.623**[b] | 1 | |
| FC | 0.146 | **-0.469**[a] | 0.401 | 0.365 | **0.772**[d] | -0.0566 | **0.532**[a] | **0.794**[d] | 0.370 | **0.452**[a] | **0.511**[a] | **0.830**[d] | **0.858**[d] | 1 |

[a] Significant correlation at 0.05 level; [b] Significant correlation at 0.01 level; [c] Significant correlation at 0.001 level; [d] Significant correlation at 0.0001 level.

## 2.4. Discussion

Urbanization can cause major changes to the freshwater systems, such as increasing chemical and microbial contamination and eutrophication (Paul and Meyer, 2001). Cumming reported that population growth and urban density can cause changes to the nature of both ecosystem and non-ecosystem services (Cumming et al., 2014). In the present study, the impact of urban intensification on the physico-chemical and microbiological characteristics of water was studied by using canals as a model system. Canals including the Grand Canal are artificial rivers and they play important roles as supply water for irrigation, transportation of goods, recreational activities and also serving as a receiving water body for source and non-point source discharges (Wang et al., 2010). Earlier studies showed that artificial water systems such as canals are sensitive to anthropogenic outputs from human and industrial activities (Owens and Niemeyer, 2006, Wang et al., 2010), Therefore, canals can be used as a good model system to study the impact of urbanization on general water quality and ecosystem function.

The climate in Suzhou is humid subtropical, which is characterized by hot and humid summers, and mild to chilly and dry weather in winter. The air temperature data reported in Suzhou Statistical Yearbook (Suzhou Statistics Bureau, 2016, Suzhou Statistics Bureau, 2017) for 2015 and 2016 were 4.2–8.9 °C for winter (Dec-Feb) and 24.4–30.1 °C for summer (Figures S2.1 and S2.2), and this is consistent with the values measured in the present study (Table 2.3). However, significant variation in the air and water temperatures with respect to sampling locations in Suzhou was observed. As the field sampling was carried out at different time points on the same day (morning, noon or afternoon), the variations observed could be due to the sampling time. We have also recorded the water temperature at 2 hours intervals temperature loggers for leaf degradation experiments (discussed in Chapter 3) from day 0 to 42, and the

average values measured at each urbanization gradient showed some variation particularly in winter. The pH values varied significantly between sampling locations but not with seasons. pH is important for aquatic life as it determines the solubility and bioavailability of chemicals including nutrients and heavy metals (USGS, 2018). In general, most freshwater systems have a pH range of 6.5 to 8 (Farrell-Poe, 2005), and the range preferred by most aquatic life is 6.5-9.0, although some organisms can live outside this range, particularly extremophiles. Factors such as interaction with rock minerals, precipitation, wastewater discharges, and $CO_2$ levels can increase the pH levels (US EPA, 2012). As per the Chinese National Standards for Surface Water Quality (GB3838-2002), the normal range of pH in surface water is 6-9. The variation in the pH between sampling locations observed in this study could be due to differences in the nutrient levels and input from the surroundings (land use). The values observed in this study are consistent with the results reported by Yu (Yu et al., 2012) for surface water quality of the Grand Canals. Electrical conductivity (EC) values were extremely high in high urbanization locations. EC is an indicator of the amount of dissolved salts, total dissolved solids and inorganic compounds present in the water samples (Farrell-Poe, 2005, Perlman, 2016). Wastewater or domestic sewage can raise the conductivity due to the presence of phosphate, nitrate and other ions (US EPA, 2017), which is likely to be the main reasons for high levels of conductivity values observed in high urbanization locations. The EC levels have been reported to be influenced by nutrients and agricultural run-off (Perlman, 2018). Relatively higher EC observed during summer was due to higher temperature which has been reported to increase the EC levels (Miller et al., 1988), although rainy weather was reported to reduce the EC levels in water due to dilution effects (Welcomme, 1985).

The nutrients such as TN, TP, $NH_4$-N and $PO_4$-P varied significantly with sampling locations, and TN, $NO_3$-N, $NO_3$-N and $PO_4$-P varied significantly with seasons (Table 2.3).

Elevated levels of nutrients were observed in high urbanization areas as compared to others. Although some of the nutrients come from natural processes, the wastewater discharge, leakage of domestic sewage, agricultural runoff (fertilizers) and industrial wastes can significantly contribute to their increase in water bodies, leading to eutrophication and excessive algal growth in lakes and reservoirs (MPCA, 2008). High concentration of multiple nutrients observed in high urbanization locations could be correlated with the land use types. As shown in Figures 2.7 and S2.4 to S2.12, the dominant land use type in the high urbanization locations was high density residential land (36-57%), followed by commercial land (8-18%). The land use type coupled with high human activities, and release of domestic wastewater or sewage in to the canals might be one of the main reasons for high concentrations of nutrients observed in these locations. Particularly, location 1-2 had highest values on most occasions. Closed canal system with reduced water flow and direction surrounded by high density residential area could be the main reason for observing high amounts at nutrients in this location. In medium urbanized locations (2-1 to 2-3), the dominant land use types were research and education institutions as well as associated residential areas including dormitories (0-63%), industrial land (0-49%) and high density residential land (3-24%). In contrast, the land use types in low urbanization locations were river and lake (11-34%), agricultural land (0-20%), public green land (6-29%) and high density residential land (7-21%) followed by industrial land (3-14%). The main source of nutrients observed in these locations could be agricultural runoff followed by domestic wastewater and industrial wastes. A high number of transportation activities by ferries were also noticed in two of the low urbanization locations (3-1 and 3-3), which might have increased the water turbidity and mixing of ionic sediments with the water.

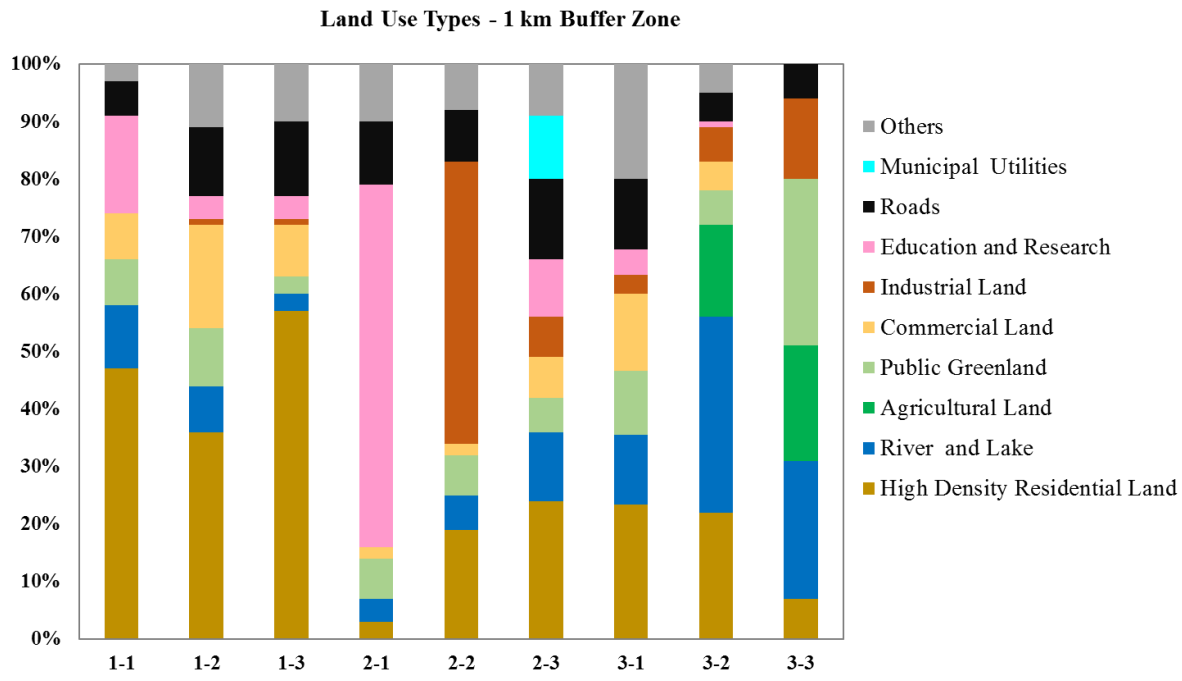



**Figure 2.7.** Land use types in high (1-1 to 1-3), medium (2-1 to 2-3) and low (3-1 to 3-3) urbanization locations. The land use type reported is for 1 km buffer zone.

An earlier study by Yu et al. reported that the surface water quality of Grand Canal was impaired by the high concentrations of nitrogen, phosphorus and various metals (Yu et al., 2012). The urban areas and also the agricultural areas were reported to be affected by the non-point source pollution. Li et al. reported that water quality deterioration in Shanghai, China was primarily due to nutrients such as ammonia nitrogen and low dissolved oxygen levels (Li et al., 2018). With continued urbanization, the wastewater and pollutants from household consumption had a huge influence on emission loads in Shanghai (Liu and Huang, 2014). Meanwhile, the land use and land cover (LULC) pattern generally has been found to have major impacts on the flow and water quality at multiple spatial scales (Zhou et al., 2012), and most of the water quality variables were correlated with landscape pattern (Eduful and Shively, 2015). The relationships between landscape and water quality varies significantly over space due to varying watershed

characteristics and pollution sources across space (Li et al., 2015). Chen et al. studied the spatial and temporal variations of water quality in four watersheds in Beijing and compared the results with LULC (Chen et al., 2016). Their study revealed that the water quality varied significantly among urban, ex-urban and non-urban sites, and they concluded that the relationship between LULC and water quality is very important in addressing the problem of non-point source pollution. The relationship between land use and lake / river water quality or impact on urban water quality has been the subject of other studies in China (Yang et al., 2007, Wu et al., 2008, Yu et al., 2013, Liu and Huang, 2014, Shen et al., 2014, Sun et al., 2016a, Li et al., 2018) and worldwide (Tu, 2011, Astaraie-Imani et al., 2012, Glinska-Lewczuk et al., 2016, Goel et al., 2018). The results and conclusions from the published studies strengthen the credibility of the water quality results obtained in this project. Among three urban intensifications (High, Medium and Low) in Suzhou, the contrasts between High vs. Medium and High vs. Low were observed for all the nutrient parameters measured. However, the significant difference between Medium vs. Low was observed for only a few nutrient parameters ($PO_4$-P), which might be related to the population density of each urban classification: High, Medium and Low were >8000, 1700-2100 and 800-1100 persons/$km^2$, respectively. The difference in the population density between Medium vs. Low is marginal as compared to High vs. Medium and High vs. Low.

The chlorophyll *a* concentration did not show significant variation between sampling locations but varied with seasons. The values were significantly higher during summer as compared to the winter and a positive correlation between chlorophyll *a* and water temperature, $NO_2$-N and $PO_4$-P was observed (Table 2.6). This result is consistent with previous studies which showed a positive relationship between chlorophyll *a* and temperature and nutrients, particularly with phosphate (Chen et al., 2003, Yu et al., 2012, Prasad et al., 2014). The chlorophyll *a*

concentration was extremely high in location 2-2 in summer 2016, which is correlated with high algal growth observed in this location during sample collection. High algal growth (bloom) is caused by eutrophication of water bodies, and the extremely high concentrations of TN and TP (6.54 mg/L, 0. 59 mg/L, respectively) recorded at location 2-2 in summer 2016 indicated serious eutrophication.

The TVC values varied significantly with sampling locations but not with seasons, and the same pattern was observed with total coliform count. The fecal coliform counts varied both with sampling locations and seasons. Since high density residential land was the dominant land use pattern in locations with high urbanization, higher population density coupled with human activities, and domestic wastewater / sewage undoubtedly contributed to high TVC, TC and FC values in high urbanization locations. While studying the impact of urban areas on the physico-chemical and microbiological characteristics in a lowland river in Poland (Glinska-Lewczuk et al., 2016) observed an increase in nutrient concentration and indicator bacteria (heterotrophic plate count and fecal coliform) in the urban areas. Santiaogo-Rodriguez et al. assessed the microbial quality of the tropical watershed in Puerto Rico with urbanization gradient, and reported that fecal indicators (*Enterococcus* spp., *Escherichia coli*, enterophages and coliphages) positively correlated with urbanization and rainfall events (Santiago-Rodriguez et al., 2016).

The correlation analysis among the variables revealed that water temperature had a strong positive correlation with $NO_2$-N and chlorophyll *a*, but had a strong negative correlation with conductivity and $NO_3$-N. It is consistent with previous reports as chlorophyll *a* is an indicator of algal growth, and most algal species have optimum growth rates at about 30 °C (Cassidy, 2011). High algal growth is usually favored in summer with warming environment (O'Reilly et al., 2015). Although photosynthesis generates oxygen, decomposition of algal biomass consumes

more oxygen when eutrophication of water bodies happens, and this decomposition reduces the dissolved oxygen levels in water environment and increase $NO_2$-N level in water samples. The pH had a moderate negative correlation with TVC and a strong negative correlation with total coliforms. By considering that the pH values in the sampling locations ranged from 7 to 8, the bacterial content particularly coliform growth was favored by the neutral pH environment rather than a weak alkaline environment. It is correct as coliform organisms prefer a near-neutral pH for their optimum growth, but they can still grow in pH ranges of 4.4–9.0 (Lee et al., 2009). Electrical conductivity values are used to estimate the amount of dissolved salts in water (Farrell-Poe, 2005), and a strong positive correlation between conductivity and TN, $NH_4$-N and $NO_3$-N indicate that $NH_4$-N and $NO_3$-N account for high percentages of the solutes in Suzhou canals. All the strong positive correlations between nutrient parameters observed in Suzhou canals indicate organic contamination, such as domestic sewage (Sakineh Lotfinasabasl, 2012). The land use type analysis showed that high density residential land predominated in most of the sampling locations, which pointed to the influence of domestic effluent as a potential source of contamination of the water system by organic matter and nutrients (Souza and Gastaldini, 2014). The strong positive correlations between nutrient parameters and TC and FC suggested bacterial contamination from domestic sewage (Nnane et al., 2011) in the canals.

## 2.5. Conclusions

In this study, physico-chemical and microbiological analyses were carried out to assess the water quality of canals in Suzhou with varying urban intensifications (High, Medium and Low) and results compared with control locations in Huangshan. Many of the parameters such as pH, EC, TN, TP, $PO_4$-P, $NH_4$-N, TVC, TC and FC varied with urban intensification; whereas water temperature, EC, TN, $PO_4$-P, $NO_3$-N, $NO_2$-N, Chl *a* and FC showed seasonal variations. Higher levels of nutrients (TN, TP, $NH_4$-N and $PO_4$-P) were usually observed in locations with high urban intensification as compared to medium and low urbanization. The nutrient values in samples from rural Huangshan were extremely low in the absence of any influence from urbanization and other anthropogenic activities as compared to the Suzhou canals. Land use types of locations with high urban intensification were mainly high density residential lands. Therefore, domestic wastewater or sewage were the main pollutants flowing into city canals, causing serious eutrophication.

TVC, TC and FC counts were higher in locations with high urbanization as compared to locations with low urbanization in Suzhou and Huangshan, which is correlated with physico-chemical parameters. Results of the correlation analysis revealed that nutrient pollution from domestic sewage in locations with high urban intensification could lead to microbial contaminations in Suzhou canals and health risks for the local residents living near the canals.

# Chapter 3 – Impact of Urbanization on Microbial Diversity and Ecosystem Function in Suzhou Canals

***Abstract***

The aim of this study was to assess the changes in microbial diversity and ecosystem function by sampling water and pre-deployed leaf samples across a gradient of urban intensification in Suzhou canals. Nine sampling locations covering three levels of urban intensification (High, Medium and Low) in Suzhou were selected for the study. Water and leaf samples were collected in winter and summer in 2015 and the study was repeated in 2016 along with control locations in Huangshan. The water and leaf associated bacterial and fungal diversity was studied by next-generation sequencing (NGS) of specific target genes (16S rRNA and ITS1). The organic matter (OM) breakdown rate (an index for ecosystem function) was assessed by ash-free dry weight (AFDW) measurements. The Operational Taxonomic Units (OTUs) and community analysis revealed obvious variations in the bacterial and fungal community between water and leaf samples and also seasonal variations. Variation in bacterial and fungal composition between samples collected from Suzhou and Huangshan was also observed with more bacterial and fungal OTUs in water and leaf samples collected from Huangshan as compared to samples from locations in Suzhou, which indicated the effect of urbanization on microbial diversity loss in freshwater ecosystems. However, the bacterial and fungal community among the sampling locations in Suzhou did not show much variation. In the bacterial community at phylum level, Proteobacteria was dominant (20-80%) in almost all the water and leaf samples and Burkholderiales was dominant (5-50%) in most samples at order level. Some bacterial genera (*Arcobacter*, *Massilia* and *Acinetobacter*), which are typically found in wastewater or associated with human/animal microbiomes were represented at high percentages in high and medium urban intensification locations in Suzhou. The fungal community analysis at phylum level showed that Ascomycota was dominant (2-99%) in most samples and Pleosporales

was dominant (1-99%) in most leaf samples at order level. Some of the fungal genera (e.g. *Trichothecium*) associated with pathogens / microbiomes and human health were represented at high percentages in high urban intensification areas, whereas natural fungal flora (e.g. *Alternaria*) associated with decomposition / ecosystem function were represented at high percentages in low urban intensification areas in Suzhou and natural reserve areas in Huangshan. The beta diversity (NMDS and cluster) analysis showed more obvious variations in bacterial and fungal community with respect to urbanization in Suzhou. The relationship between bacterial / fungal community and temperature / nutrient parameters was confirmed by Redundancy analysis or Canonical correspondence analysis (RDA / CCA) that nutrient parameters could affect bacterial / fungal composition. Ecosystem function (AFDW loss rate) was significantly affected by season rather than urbanization; positive correlation between AFDW loss rate and temperature was observed and high temperature accelerated AFDW loss rate. The results showed that microbial diversity and ecosystem function in Suzhou canals was influenced by both season and urbanization, but season had a greater influence than urbanization.

### 3.1. Introduction

Microbial communities are fundamental components of freshwater ecosystems and can indicate shifts in ecosystem structure (Paerl et al., 2003), as microbial communities play major roles in the function such as primary production and decomposition (Naeem et al., 2009, Kim et al., 2014). Linking the specific microbial populations to environmental processes is a key aspect to investigate the relationship between microbial biodiversity and ecosystem function (Morales and Holben, 2011), which is a fundamental research goal in microbial ecology (Bernhard and Kelly, 2016). Freshwater ecosystems with diversified species are more efficient in removing

nutrients than those with fewer species, so that conservation of biodiversity could be a useful tool for controlling nutrient levels in watersheds (Cardinale et al., 2012). Therefore, we need to not only consider how urbanization impacts water quality, but also how these changes can in turn affect the microbial diversity and the function of these ecological communities.

The research focused on freshwater resources has been placed on assessing physical and chemical variables, and also on the ecosystem responses to these environmental stressors (Huang et al., 2013a, Vogt et al., 2016). Many contaminants in waterways also had flow-on effects on freshwater biodiversity, and changed activity and diversity of the functional groups (Wang et al., 2011b, Zhang et al., 2015). Multiple ecosystem functions require diversified communities, and different ecosystem functions are influenced by significantly different sets of species (Hector and Bagchi, 2007).

There has been a push to assess the structure of freshwater communities and the function of these communities (Duarte et al., 2010, Delgado-Baquerizo et al., 2016). Biodiversity-ecosystem functioning (BEF) research has been successfully applied for trait-based approaches in ecology, and combining eco-physiological studies with bio-molecular techniques reinforced the ability to link microbial diversity to ecosystem processes (Krause et al., 2014, Meyer et al., 2015, Daam et al., 2019). One of the more regularly used measures for river ecosystem function is leaf breakdown rates (Clapcott et al., 2012, Collier et al., 2013, Thompson et al., 2016), which can be indicative of a number of important biological and ecosystem features. Therefore, leaf litter placed in bags (also known as leaf bag experiment) were deployed within the water systems and collected at different time intervals to measure the leaf litter decay rate to assess the ecosystem function (Young et al., 2008). Leaf litter decomposition in streams is processed by physical forces, chemical interactions, microorganisms and larger invertebrates (Hill and Perrotte, 1995).

Once the leaves enter into water, soluble organic and inorganic compounds leach with 1-2 days at the beginning. Following the initial leaching period, fungi and bacteria colonize the leaves for microbial conditioning. During this process, the leaves are broken down due to microbial activity and secretion of enzymes to degrade the structural materials of leaves such as cellulose and lignin (Suberkropp and Klug, 1976, Das et al., 2007). The microbial conditioning is followed by the colonization of macroinvertebrates on the decomposing leaf packs therefore fine mesh are used in the leaf bag experiments (focused on microbial diversity / activity) to avoid the macroinvertebrate colonization. Microbial conditioning is the key step for leaf decomposition process, which is the combined process for various microorganisms, including fungi and bacteria. Fungi are considered as the main decomposer of leaves by the release of extracellular enzymes (Kuehn et al., 2000, Findlay et al., 2002, Kim et al., 2014); whereas bacteria are known to participate in litter decomposition with less importance (Kominkova et al., 2000, Duarte et al., 2010), and they may use decomposition products released by fungi (Romani et al., 2006, Johnston et al., 2016, Soares et al., 2017). However, studying the leaf associated bacterial and fungal diversity and their role in the decomposition is a great interest to the microbial ecologists.

Freshwater fungi are a diverse group of organisms and fulfil important functions in the food web dynamics of surface water ecosystems, as they play a key role in the breakdown of organic matters such as leaves, which accounts for 99% of the total input energy of the surface water (Bärlocher and Kendrick, 1974). Therefore, the colonization of organic matter by aquatic fungi and other microorganisms represents an essential component in the food web of running water (Ittner et al., 2018). As an indicator of ecosystem function, leaf breakdown rates have been shown to respond to land-use change in varying ways (Young and Collier, 2009, Gardeström et al., 2016). Leaf microbial decomposition in streams subjected to stressors of multiple chemical

contaminations, including pesticides and pharmaceuticals and environmental parameters (i.e. temperature, nutrient levels) (Foulquier et al., 2015, Tant et al., 2015, Emilson et al., 2016, Rossi et al., 2019).

Previous research showed that microbial diversity, especially the richness and diversity of the fungal community, increased with urbanization in vernal pools of the Cuyahoga River watershed (USA) (Carrino-Kyker et al., 2011), and urban-influenced waterways in an urbanized estuary harboured significantly greater bacterial diversity than an oligotrophic lake (Newton and McLellan, 2015). Land-use pressures and eco-genomics reveal that chemicals / metals such as potassium, copper and aluminum are major drivers of microbial communities and their functions in the waterways of a megacity (Saxena et al., 2015). Shifts in compositional communities in urbanized streams indicated an increase in taxa associated with eutrophication and human activities (Hosen et al., 2017). Watershed urbanization not only altered the total composition of stream bacterial communities, but also changed composition of denitrifying bacterial communities, which may have effect on a critical ecosystem function such as denitrification (Wang et al., 2011b). A recent study reported that the dynamic of aquatic fungal communities in a heavily-contaminated tropical river was mainly driven by seasonality, geographical distance and physico-chemical parameters (e.g. pH, dissolved iron, dissolved oxygen, nitrate, biological oxygen demand, total aluminum, total organic carbon and total iron) (Ortiz-Vera et al., 2018). Iniguez-Armijos et al. reported that riparian land use changed from natural forest sites to urban sites with anthropogenically disturbed conditions, the fungal richness and biomass were found to be lower and leaf litter breakdown rates became slower (Iniguez-Armijos et al., 2016). Complex interactions between nutrients, xenobiotics and global change negatively affected the microbial communities associated with leaf decomposition in streams (Rossi et al., 2018) and the leaf

microbial decomposition rates were higher in urbanized watersheds than in forested watersheds. However, the study showed that pesticide toxicity lowered the fungal biomass in decomposed leaves in the urbanized watersheds (Rossi et al., 2019). It has been reported that urbanization had severe impacts on ecosystem function in Ampang River (Yule et al., 2015).

Freshwater microorganisms are important for recycling of nutrients, balance the trophic chains, physiological activities of plants and animals and conservation of natural habitats (Panizzon et al., 2015). Therefore, it is very important to study the microbial diversity and ecosystem function in the freshwater environments such as canals. The main scope of this research is to investigate the influence of urbanization on microbial diversity and ecosystem function in Suzhou canals. The specific objectives are 1) to assess the pattern of bacterial and fungal community in water and leaf samples across the urban intensifications (High, Medium and Low) in Suzhou canals and 2) to assess the organic matter (leaf litter) breakdown rate in Suzhou canals across three urban intensifications.

## 3.2. Materials and Methods

### 3.2.1. Field sampling methods

Water samples (5 L) were collected from each sampling location at each season as mentioned in Chapter 2. The water samples were collected in sterile containers and used for microbial community analysis.

Leaf litter breakdown was assessed by measuring the decay rate of leaf litter placed in bags deployed within the canals. Prior to the field experiments, willow (*Salix* sp.) leaves were collected from trees lining local canals. Leaves were dried in the laboratory, weighed approximately 5 g using a digital balance and the exact readings were noted down. The leaves

were then placed in ready-made nylon bags of 15 x 10 cm dimension with 0.5-1.0 mm mesh. Fine mesh was used to exclude macroinvertebrates from colonizing the leaves and increasing breakdown rate, but it was coarse enough to ensure adequate pore size for microbial colonization and flow through of water.

At each sampling site, 12 leaf bags were deployed at the beginning of each sampling occasion. Due to the importance of temperature on microbial activity and leaf breakdown rates, one waterproof temperature logger (HOBO® Pendant UA-001-08 Temperature/Alarm Data Logger, HOBO, USA) was placed at each sampling location to record water temperature every 2 hours over the 42-day period.

Fourteen days after initial deployment, 6 leaf bags were collected from each site. Three bags were used for analyzing the leaf litter breakdown rate, and three for microbial community analysis. This step was repeated on day 42. This process was done for winter and summer 2015 and 2016 to assess the seasonal dynamics in the microbial diversity and their ecosystem function. In summer 2016, sampling in Huangshan area, as a control group, was conducted to compare with high to low urbanization locations in Suzhou.

### 3.2.2. Sample processing and laboratory methods

#### 3.2.2.1. Microbial community analysis

*DNA extraction from water and leaf samples:*

Water samples (500 mL for each) were filtered through 0.22 μm polycarbonate membrane filters (Millipore, UK) in triplicate to collect microorganisms for DNA extraction. Membrane filters (water samples) and 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 membrane filters were cut into pieces and placed into the PowerBead tubes aseptically; the leaf samples (approximately 0.5 g) were grinded in liquid nitrogen. The extracted DNA was quantified using NanoDrop ND 2000C spectrophotometer (Thermo Scientific, US), verified by gel electrophoresis and stored at -20 °C until further processing.

*Bacterial community analysis:*

The bacterial community in water and leaf samples collected in winter and summer of 2015 and 2016 were studied by next generation sequencing (NGS) (Illumina MiSeqPE250). Primer sequences (515F 5'barcode GTGCCAGCMGCCGCGG and 806R GGACTACHVGGGTWTCTAAT) (Walters et al., 2016) which target V4 region of the 16S rRNA gene were used to study the bacterial diversity using MiSeq250 platform. Illumina sequencing applies clonal array assays for rapid and accurate large-scale sequencing, which is widely used for community studies (Xie et al., 2015, Wang et al., 2018, Wang et al., 2019).

The PCR reactions were performed in triplicate. The PCR mixture (20 µL) contained 4 µL of 5 × FastPfu buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase, 0.2 µL of BSA, 10 ng of template DNA and supplement of $ddH_2O$. The PCR cycling conditions used were: 95 °C for 3 mins, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 10 mins. The PCR amplified products were extracted and purified using AxyPrep DNA extraction kit and sequenced using Illumina MiSeq platform according to the standard protocols. The sequencing and the preliminary data analyses were carried by the Majorbio Pharmaceutical Technology Ltd, Shanghai, China. The sequences were analyzed using Quantitative Insights Into Microbial

Ecology (QIIME) software (Caporaso et al., 2012, Badapanda, 2017) and operational taxonomic units (OTUs) were clustered with 97% similarity cut-off. The phylogenetic affiliations (phylum, order and genus levels) were analyzed and cluster analyses were performed to compare samples collected at different urban intensifications and seasons.

*Fungal community analysis:*

The fungal community in water and leaf samples collected in winter and summer of 2015 and 2016 were studied by next generation sequencing (NGS) (Illumina MiSeqPE250). Primer sequences (ITS1F 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS2R 5'-GCTGCGTTCTTCATCGATGC-3') (Reazin et al., 2016, French et al., 2017) which target fungal Internal Transcribed Spacer 1 (ITS1) region of the rRNA gene were used to study the fungal diversity using MiSeq250 platform. The procedure used for of sequencing and preliminary data analyses were the same as mentioned for bacterial community analysis but Fungal High-throughput Taxonomic Identification tool for use with Next-Generation Sequencing (FHiTINGS) (Dannemiller et al., 2014, McTaggart et al., 2019) database was used for fungal community analysis.

The 16S rRNA genes and ITS1 sequences obtained to study the bacterial and fungal communities, respectively were submitted to the National Center for Biotechnological Information (NCBI) Short Read Archive (SRA) database under the accession numbers SAMN09907924 to SAMN09908036 and SAMN10688512 to SAMN10688590.

#### 3.2.2.2. Leaf litter breakdown

Leaf breakdown was analyzed from the triplicate leaf bags collected from each sampling location. Leaf breakdown rate is simply the percent of leaf dry weight loss over the period they were deployed. Leaf samples collected from the field were dried at 30 °C until the weight measurements were constant and then the values were compared with the initial weight. The leaves were then ashed at 550 °C for 6 hours (Duarte et al., 2010) and re-weighed to account for inorganic matter deposition on leaf surfaces and converted to ash free dry weight (AFDW).

### 3.2.3. Statistical analyses

Based on OTU analysis results (Venn diagrams), the bacterial and fungal diversity and richness within each sample (alpha diversity) was measured by diversity indices (Shannon, Simpson, abundance-based coverage estimator (ACE) and Chao1 richness estimator). Alpha diversity means species diversity in habitats or sites at a local scale, which was introduced by Whittaker (Whittaker, 1960, Whittaker, 1972); Shannon and Simpson indices are the simple mathematical equations used for calculating the alpha diversity (Sagar and Sharma, 2012). The variations in the diversity between samples (beta diversity) were studied by Non-Metric Multidimensional Scaling Analysis (NMDS) and cluster tree analysis. Beta diversity is the ratio between regional and local species diversity (Whittaker, 1960). The variations with seasons and urban intensifications on the composition of bacterial and fungal community in both water and leaf samples were researched by these analyses. The relationship between bacterial or fungal diversity and the environmental parameters (e.g. temperature and multiple nutrients) were studied by Redundancy analysis (RDA) or Canonical correspondence analysis (CCA) (Sheik et al., 2012, Huang et al., 2013a). RDA or CCA is an ordering method developed based on

correspondence analysis, combining correspondence with multivariate regression analysis, each calculation steps all regress with environmental factors, also called multivariate direct gradient analysis. All these analyses were carried out through the *R* statistics software package.

AFDW loss rate was analyzed statistically with "IBM SPSS Statistics" and "Graphpad Prism 5" software. Tukey two-way ANOVA was used to analyze variations within seasons (winter and summer) and urban intensifications (High, Medium and Low). Pearson's correlation analysis was used to study the correlation between AFDW loss rate (organic matter breakdown rate) and temperature that were measured using waterproof temperature loggers.

## 3.3. Results

### 3.3.1. Bacterial diversity in water and leaf samples

The bacterial community in water and leaf samples collected from different sampling locations in winter and summer of 2015 and 2016 were investigated by next-generation sequencing (NGS) of bacterial specific 16S rRNA gene amplicons. Seventy samples (36 water samples and 34 leaf samples from different urbanization gradients in Suzhou) collected in 2015 and 43 representative samples (15 water and 28 leaf samples from sampling locations in Suzhou and Huangshan) collected in 2016 were sequenced. In total 1,184,750 and 1,202,538 reads were obtained from water and leaf samples collected in 2015 and 2016, respectively. The sequencing data were normalized to 16925 reads per sample for samples collected in 2015 and 27966 for samples collected in 2016 and this was done to keep the lowest sequencing reads as cut-off for these two libraries, which was conducive to comparison of community profiles between sampling locations (H, M, L), sampling dates (0 day and 42 day - 2015), water and leaf samples and seasons (Winter and Summer). The rarefaction curves for samples collected in 2015 and

2016 are shown in Figure 3.1. High Good's coverage (0.959-0.997) (Tables 3.1, 3.2, S3.1 and S3.2) were obtained for all the water and leaf samples, which indicate that the sequencing depths for all the samples were sufficient. In general, the number of OTUs in water samples were high in summer as compared to winter and the same trend was observed with leaf samples in 2015 (Table 3.1). The same pattern was observed for sequences obtained from water and leaf samples collected in 2016 (Table 3.2). Although no major difference in number of OTUs observed between the sampling locations, the number of OTUs observed between water and leaf samples highly varied. The same pattern was observed with Shannon diversity and other alpha diversity indices; the values were higher in summer as compared to winter, particularly in leaf samples (Tables 3.1, 3.2, S3.1 and S3.2).

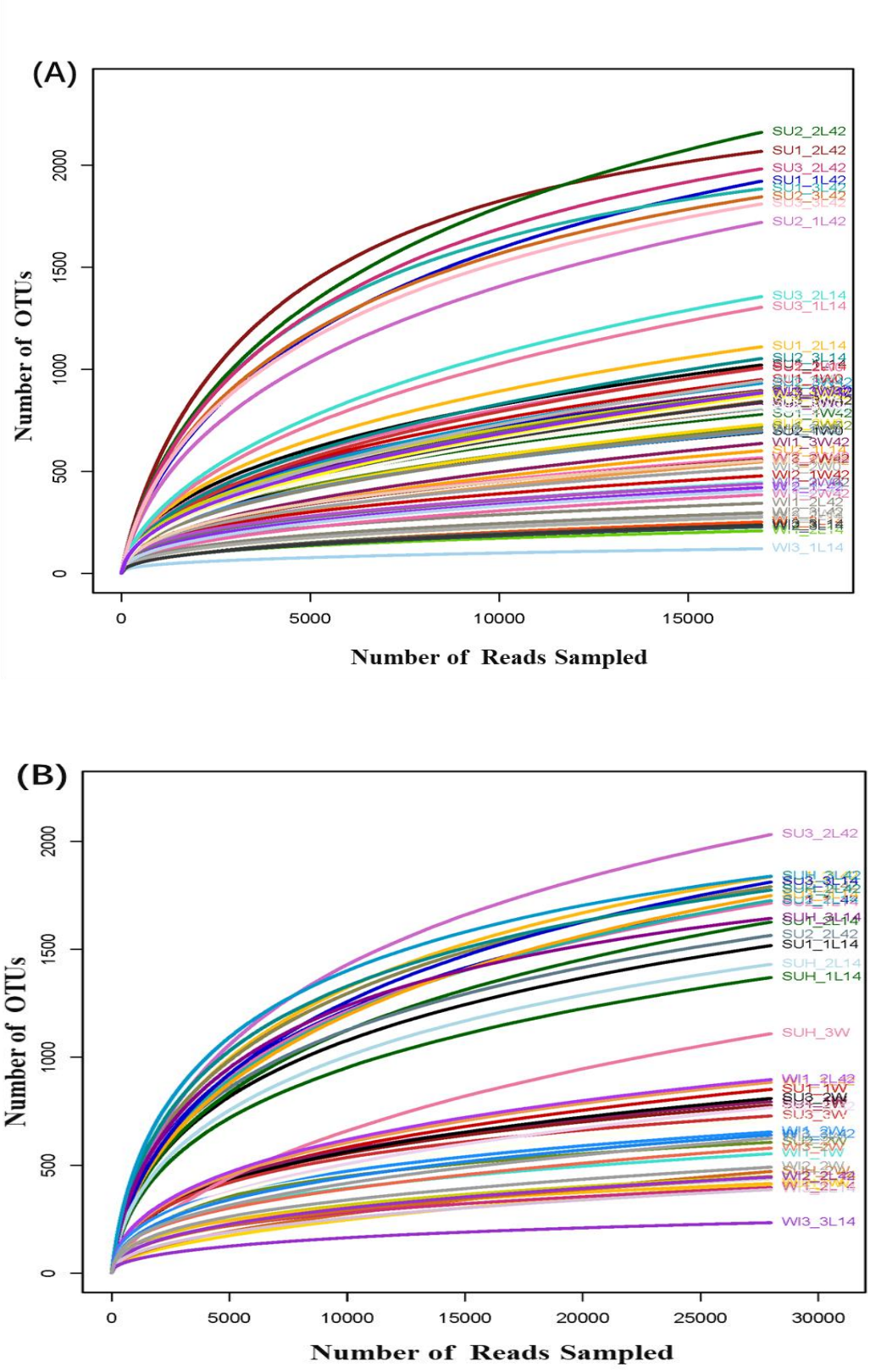



**Figure 3.1.** Rarefaction curves generated for bacterial OTUs in water and leaf samples collected in 2015 (A) and 2016 (B).

**Table 3.1.** Similarity-based OTUs and species richness and diversity estimates (Mean ± SD) for bacterial community in water and leaf samples collected from different urban gradients (High, Medium and Low) in winter and summer 2015. (The number of reads was normalized to 16925 per sample).

| Sample Group | OTUs | ACE | Chao1 | Shannon | Simpson | Coverage |
|---|---|---|---|---|---|---|
| Winter, Water, High urbanization | 614±264 | 1428±652 | 1120±506 | 3.95±0.63 | 0.059±0.032 | 0.983±0.008 |
| Winter, Water, Medium urbanization | 554±122 | 1174±275 | 901±217 | 4.35±0.21 | 0.033±0.013 | 0.987±0.004 |
| Winter, Water, Low urbanization | 752±168 | 1833±571 | 1364±391 | 4.58±0.16 | 0.027±0.007 | 0.980±0.006 |
| Summer, Water, High urbanization | 835±120 | 1648±251 | 1364±223 | 4.69±0.26 | 0.027±0.010 | 0.980±0.003 |
| Summer, Water, Medium urbanization | 812±92 | 1595±164 | 1283±104 | 4.64±0.21 | 0.032±0.014 | 0.980±0.002 |
| Summer, Water, Low urbanization | 841±41 | 1747±232 | 1416±162 | 4.49±0.28 | 0.040±0.022 | 0.978±0.002 |
| Winter, Leaf, High urbanization | 364±118 | 721±308 | 596±228 | 3.64±0.38 | 0.062±0.022 | 0.991±0.004 |
| Winter, Leaf, Medium urbanization | 305±94 | 589±200 | 493±147 | 3.46±0.40 | 0.071±0.023 | 0.993±0.002 |
| Winter, Leaf, Low urbanization | 310±149 | 572±258 | 494±242 | 3.26±0.71 | 0.102±0.058 | 0.993±0.003 |
| Summer, Leaf, High urbanization | 1484±528 | 2074±292 | 1898±460 | 5.69±0.83 | 0.016±0.012 | 0.974±0.005 |
| Summer, Leaf, Medium urbanization | 1398±599 | 2161±520 | 1960±610 | 5.23±1.21 | 0.044±0.069 | 0.971±0.009 |
| Summer, Leaf, Low urbanization | 1613±335 | 2300±192 | 2138±204 | 5.77±0.67 | 0.014±0.010 | 0.968±0.001 |

**Table 3.2.** Similarity-based OTUs and species richness and diversity estimates (Mean ± SD) for bacterial community in water and leaf samples collected from different urban gradients (High, Medium and Low) in winter and summer 2016. The alpha diversity indices for bacterial community in water and leaf samples in Huangshan in summer 2016 is also included. (The number of reads was normalized to 27966 per sample.)

| Sample Group | OTUs | ACE | Chao1 | Shannon | Simpson | Coverage |
|---|---|---|---|---|---|---|
| Winter, Water, High urbanization | 604±71 | 973±40 | 899±3 | 4.49±0.23 | 0.026±0.007 | 0.993±0.000 |
| Winter, Water, Medium urbanization | 453±54 | 798±165 | 679±106 | 3.76±0.18 | 0.065±0.009 | 0.995±0.001 |
| Winter, Water, Low urbanization | 602±33 | 1197±24 | 1006±76 | 4.20±0.21 | 0.043±0.021 | 0.992±0.001 |
| **S**ummer, Water, High urbanization | 815±52 | 1226±264 | 1141±135 | 4.63±0.13 | 0.035±0.015 | 0.990±0.002 |
| Summer, Water, Medium urbanization | 700±133 | 924±212 | 938±192 | 4.38±0.28 | 0.039±0.012 | 0.993±0.002 |
| Summer, Water and Low urbanization | 769±57 | 1022±135 | 1052±124 | 4.63±0.06 | 0.028±0.005 | 0.992±0.002 |
| Summer, Water, Huangshan | 664±387 | 1564±625 | 1182±569 | 3.17±0.25 | 0.122±0.010 | 0.989±0.006 |
| Winter, Leaf, High urbanization | 656±271 | 991±407 | 926±350 | 4.37±0.55 | 0.032±0.015 | 0.992±0.003 |
| Water, Leaf Medium urbanization | 524±162 | 1003±317 | 819±226 | 3.85±0.31 | 0.051±0.010 | 0.993±0.002 |
| Winter, Leaf Low urbanization | 492±220 | 731±298 | 703±298 | 3.73±0.73 | 0.060±0.035 | 0.994±0.003 |
| Summer, Leaf, High urbanization | 1675±136 | 2152±122 | 2178±104 | 5.78±0.19 | 0.011±0.003 | 0.982±0.001 |
| Summer, Leaf, Medium urbanization | 1698±96 | 2175±117 | 2219±93 | 5.86±0.22 | 0.012±0.009 | 0.982±0.001 |
| Summer, Leaf, Low urbanization | 1863±149 | 2425±176 | 2457±261 | 5.79±0.09 | 0.014±0.004 | 0.979±0.001 |
| Summer, Leaf, Huangshan | 1611±206 | 1970±136 | 1990±141 | 5.82±0.52 | 0.015±0.015 | 0.986±0.001 |

### 3.3.2. Bacterial community composition in water and leaf samples

The bacterial specific 16S rRNA gene sequences obtained from water and leaf samples collected from different sampling locations in winter and summer 2015 and 2016 were analysed at phylum, order and genus levels. In general, the bacterial community in water samples was quite different from the community in leaf samples. In water samples, the phylum Proteobacteria were dominant in both winter and summer 2015 and 2016 and followed by either Bacteriodetes in winter or Actinobacteria during summer. A significant proportion of Cyanobacteria were also observed in the water samples collected during winter 2015 and summer 2016. Although the differences in the bacterial community in water samples across the sampling locations were less obvious, marked difference in the composition was observed at one of the high urbanization locations (1-2) during winter 2015 and all three control locations (Huangshan) during summer 2016. In leaf samples, the Proteobacteria were dominant regardless of the locations and seasons followed by dominance of Bacteroidetes during winter and Firmicutes and / or Chloroflexi during summer. Notably, no marked difference in the community in leaf samples collected across the sampling locations (including control locations) were observed (Figures 3.2. and 3.5).

At order level, high variations in the bacterial composition between water and leaf samples and also between seasons were observed. In water samples collected in winter (2015), the samples were co-dominated by Burkholderiales, Flavobacteriales, Pseudomonadales, Frankiales whereas in the water samples collected in the same year in summer were dominated by Burholderiales and Frankiales followed by Rhodocyclales and Sphingobacteriales (Figure 3.3). Interestingly, the community composition in the water samples collected from location 1-2 (one of the high urbanization locations) in winter 2015 and in control locations (H-1 to H-3) in summer 2016 were highly different from the community observed in other locations. Similarly,

the community observed in the leaf samples in both seasons and year were different from the water samples. The leaf samples were dominated by Burkholderiales, Flavobacteriales and Pseudomonadales during winter 2015; In summer, Pseudomonadales dominated in the leaf samples from some locations, but in other locations, no clear pattern was observed, as none of the above orders were dominant. The pattern was similar for the samples collected in 2016 (Figure 3.6).

At genus level, differences in the bacterial composition between winter and summer samples and also between water and leaf samples were observed. *Flavobacterium*, *Pseudomonas* and members of Comamonadaceae were represented at high percentages in water and leaf samples in winter (3-47%; 0.3-26%) but they were low in summer 2015 (0-11%; < 3%) (Figure 3.4 and Tables S3.5-3.8). *Flavobacterium* was dominant (3-47%) in most water and leaf samples in winter 2015. The genera *Malikia*, *Arcobacter* and *Polynucleobacter* were represented at higher percentages in water samples (0.2-16%; 0.1-50%; 0.5-5.8%) than in leaf samples (< 1%; < 1.2%; < 0.1%), whereas *Acinetobacter* was represented at higher percentage (0.1-49%) in leaf samples than in water samples (< 2.4%). Some bacterial genera (*Arcobacter*, *Massilia* and *Acinetobacter*) were represented at extremely high percentages in some samples collected in locations with high or medium urban intensifications: *Arcobacter* was represented at 41-50% in water samples collected from 1-2 (high urban intensification) in winter 2015; *Massilia* was represented at 31% in leaf samples collected from location 2-1 (medium urban intensification) on 14$^{th}$ day in winter 2015; *Acinetobacter* was represented at 49% in leaf samples collected from 2-1 on 14$^{th}$ day in summer 2015. Although the abundance in the bacterial genera varied with the samples collected in winter and summer 2016, similar pattern was observed (Figure 3.7 and Tables S3.9-S3.12).

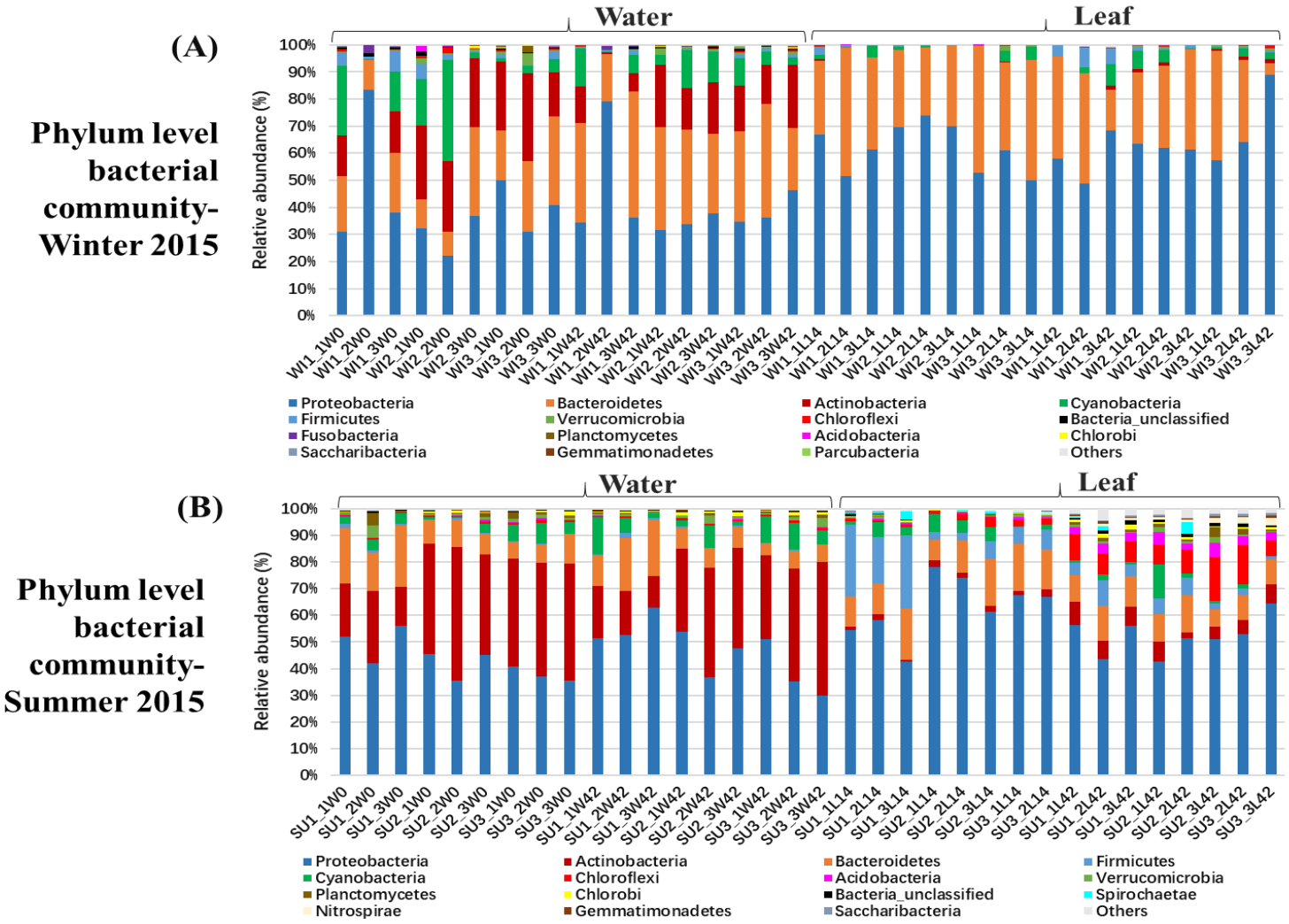



**Figure 3.2.** Relative abundance of bacterial phyla in water and leaf samples collected from different locations in winter (A) and summer (B) 2015. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; W0 and W42=Water samples collected on day 0 and 42, respectively; L14 and L42=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively.)

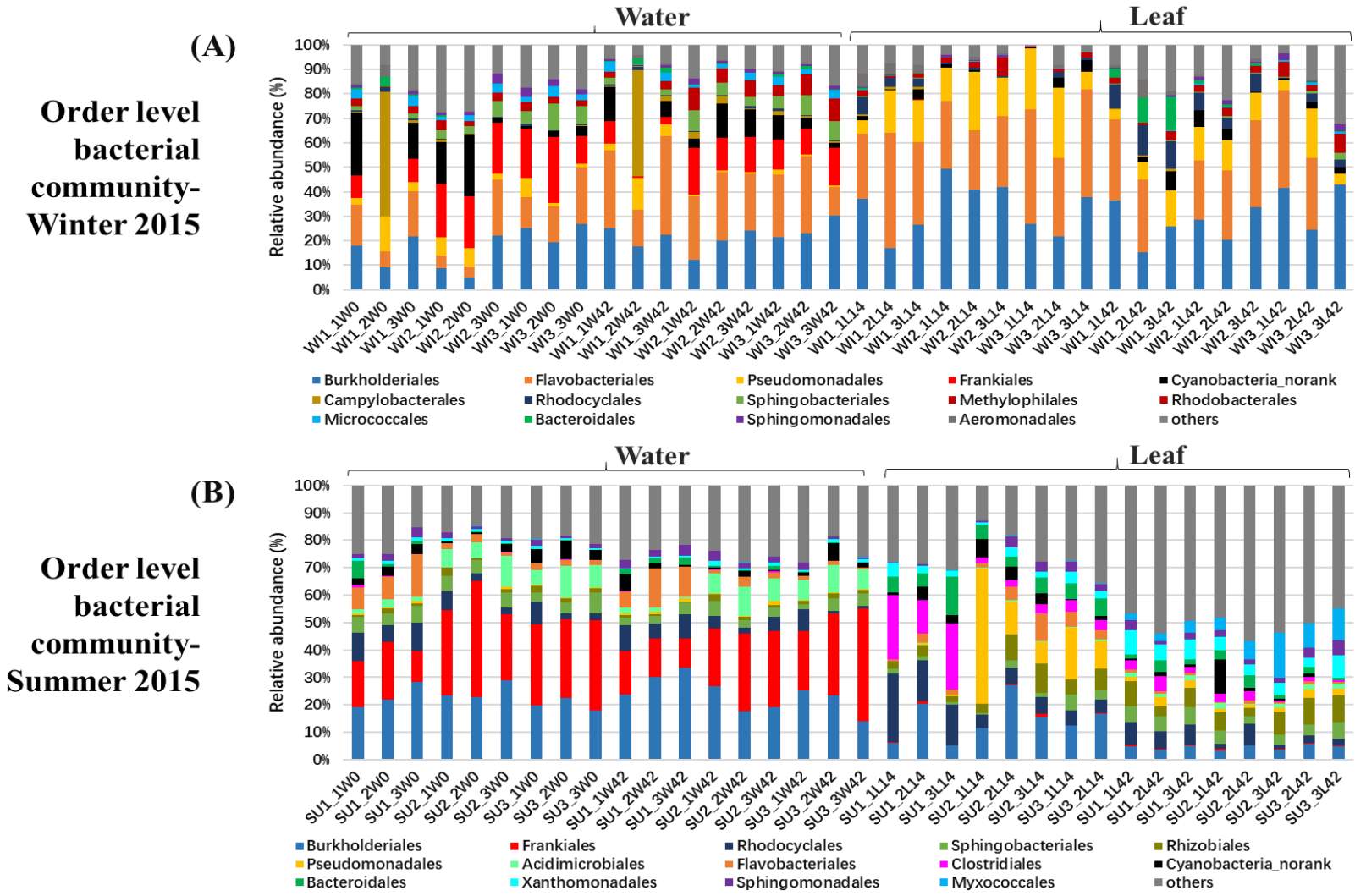



**Figure 3.3.** Relative abundance of bacterial orders in water and leaf samples collected from different locations in winter (A) and summer (B) 2015. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; W0 and W42=Water samples collected on day 0 and 42, respectively; L14 and L42=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively.)

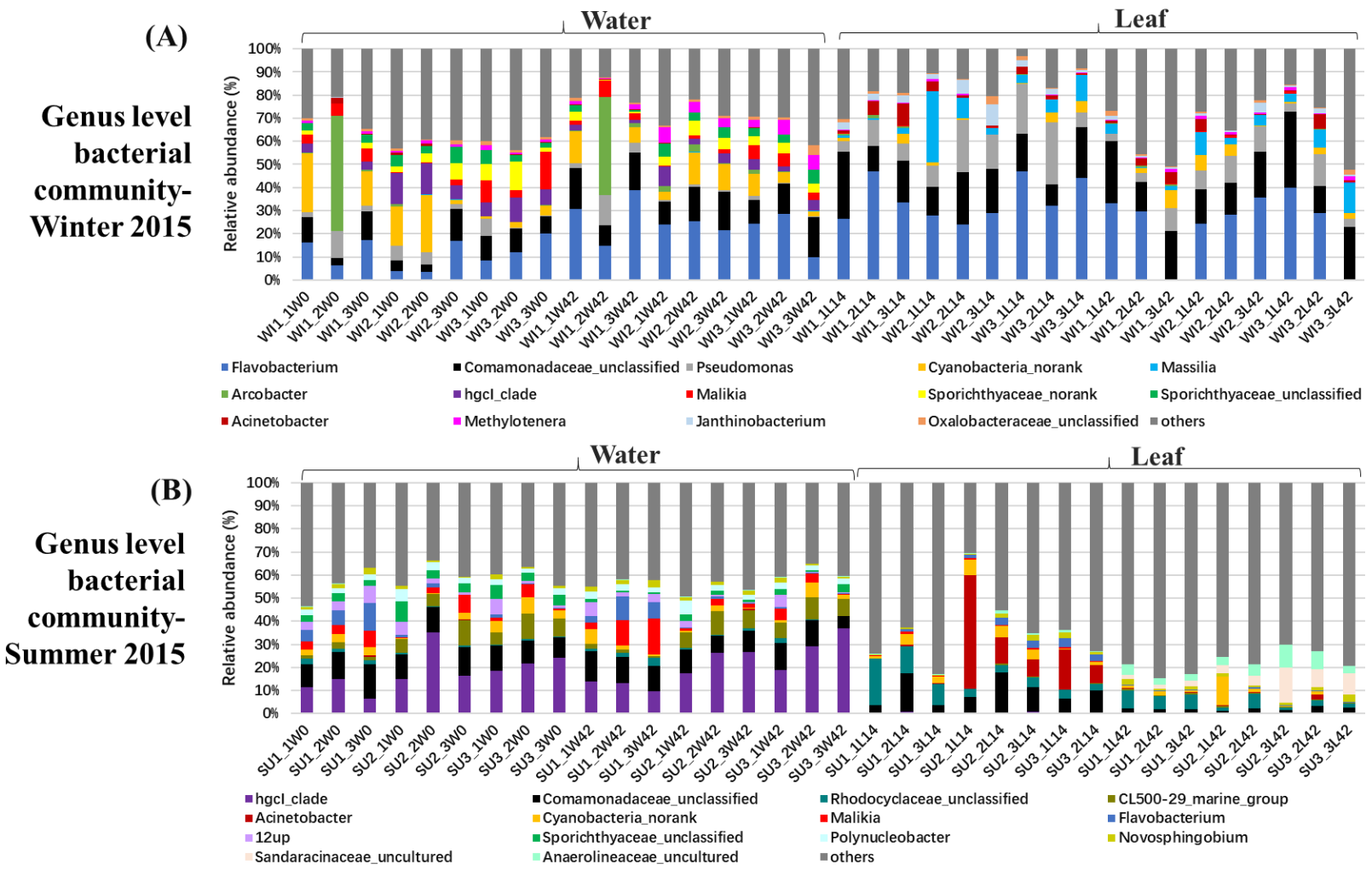



**Figure 3.4.** Relative abundance of bacterial genera in water and leaf samples collected from different locations in winter (A) and summer (B) 2015. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; W0 and W42=Water samples collected on day 0 and 42, respectively; L14 and L42=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively.)

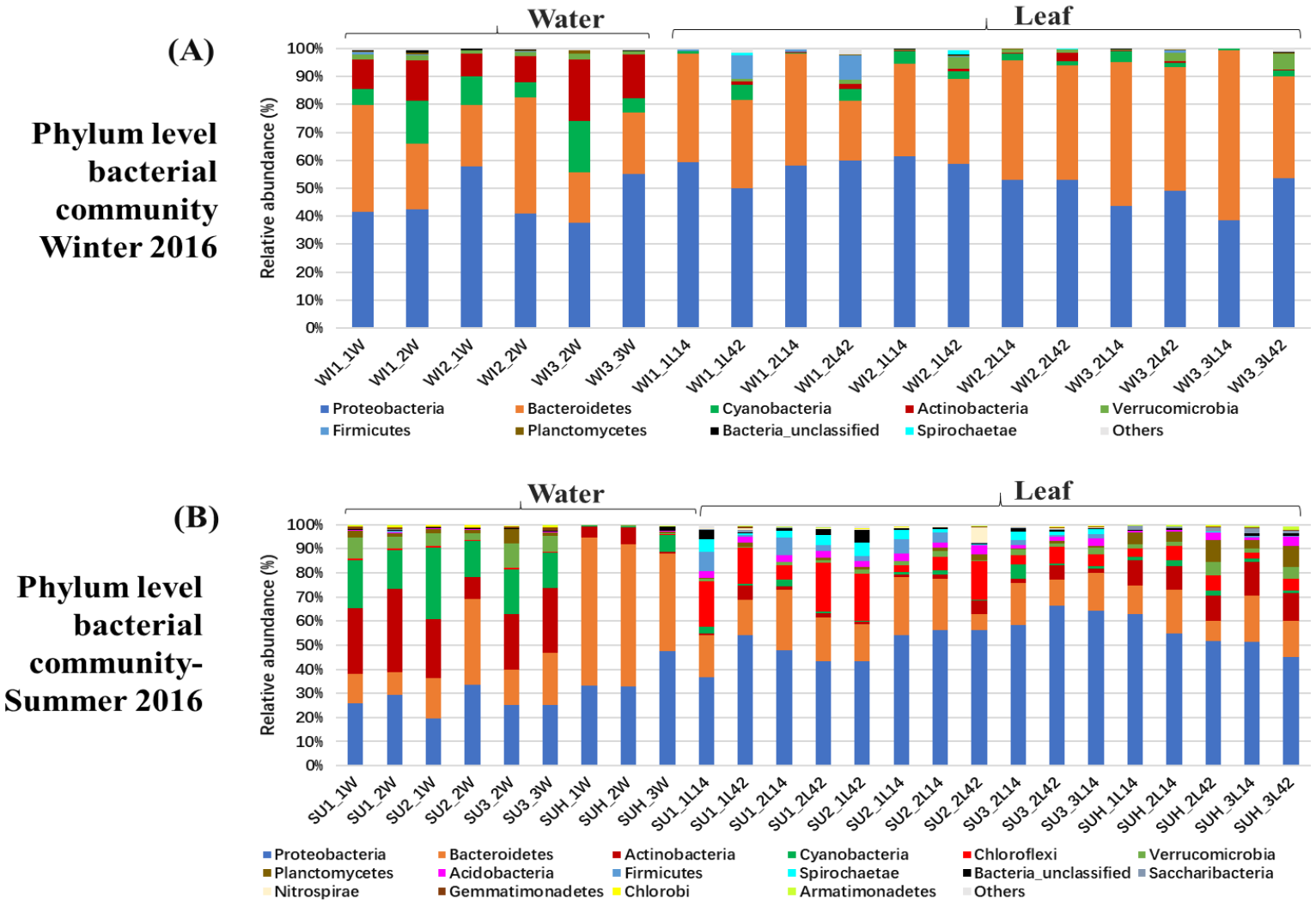



**Figure 3.5.** Relative abundance of bacterial phyla in water and leaf samples collected from different locations in winter (A) and summer (B) 2016. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; H-1 to H-3=Control locations in Huangshan; L14 and L42=Leaf samples collected on $14^{th}$ and $42^{nd}$ day, respectively.)

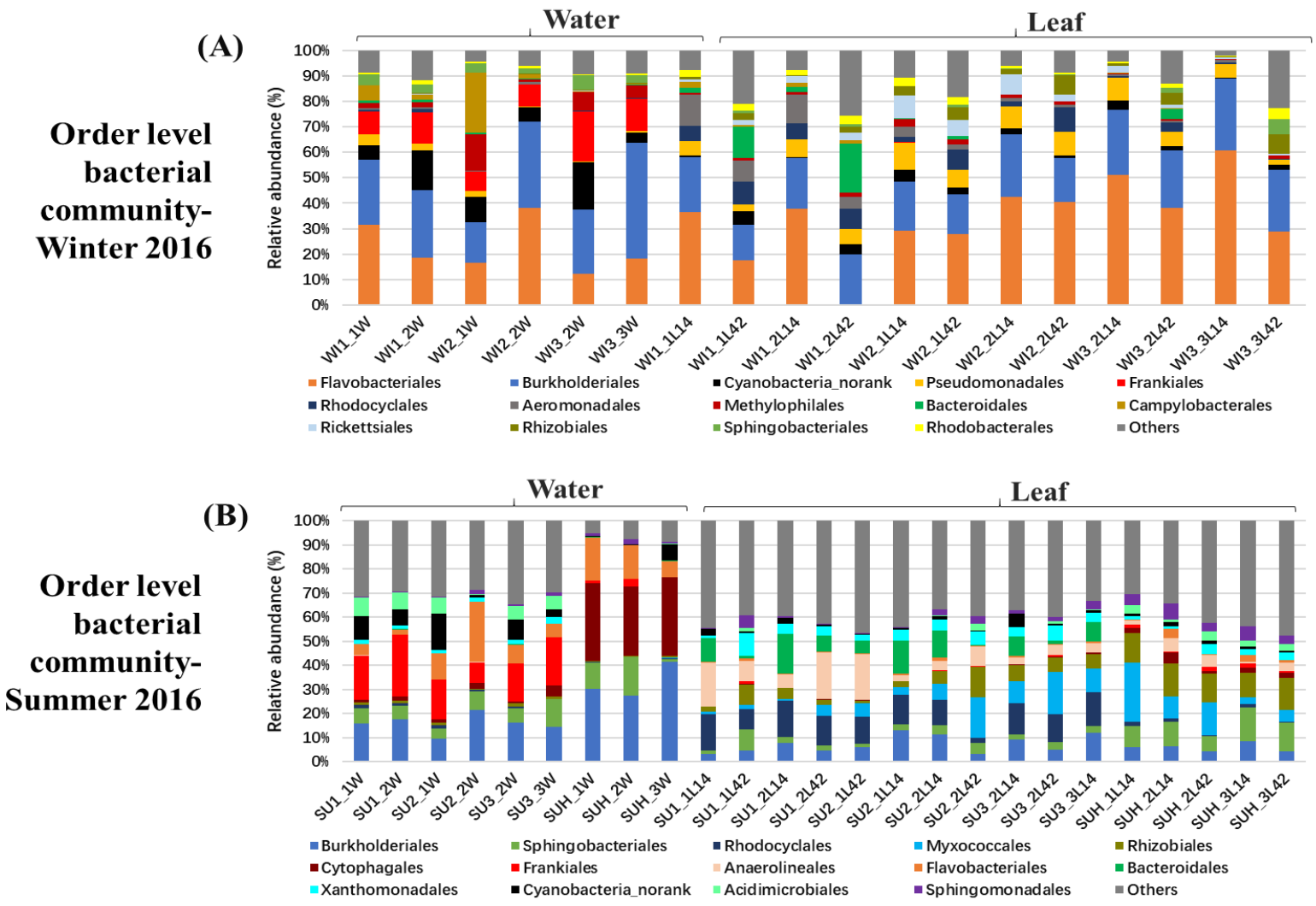



**Figure 3.6.** Relative abundance of bacterial orders in water and leaf samples collected from different locations in winter (A) and summer (B) 2016. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; H-1 to H-3=Control locations in Huangshan; L14 and L42=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively.)

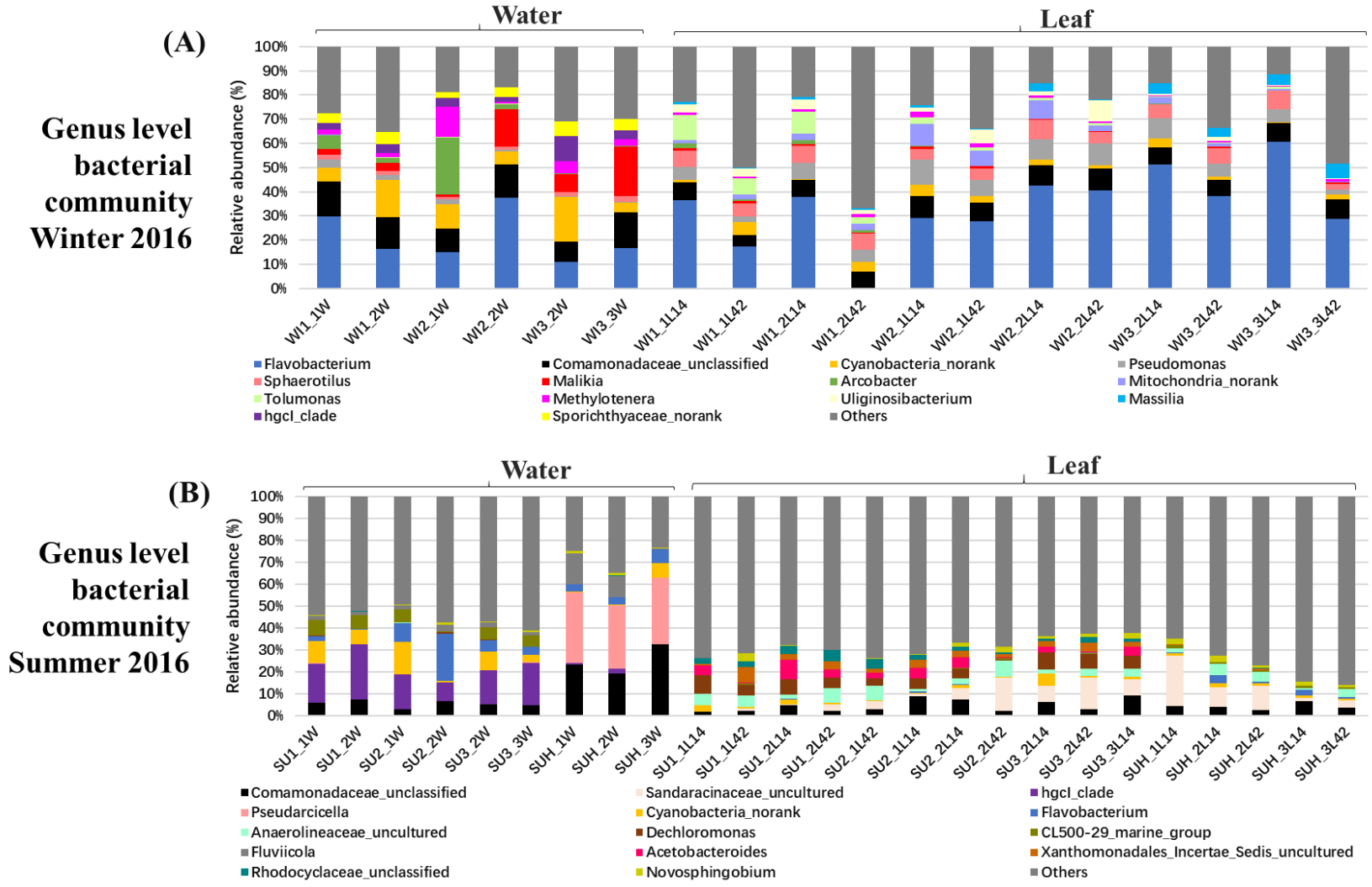



**Figure 3.7.** Relative abundance of bacterial genera in water and leaf samples collected from different locations in winter (A) and summer (B) 2016. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; H-1 to H-3=Control locations in Huangshan; L14 and L42=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively.)

### 3.3.3. Comparison of bacterial community in water and leaf samples collected from different sampling locations

*Venn diagram analysis:*

Venn diagram analysis was performed to find out the shared and unique OTUs present in water and leaf samples collected from different sampling locations in different seasons. When the water (W) and leaf (L) samples collected in winter (WI) and summer (SU) were analysed, the results showed that the number of unique OTUs were more in leaf samples (46-50%) as compared to water samples (8–16%) and the shared OTUs were in the range of 38-42% (Figures 3.8 A and C). The comparison of OTUs between summer and winter samples revealed that the unique OTUs in summer samples were extremely high (57-59%) as compared to winter (3-7%) and the shared OTUs were 37-38% (Figures 3.8 B and D).

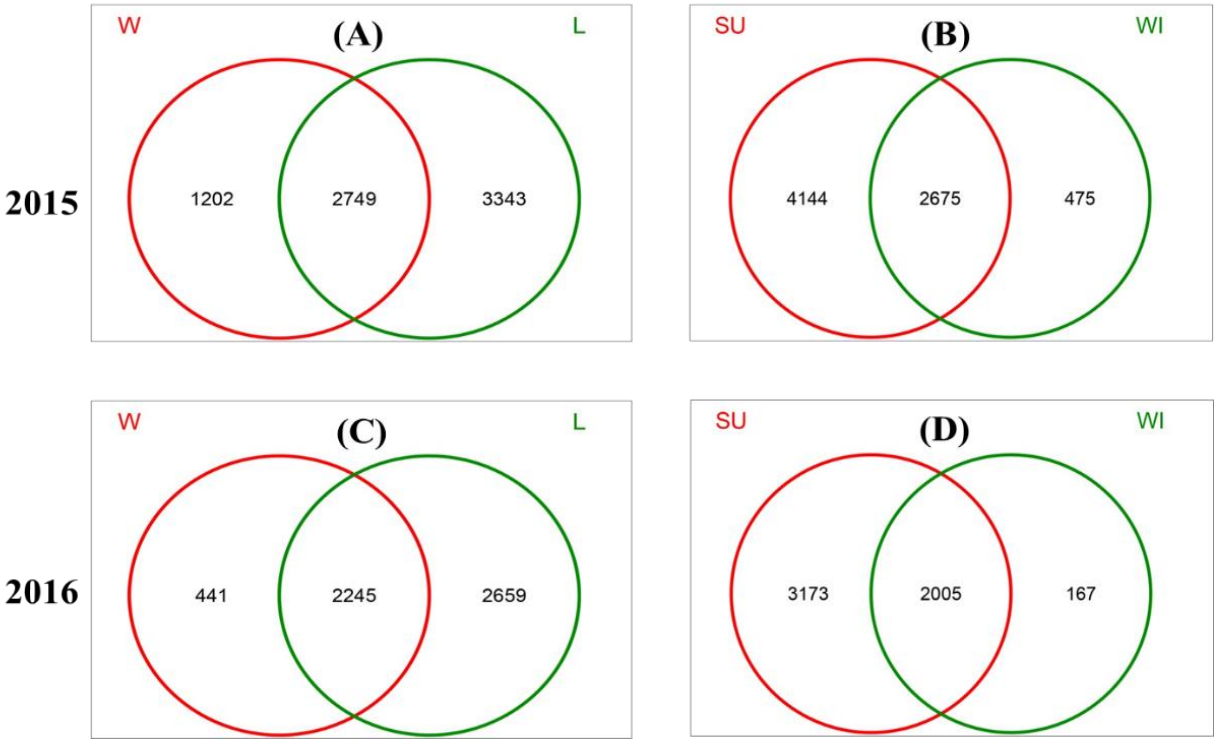



**Figure 3.8.** Venn diagram showing unique and shared bacterial OTUs when the sequences were compared between water and leaf samples (A, C) and between winter and summer (B, D) 2015 and 2016. (W=Water; L= Leaf; SU=Summer and WI=Winter.)

Several comparisons were made to find out the unique and shared OTUs in the sequences obtained from water and leaf samples from different sampling locations (high, medium and low urbanization). In the samples collected in 2015 (Figure 3.9), no obvious pattern with regards to urbanization was found. More unique OTUs were higher in high urbanization locations (WIL1 and SUL1) as compared to medium (WIL2 and SUL2) and low (WIL3 and SUL3) urbanization locations for leaf samples, but it was opposite for water samples. Similar comparisons were made for the samples collected in 2016, and the results were compared with the water and leaf samples collected from control locations (Huangshan, SUW4_C and SUL4_C). The analysis revealed that the number of unique OTUs were extremely high in the control locations as compared to all others (Figure 3.10).

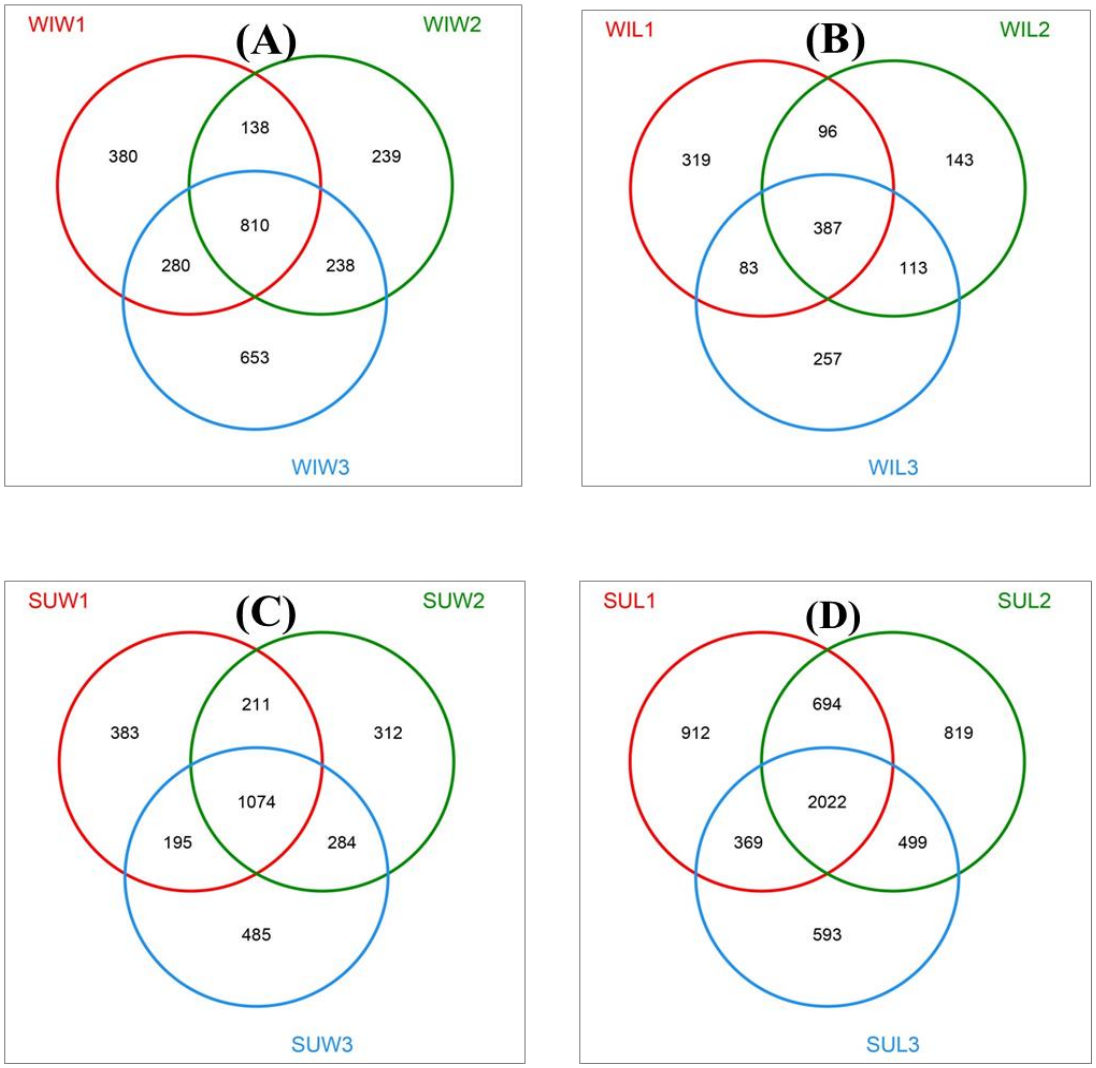



**Figure 3.9.** Venn diagram showing unique and shared bacterial OTUs in the sequences compared between water and leaf samples collected from different sampling locations in winter (A, B) and summer (C, D) 2015. (WIW=Winter Water; WIL=Winter Leaf; SUW=Summer Water; SUL=Summer Leaf; 1,2,3= High, Medium and Low urbanization locations, respectively.)

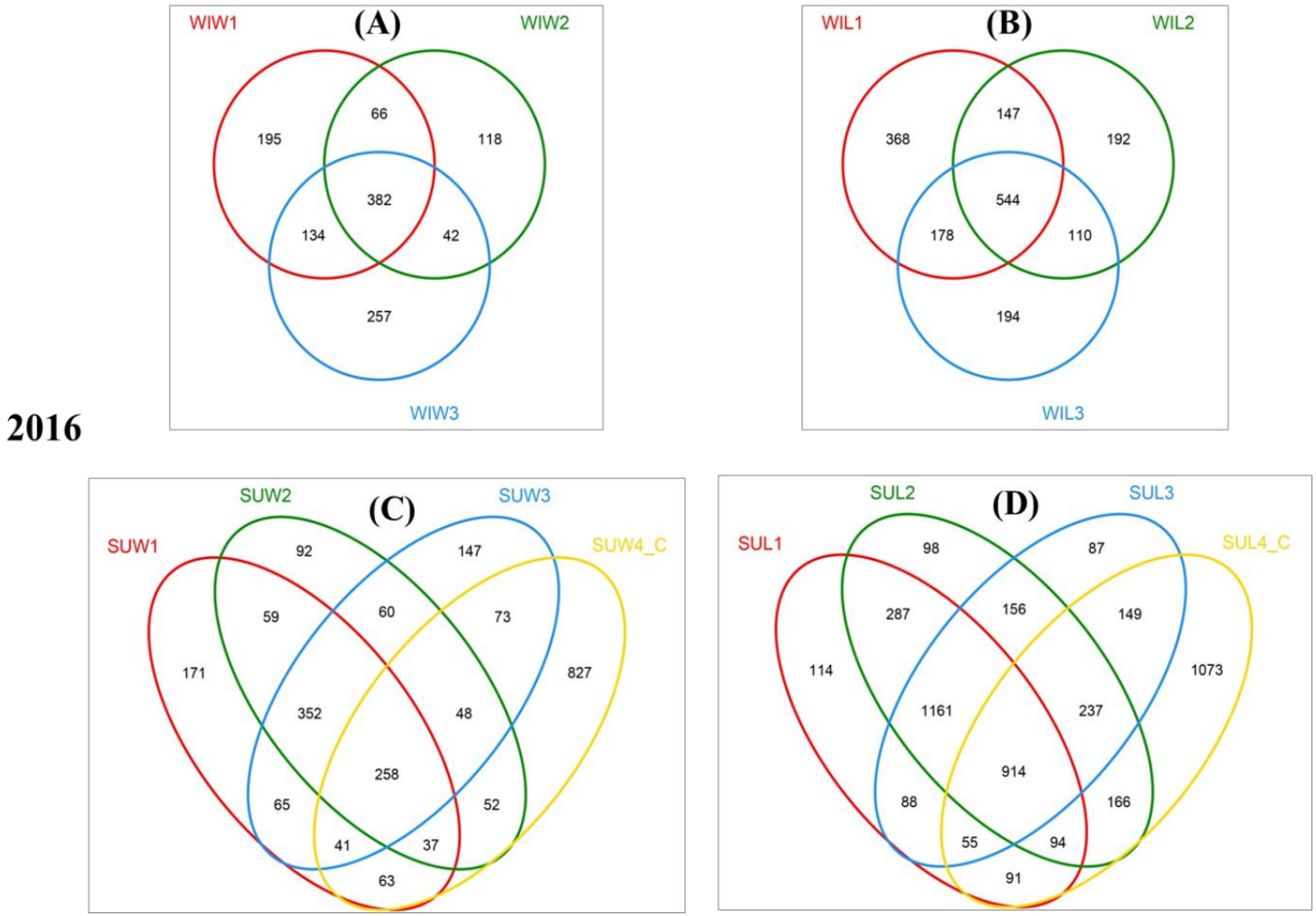



**Figure 3.10.** Venn diagram showing unique and shared bacterial OTUs in the sequences compared between water and leaf samples collected from different sampling locations in winter (A, B) and summer (C, D) 2016. (WIW=Winter Water; WIL=Winter Leaf; SUW=Summer Water; SUL=Summer Leaf; 1,2,3=High, Medium and Low urbanization locations, respectively; SUW4_C and SUL4_C=Water and Leaf samples collected in control locations in Huangshan.)

*Beta diversity analysis (Cluster and NMDS analyses):*

Cluster analysis was used to find out the similarities between the bacterial community in water and leaf samples collected from different sampling locations across the urbanization gradients. Although distinct clusters with respect to sampling locations were not observed, in water and leaf samples collected in winter and summer 2015 (Figure 3.11), most of the samples (water or leaf samples) collected from the highly urbanized locations (1-1 to 1-3) formed a separate cluster. Only in winter 2015, the samples collected from the location 1-2 formed a distinct cluster (Figure 3.11A). Similarly, most of the water or leaf samples collected from low urbanization locations (3-1 to 3-3) formed a separate cluster. The pattern observed for the samples collected from winter and summer 2016 (Figure 3.12) was the same. However, interestingly, the water and leaf samples from the control locations (H-1 to H-3) formed distinct clusters, which clearly indicate that the bacterial community was entirely different in those locations as compared to high to low urbanization locations (Figures 3.12 C and D).

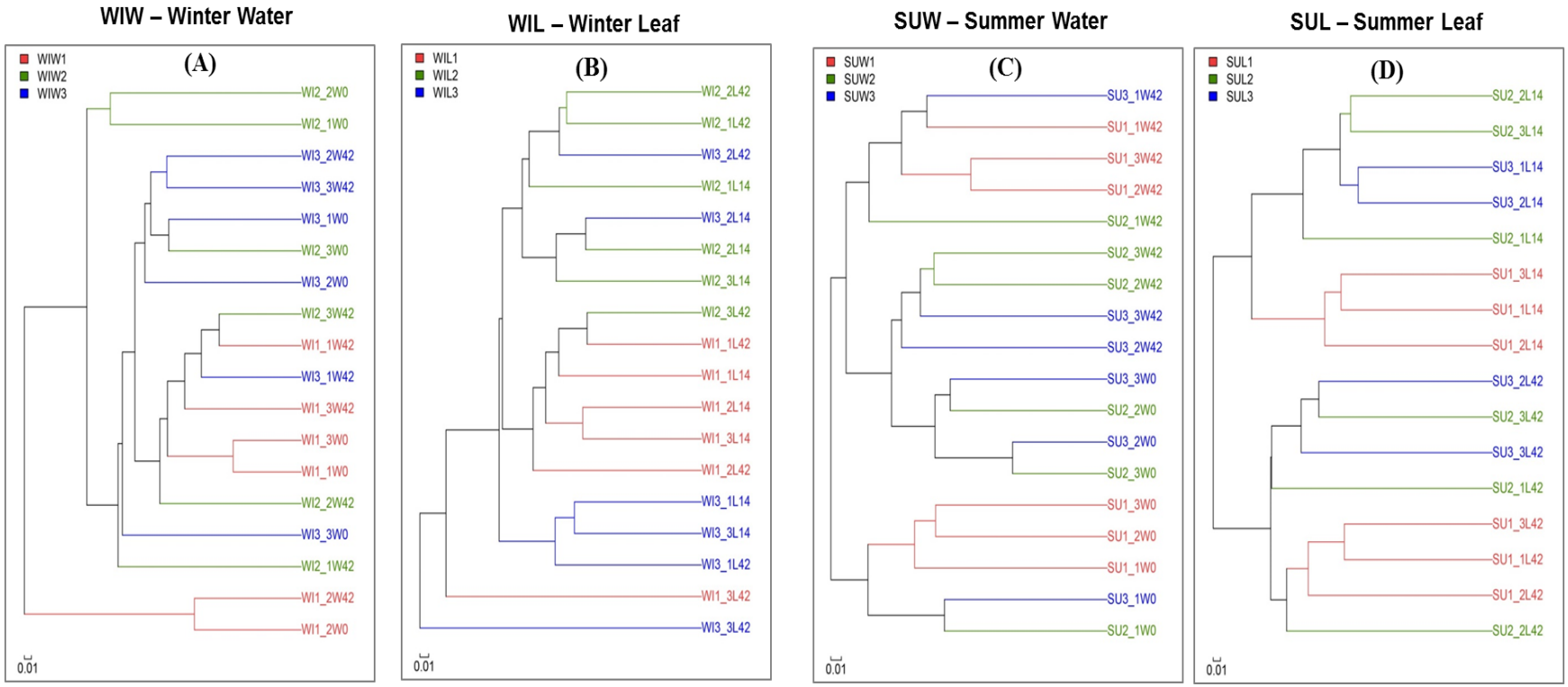



**Figure 3.11.** Cluster analysis showing the similarity in bacterial diversity in water and leaf samples collected from sampling locations across urbanization gradient in winter and summer 2015. Comparison of bacterial diversity in water (A, C) and leaf (B, D) samples collected from different locations across urbanization gradients in winter (A, B) and summer (C, D) in 2015. (WIW=Winter Water; WIL=Winter Leaf; SUW=Summer Water; SUL=Summer Leaf; 1,2,3=High, Medium and Low urbanization locations, respectively.)

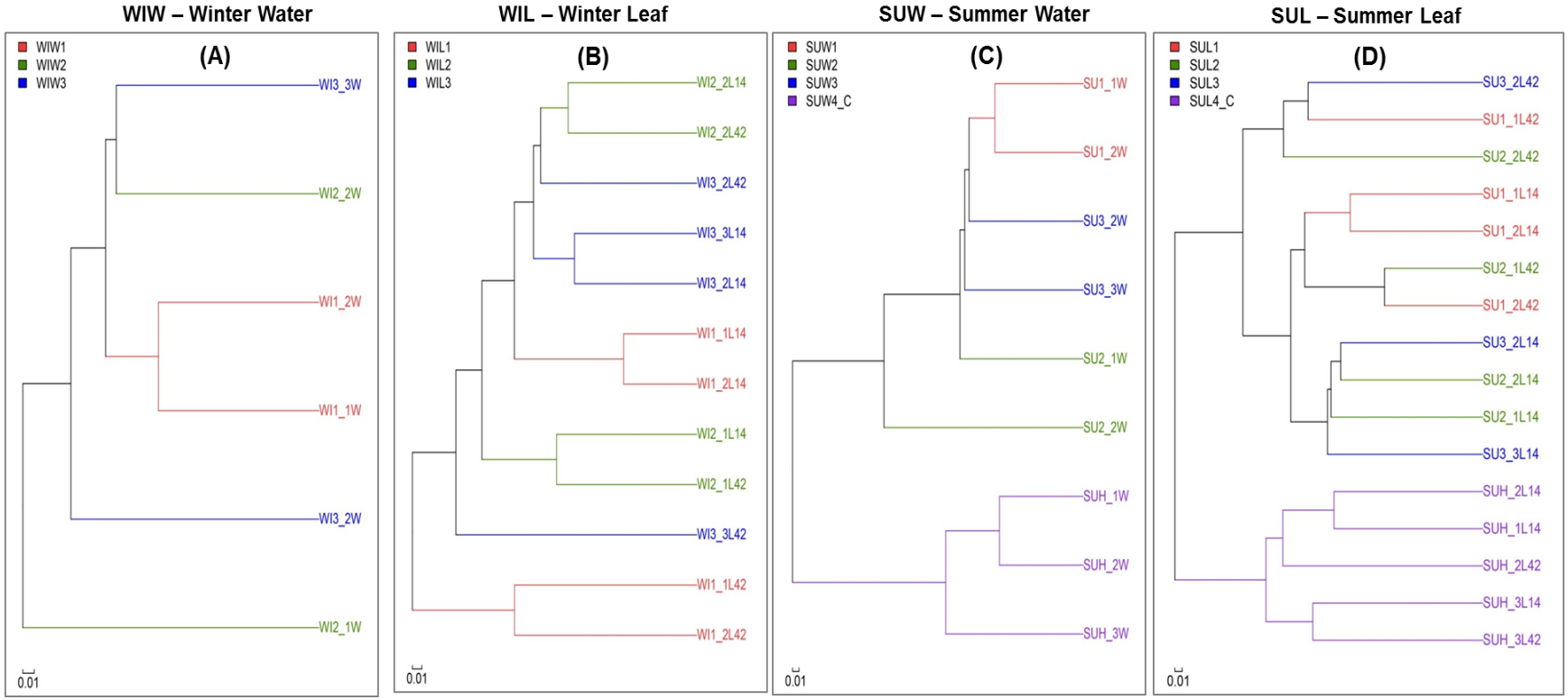
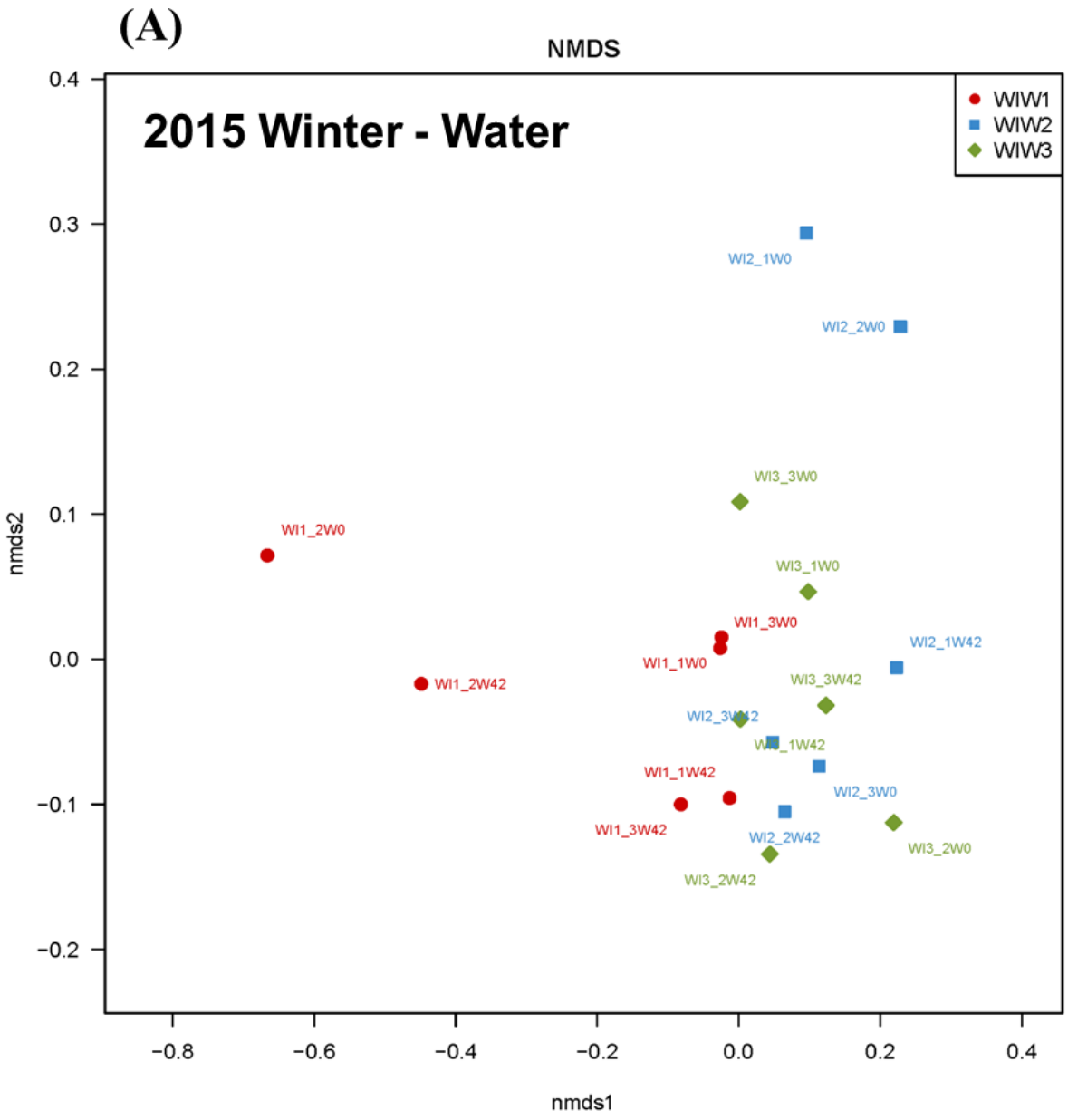


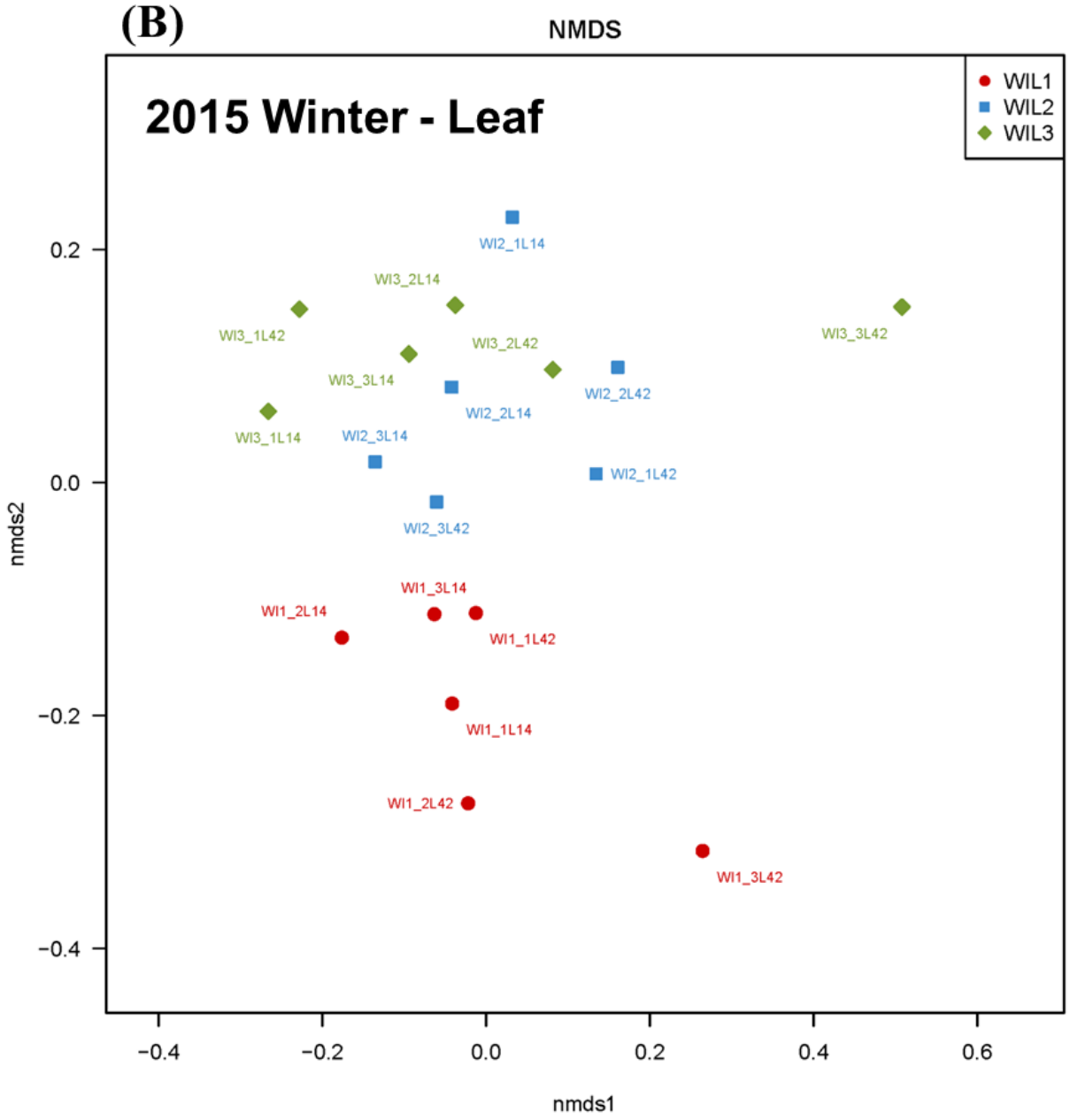

**Figure 3.12.** Cluster analysis showing the similarity in bacterial diversity in water and leaf samples collected from sampling locations across urbanization gradient in winter and summer 2016. Comparison of bacterial diversity in water (A, C) and leaf (B, D) samples collected from different locations across urbanization gradients in winter (A, B) and summer (C, D) in 2016. (WIW=Winter Water; WIL=Winter Leaf; SUW=Summer Water; SUL=Summer Leaf; 1,2,3=High, Medium and Low urbanization locations, respectively; SUW4_C and SUL4_C=Water and Leaf samples collected from control locations in Huangshan.)

The NMDS analysis showed that the diversity in water samples collected from some of the high (1-2 and 1-3) and medium urbanization (2-1 and 2-2) in winter 2015 were different from water samples collected from other locations (Figure 3.13A). In leaf samples collected in winter 2015, clear pattern was observed. The samples collected from high urbanization locations clustered together as compared to the medium and low urbanization locations (Figure 3.13B). In the water samples collected in summer 2015, the samples from high urbanization locations formed two distinct clusters which were based on dates (0 and 42$^{nd}$ day) when the samples were collected (Figure 3.13C). The same pattern was observed with the leaf samples collected in summer (14$^{th}$ and 42$^{nd}$ day) (Figure 3.13D).

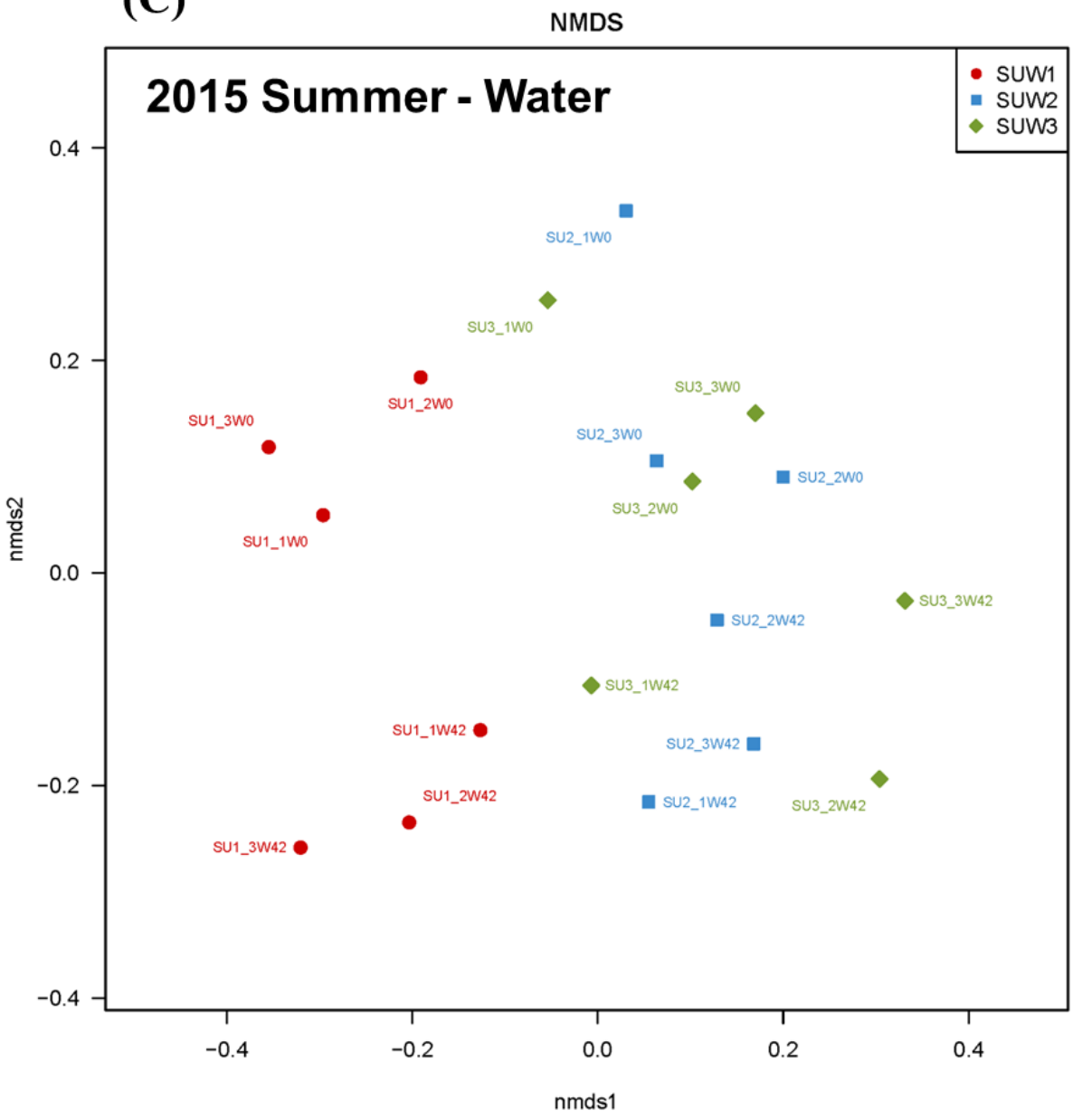
The bacterial diversity in water samples collected in winter 2016 (Figure 3.14A) did not show any clear pattern. However, in leaf samples, clustering based on the sampling locations (urbanization) was observed (Figure 3.14B). Interestingly, when the data obtained from different sampling locations during summer 2016 were analysed together with the data from the control locations (Huangshan, H-1 to H-3), the bacterial community in control locations were distinct from the high, medium and low urbanization locations (Figure 3.14 C and D), which clearly indicate how the urbanization is influencing the bacterial community in the aquatic systems.

Some variations between sampling locations with varying urbanization were observed by NMDS, especially variations between High vs. Medium and Low vs. Huangshan. This is correlated to water quality results in Chapter 2, as extremely high nutrient levels were observed in high urbanized locations and very low nutrient pollution levels were observed in control areas (Huangshan). Therefore, NMDS1 (x-axis) and NMDS2 (y-axis) in figures were contributed to by multiple nutrient parameters, such as TN, TP, $NH_4$-N and $PO_4$-P.


(A)
NMDS
2015 Winter - Water
WIW1
WIW2
WIW3
WI2_1W0
WI2_2W0
WI3_3W0
WI1_2W0
WI3_1W0
WI1_3W0
WI2_1W42
WI1_1W0
WI1_2W42
WI3_3W42
WI2_3W42
WI3_1W42
WI1_1W42
WI2_3W0
WI1_3W42
WI2_2W42
WI3_2W0
WI3_2W42
nmds2
nmds1
0.4
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.8
−0.6
−0.4
−0.2
0.0
0.2
0.4


(B)
NMDS
2015 Winter - Leaf
WIL1
WIL2
WIL3
WI2_1L14
WI3_2L14
WI3_1L42
WI3_2L42
WI3_3L42
WI3_3L14
WI2_2L42
WI2_2L14
WI3_1L14
WI2_3L14
WI2_1L42
WI2_3L42
WI1_3L14
WI1_2L14
WI1_1L42
WI1_1L14
WI1_2L42
WI1_3L42
0.2
0.0
−0.2
−0.4
nmds2
−0.4
−0.2
0.0
0.2
0.4
0.6
nmds1

(C)


NMDS
2015 Summer - Water
SUW1
SUW2
SUW3
SU2_1W0
SU3_1W0
SU1_2W0
SU3_3W0
SU1_3W0
SU2_3W0
SU2_2W0
SU3_2W0
SU1_1W0
SU3_3W42
SU2_2W42
SU3_1W42
SU1_1W42
SU2_3W42
SU1_2W42
SU2_1W42
SU3_2W42
SU1_3W42
0.4
0.2
0.0
−0.2
−0.4
nmds2
−0.4
−0.2
0.0
0.2
0.4
nmds1

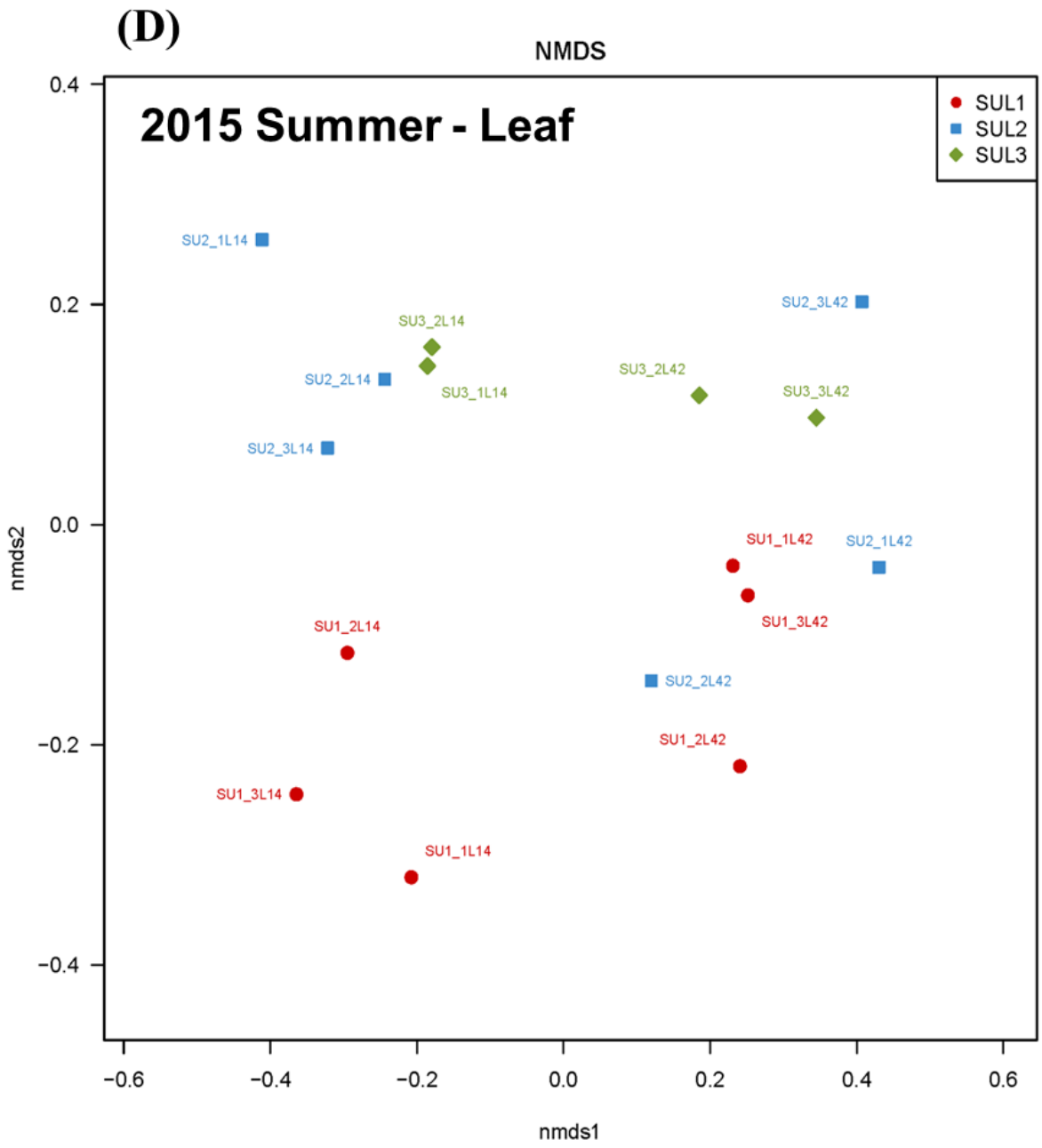
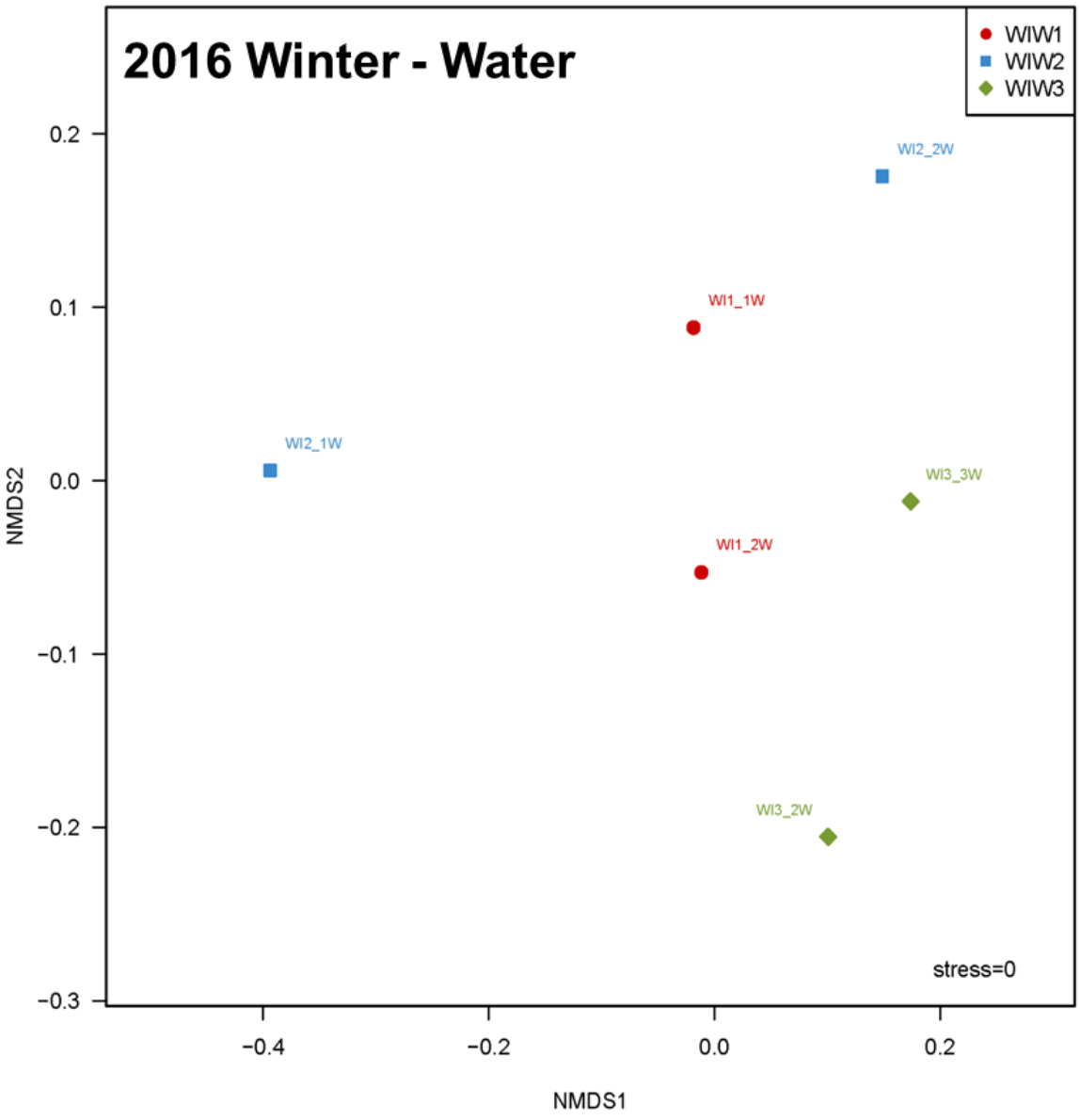
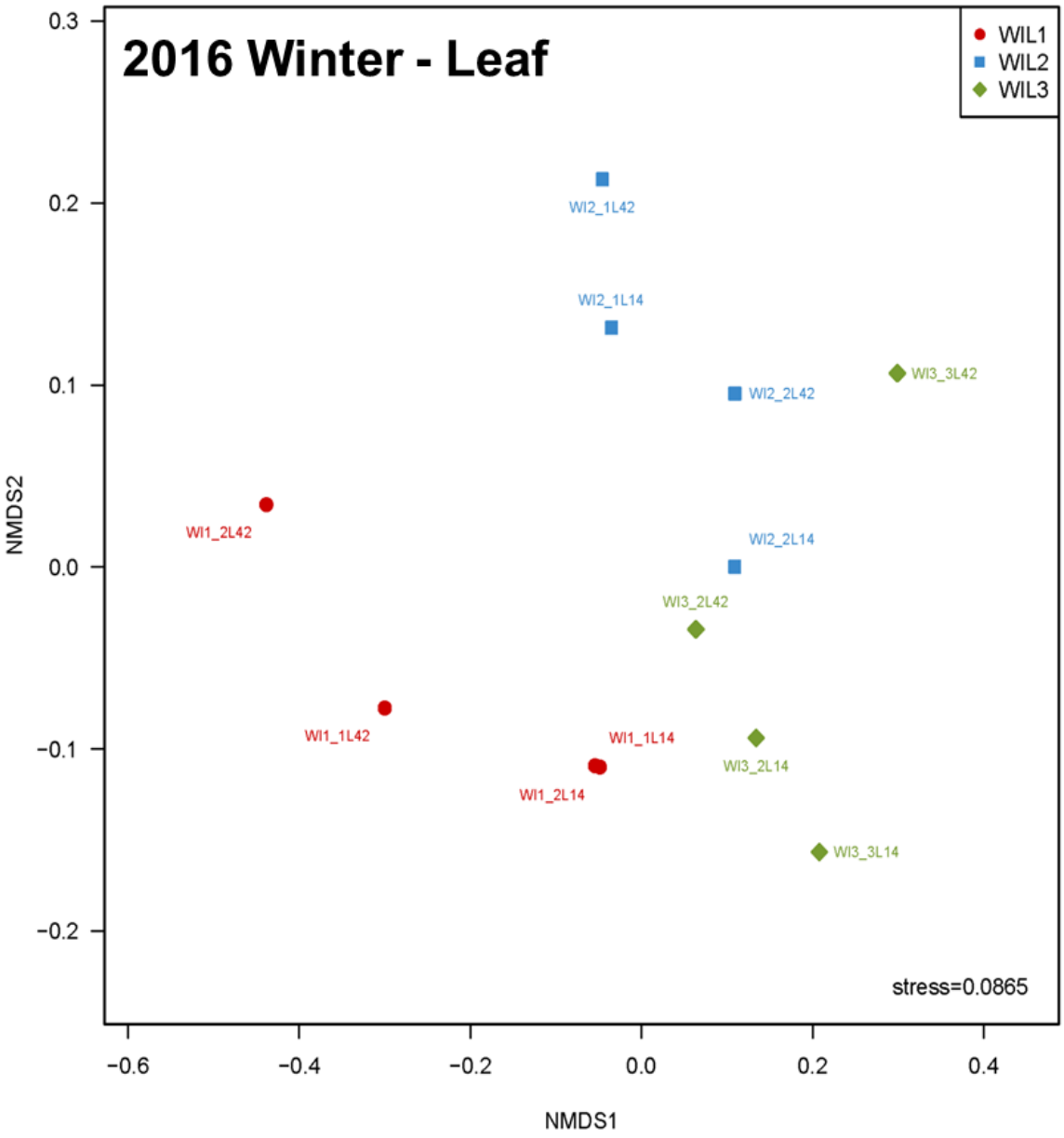



**Figure 3.13.** Visualization of bacterial beta-diversity among water and leaf samples collected from different locations in winter and summer 2015 by non-metric multidimensional scaling (NMDS) analysis. (A and C - Water samples collected from different sampling locations in winter and summer 2015, respectively; B and D - Leaf samples collected from different sampling locations in winter and summer 2015, respectively; WIW=Winter Water; WIL=Winter Leaf; SUW=Summer Water; SUL=Summer Leaf; 1,2,3=High, Medium and Low urbanization locations, respectively.)

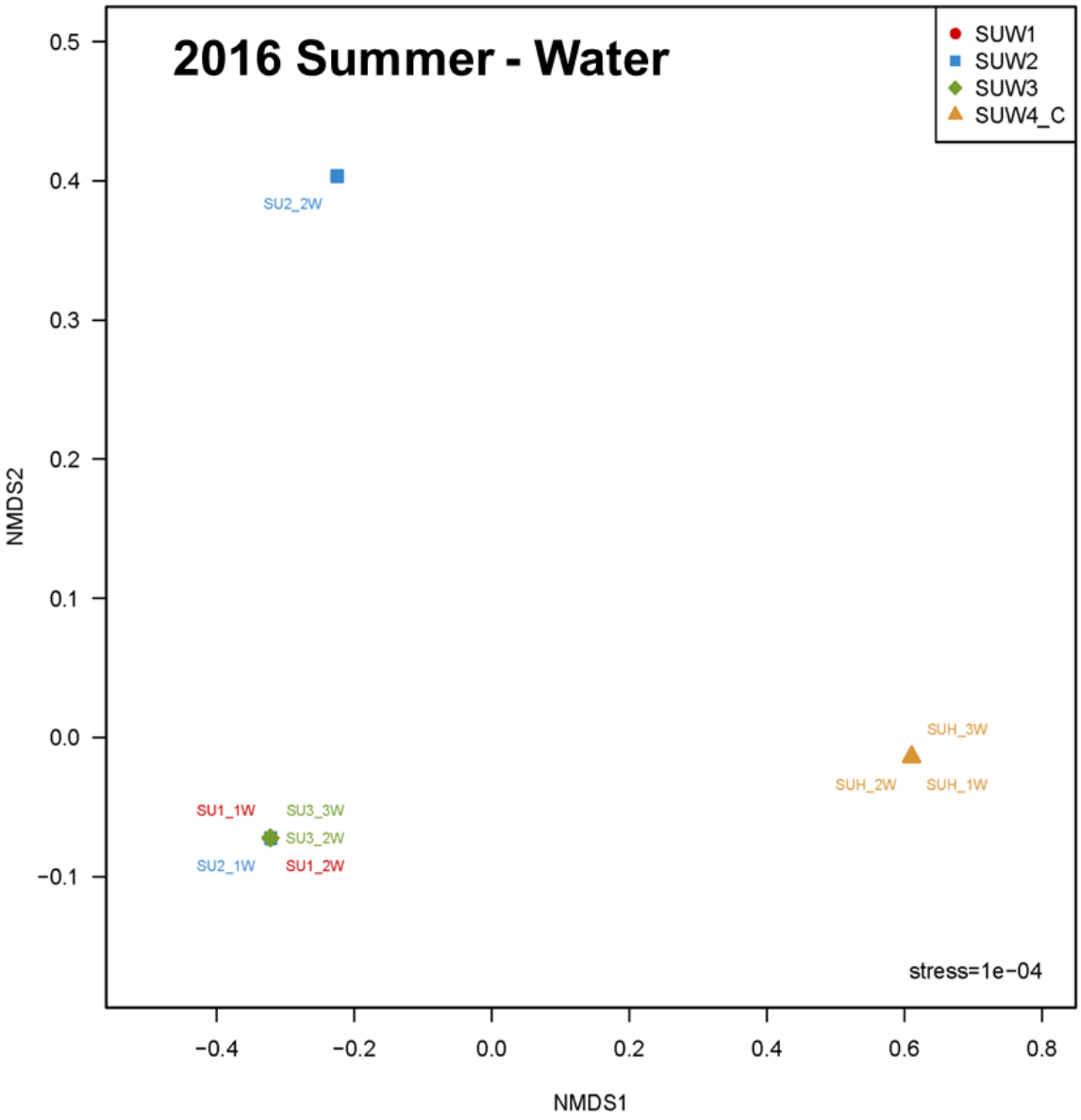
**(A)**


2016 Winter - Water
WIW1
WIW2
WIW3
WI2_2W
WI1_1W
WI2_1W
WI3_3W
WI1_2W
WI3_2W
stress=0
NMDS2
NMDS1
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.4
−0.2
0.0
0.2

**(B)**


2016 Winter - Leaf
WIL1
WIL2
WIL3
WI2_1L42
WI2_1L14
WI3_3L42
WI2_2L42
WI1_2L42
WI2_2L14
WI3_2L42
WI1_1L42
WI1_1L14
WI3_2L14
WI1_2L14
WI3_3L14
stress=0.0865
NMDS1
NMDS2
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.6
−0.4
−0.2
0.0
0.2
0.4

(C)


2016 Summer - Water
SUW1
SUW2
SUW3
SUW4_C
SU2_2W
SUH_3W
SUH_2W
SUH_1W
SU1_1W
SU3_3W
SU3_2W
SU2_1W
SU1_2W
stress=1e-04
NMDS1
NMDS2
0.5
0.4
0.3
0.2
0.1
0.0
−0.1
−0.4
−0.2
0.0
0.2
0.4
0.6
0.8

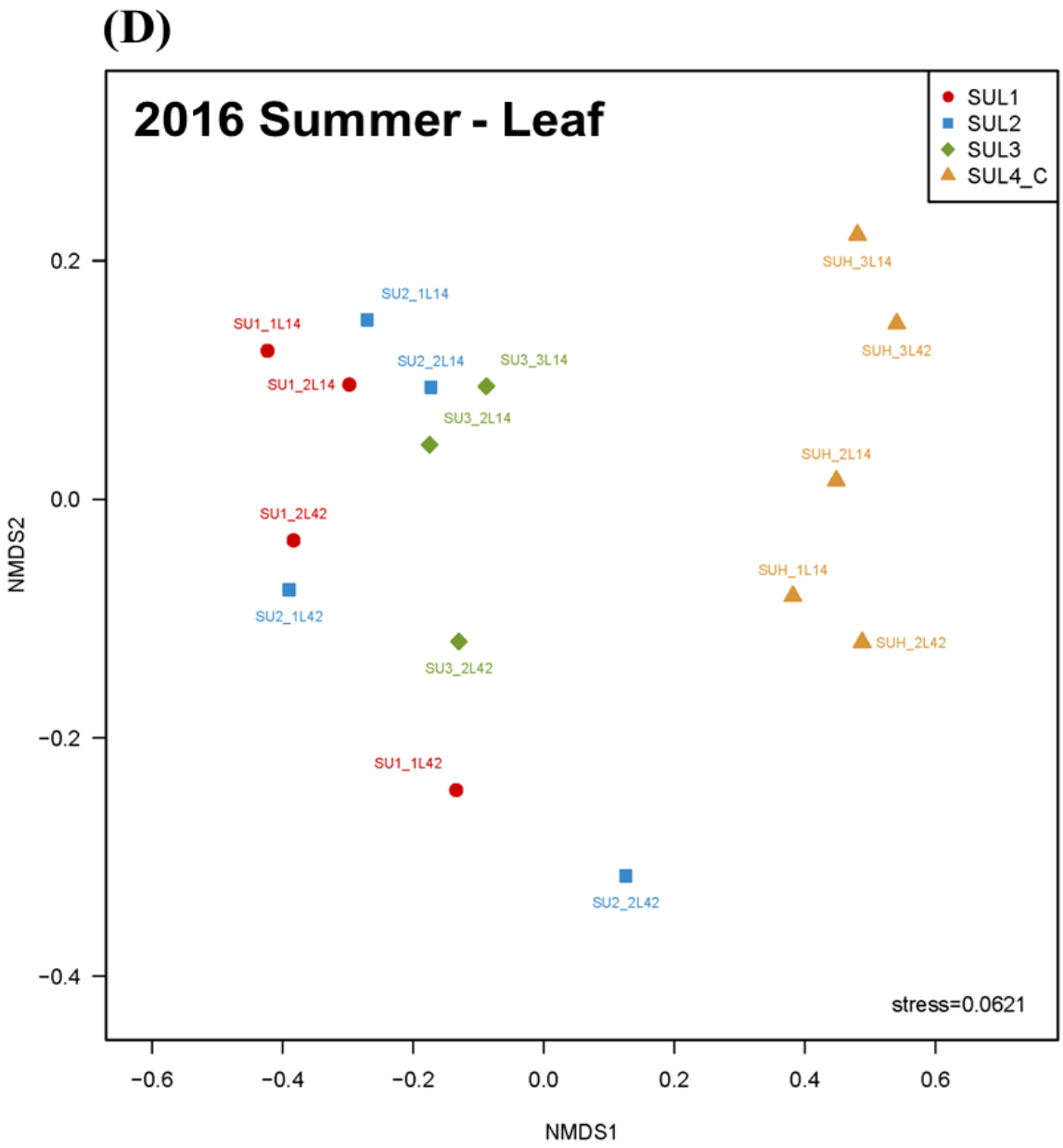


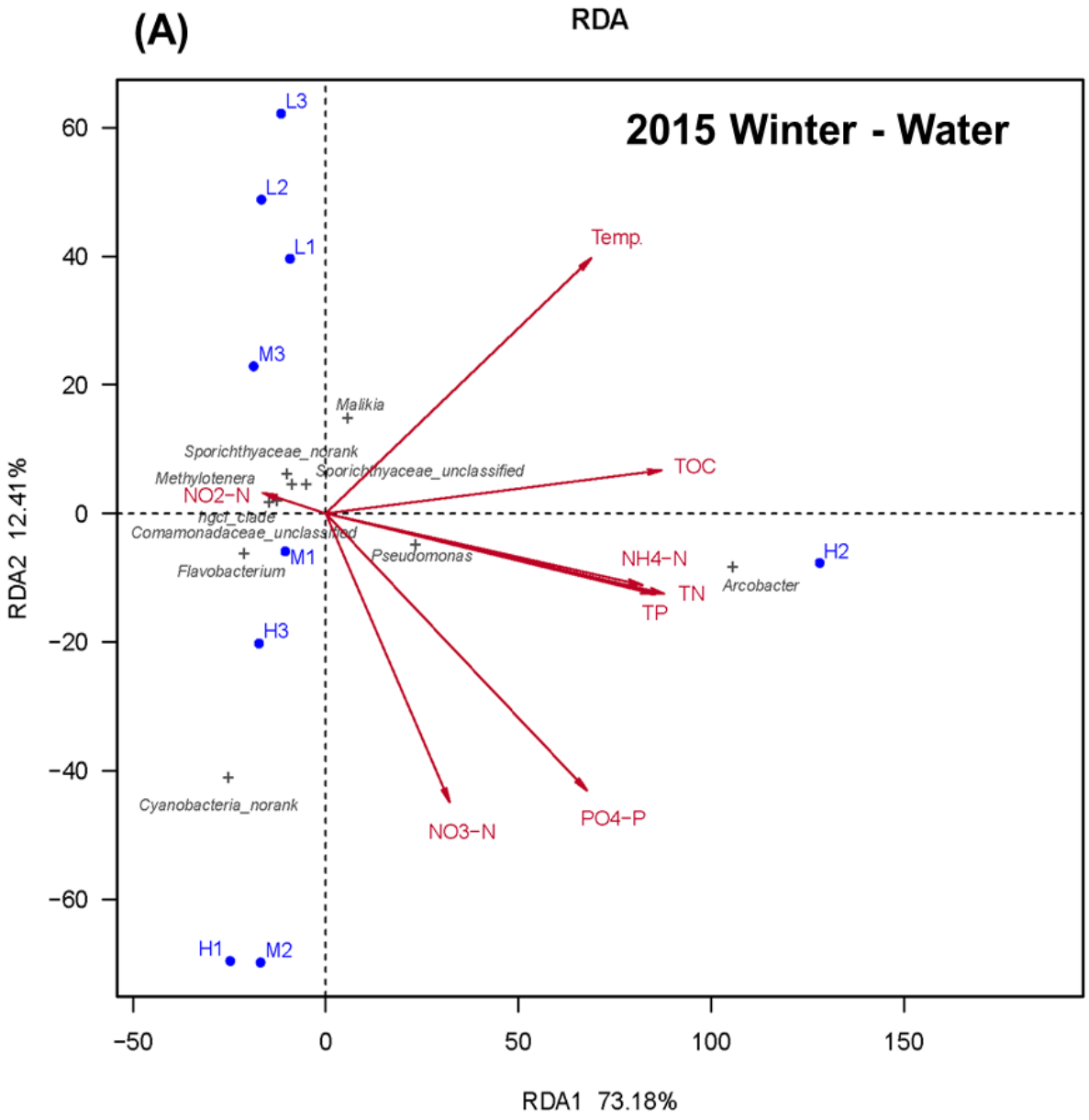

**Figure 3.14.** Visualization of bacterial beta-diversity among water and leaf samples collected from different locations in winter and summer 2016 by non-metric multidimensional scaling (NMDS) analysis. (A and C - Water samples collected from different sampling locations in winter and summer 2016, respectively; B and D - Leaf samples collected from different sampling locations in winter and summer 2016, respectively; WIW=Winter Water; WIL=Winter Leaf; SUW=Summer Water; SUL=Summer Leaf; 1,2,3=High, Medium and Low urbanization locations, respectively; SUW4_C and SUL4_C=Water and Leaf samples collected from control locations in Huangshan.)

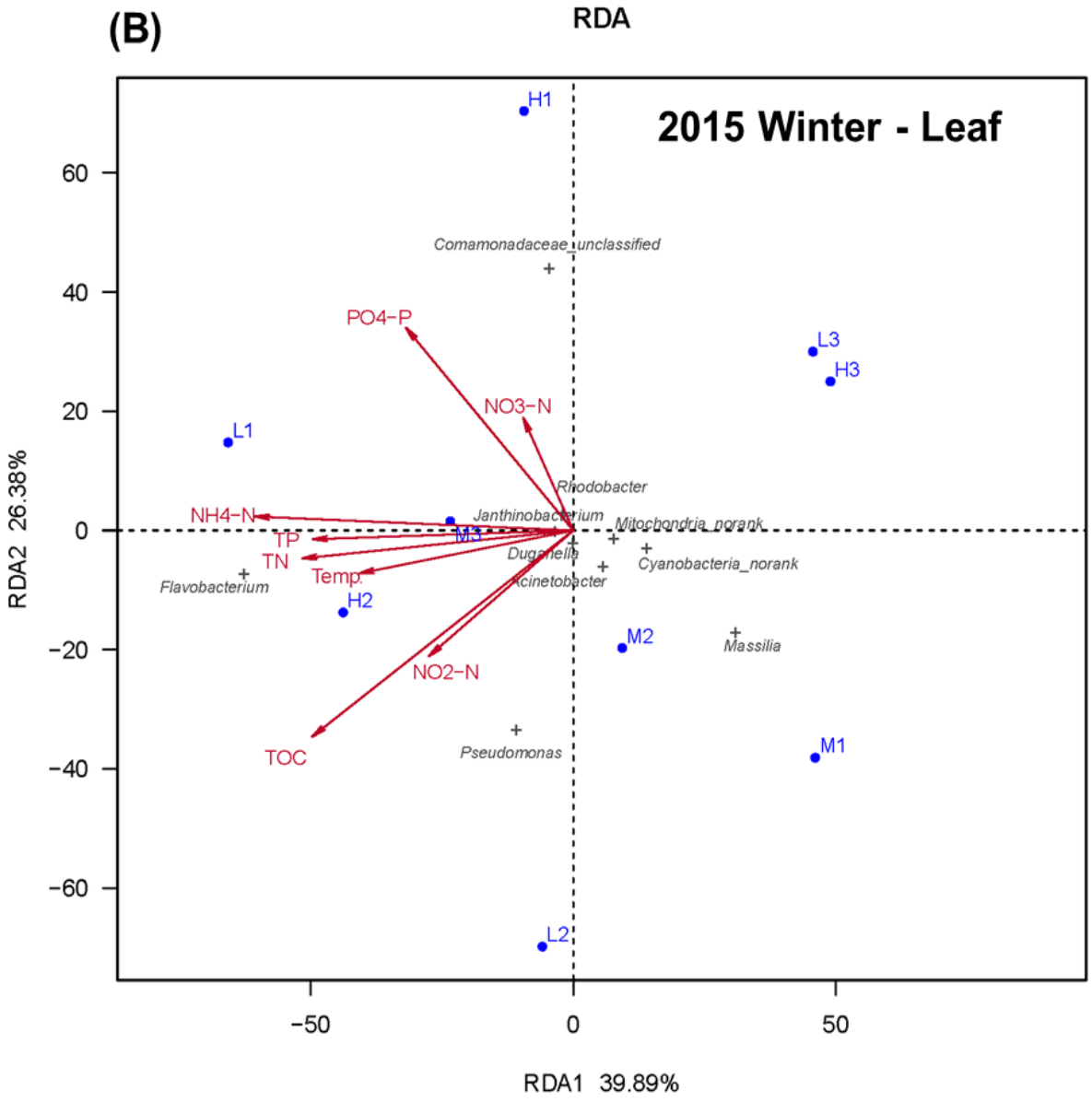
*Redundancy analysis (RDA):*

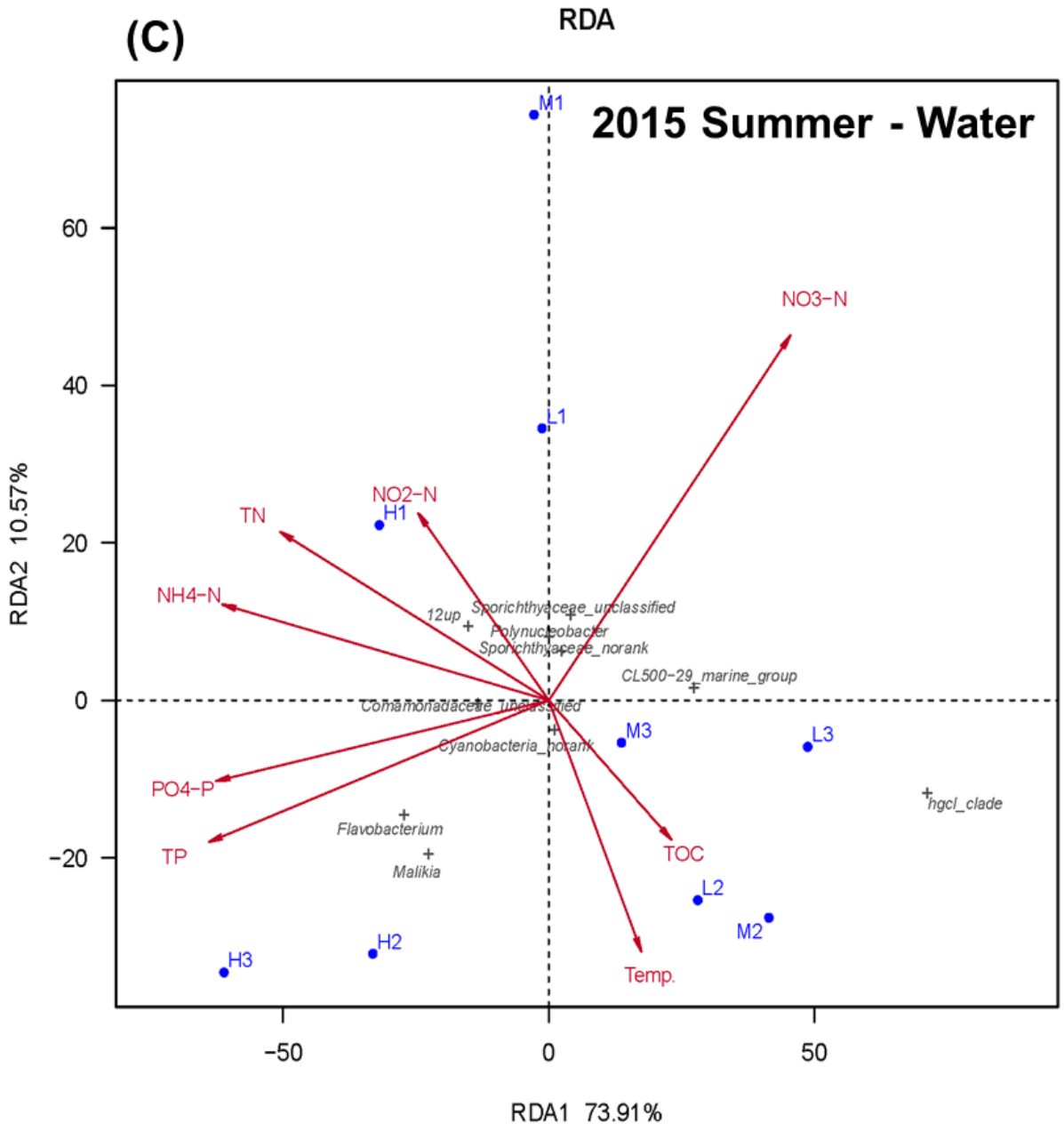
RDA analysis was carried out to find out the relationship between environmental parameters including temperature, total organic carbon (TOC) and multiple nutrients (TN, TP, $NO_3$-N, $NO_2$-N, $PO_4$-N and $NH_4$-N) and bacterial community composition in water and leaf samples collected from different sampling locations. The analysis was carried out for samples collected during winter and summer in 2015 and 2016.

The RDA1 (x-axis) and RDA2 (y-axis) explained 73.18% and 12.41% of variations observed, respectively in bacterial community in water samples collected in winter 2015 (Figure 3.15A). Particularly, the parameters such as temperature, TOC and multiple nutrients influenced the community composition in location 1-2 (H2, high urbanization location), and the bacterial genera such as *Arcobacter*, *Pseudomonas* and *Malikia* were dominant in that location. In the leaf samples collected in winter 2015, the RDA1 and RDA2 explained 39.89% and 26.38% of the total variations in the community, and parameters such as TOC, $NH_4$-N, $PO_4$-P, TN, TP and temperature were found to be major factors influencing the bacterial community in some of the high (H2=1-2), medium (M3=2-3) low (L1=3-1) urbanization locations (Figure 3.15B). The results correlate with relative abundance of some of the bacterial genera, *Flavobacterium*, *Pseudomonas* and *Acinetobacter* in those locations. The RDA biplot pattern was different for water samples collected in summer 2015 (Figure 3.15C). The RDA1 and RDA2 explained 73.91% and 10.57% of the total variations observed in the water samples, and multiple nutrients (TN, TP, $NH_4$-N, $PO_4$-P and $NO_2$-N) contributed to the variations observed in the high urbanization locations (H1 to H3=1-1 to 1-3). High relative abundances of members of Comamonadaceae, *Flavobacterium* and *Malikia* were observed in those locations. The RDA1 and RDA2 explained 59.17% and 18.87% of the variations in the bacterial community observed in leaf samples

collected in summer 2015 (Figure 3.15D), and the above multiple nutrients contributed to the observed variations in the high urbanization locations (H1 to H3=1-1 to 1-3). The overall RDA analysis results indicated that the parameters such as temperature, TOC and multiple nutrients were main parameters which had influence on the bacterial community in water and leaf samples collected from different urban gradients. Similar pattern was observed in the samples collected in 2016 (RDA plots not shown).


(A)
RDA
2015 Winter - Water
L3
L2
L1
M3
H3
H1
M2
M1
H2
Temp.
TOC
NH4−N
TN
TP
NO2−N
NO3−N
PO4−P
Malikia
Sporichthyaceae_norank
Sporichthyaceae_unclassified
Methylotenera
hgcI_clade
Comamonadaceae_unclassified
Flavobacterium
Pseudomonas
Arcobacter
Cyanobacteria_norank
RDA2 12.41%
RDA1 73.18%
60
40
20
0
−20
−40
−60
−50
0
50
100
150


(B)
RDA
2015 Winter - Leaf
H1
L3
H3
L1
M3
M2
M1
L2
PO4-P
NO3-N
NH4-N
TP
TN
Temp.
NO2-N
TOC
Comamonadaceae_unclassified
Rhodobacter
Janthinobacterium
Mitochondria_norank
Dugaella
Acinetobacter
Cyanobacteria_norank
Flavobacterium
Massilia
Pseudomonas
H2
60
40
20
0
−20
−40
−60
−50
0
50
RDA2 26.38%
RDA1 39.89%


(C)
RDA
2015 Summer - Water
M1
L1
H1
H2
H3
M2
M3
L2
L3
NO3−N
NO2−N
TN
NH4−N
PO4−P
TP
TOC
Temp.
12up
Sporichthyaceae_unclassified
Polynucleobacter
Sporichthyaceae_norank
CL500−29_marine_group
Comamonadaceae_unclassified
Cyanobacteria_norank
Flavobacterium
Malikia
hgcI_clade
RDA2 10.57%
RDA1 73.91%
60
40
20
0
−20
−50
0
50

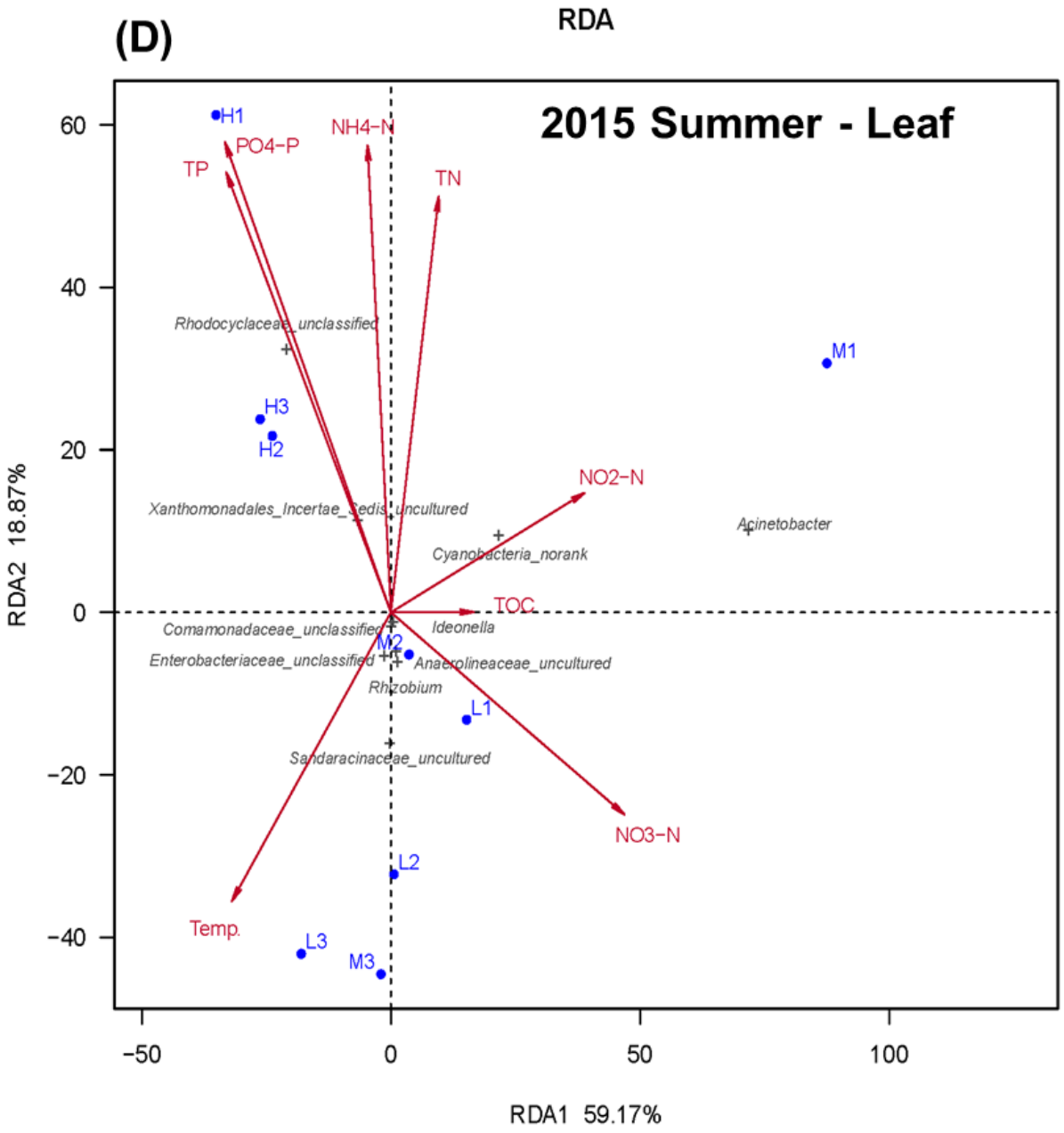



**Figure 3.15.** Redundancy analysis (RDA) showing correlation of physico-chemical parameters with bacterial community composition in water and leaf samples collected from different sampling locations in winter and summer 2015. A) Water samples collected in winter 2015, B) Leaf samples collected in winter 2015, C) Water samples collected in summer 2015, and D) Leaf samples collected in summer 2015.

### 3.3.4. Fungal diversity in water and leaf samples

The fungal community in water and leaf samples collected from different sampling locations in winter and summer of 2015 and 2016 were investigated by NGS of fungal specific ITS1 region amplicons. Thirty six samples (12 water samples and 24 leaf samples from different urbanization gradients in Suzhou) collected in 2015 and 43 representative samples (15 water and 28 leaf samples from sampling locations in Suzhou and Huangshan) collected in 2016 were sequenced. In total 1,065,600 and 1,617,660 reads were obtained from water and leaf samples collected in 2015 and 2016, respectively. The sequencing data were normalized to 29600 reads per sample for samples collected in 2015 and 37620 for samples collected in 2016. High Good's coverage (0.991-1.000) (Tables 3.3, 3.4, S3.3 and S3.4) were obtained for all the water and leaf samples, which indicated that the sequencing depths for all the samples were sufficient. In general, the number of OTUs in water samples were high in summer as compared to winter in 2015 (Table 3.3), and the same pattern was observed for sequences obtained from water and leaf samples collected in 2016 (Table 3.4). The Shannon diversity and other alpha diversity indices showed that the values were higher in summer as compared to winter (Tables 3.3, 3.4, S3.3 and S3.4).

**Table 3.3.** Similarity-based OTUs and species richness and diversity estimates (Mean ± SD) for fungal community in water and leaf samples collected from different urban gradients (High, Medium and Low) in winter and summer 2015. (The number of reads were normalized to 29600 per sample).

| Sample Group | OTUs | ACE | Chao1 | Shannon | Simpson | Coverage |
|---|---|---|---|---|---|---|
| Winter, Water, High urbanization | 439±81 | 572±33 | 560±54 | 3.66±0.41 | 0.060±0.025 | 0.996±0.000 |
| Winter, Water, Medium urbanization | 206±52 | 286±83 | 269±70 | 1.26±0.01 | 0.519±0.057 | 0.998±0.001 |
| Winter, Water, Low urbanization | 539±286 | 663±296 | 664±280 | 2.94±1.11 | 0.230±0.168 | 0.995±0.001 |
| **S**ummer, Water, High urbanization | 1063±69 | 1128±54 | 1128±64 | 5.29±0.25 | 0.014±0.005 | 0.995±0.001 |
| Summer, Water, Medium urbanization | 919±142 | 999±122 | 991±127 | 4.60±0.32 | 0.050±0.020 | 0.995±0.000 |
| Summer, Water and Low urbanization | 798±215 | 917±144 | 905±141 | 4.00±0.92 | 0.093±0.067 | 0.995±0.001 |
| Winter, Leaf, High urbanization | 482±123 | 638±147 | 616±149 | 3.41±0.43 | 0.097±0.044 | 0.995±0.001 |
| Water, Leaf Medium urbanization | 69±15 | 125±55 | 109±37 | 1.02±0.26 | 0.438±0.059 | 0.999±0.000 |
| Winter, Leaf Low urbanization | 99±27 | 223±84 | 158±44 | 1.45±0.22 | 0.374±0.109 | 0.999±0.000 |
| Summer, Leaf, High urbanization | 621±339 | 870±316 | 797±324 | 2.65±1.29 | 0.265±0.148 | 0.994±0.003 |
| Summer, Leaf, Medium urbanization | 628±323 | 933±204 | 814±272 | 2.85±1.07 | 0.209±0.122 | 0.993±0.002 |
| Summer, Leaf, Low urbanization | 544±323 | 765±348 | 723±373 | 3.27±0.95 | 0.104±0.055 | 0.994±0.003 |

**Table 3.4.** Similarity-based OTUs and species richness and diversity estimates (Mean ± SD) for fungal community in water and leaf samples collected from different urban gradients (High, Medium and Low) in winter and summer 2016. The alpha diversity indices for bacterial community in water and leaf samples in Huangshan in summer 2016 are also included. (The number of reads was normalized to 37620 per sample).

| Sample Group | OTUs | ACE | Chao1 | Shannon | Simpson | Coverage |
|---|---|---|---|---|---|---|
| Winter, Water, High urbanization | 418±30 | 532±24 | 541±8 | 3.36±0.04 | 0.065±0.001 | 0.997±0.000 |
| Winter, Water, Medium urbanization | 409±20 | 495±6 | 500±0 | 2.85±0.52 | 0.199±0.093 | 0.997±0.000 |
| Winter, Water, Low urbanization | 621±319 | 746±258 | 742±257 | 3.71±1.46 | 0.099±0.102 | 0.996±0.000 |
| **S**ummer, Water, High urbanization | 908±115 | 1011±151 | 1011±132 | 4.88±0.28 | 0.028±0.014 | 0.996±0.001 |
| Summer, Water, Medium urbanization | 755±310 | 891±385 | 887±375 | 4.36±0.60 | 0.038±0.021 | 0.996±0.002 |
| Summer, Water and Low urbanization | 853±236 | 979±253 | 969±250 | 4.63±0.66 | 0.028±0.017 | 0.995±0.001 |
| Summer, Water, Huangshan | 949±58 | 1065±91 | 1053±93 | 4.37±0.35 | 0.048±0.023 | 0.995±0.001 |
| Winter, Leaf, High urbanization | 57±10 | 108±25 | 88±12 | 0.48±0.52 | 0.797±0.274 | 0.999±0.000 |
| Water, Leaf Medium urbanization | 108±24 | 208±40 | 159±30 | 1.53±0.43 | 0.323±0.072 | 0.999±0.000 |
| Winter, Leaf Low urbanization | 89±32 | 188±63 | 138±55 | 1.23±0.33 | 0.434±0.176 | 0.999±0.000 |
| Summer, Leaf, High urbanization | 958±280 | 1165±279 | 1151±276 | 3.74±1.41 | 0.161±0.216 | 0.994±0.001 |
| Summer, Leaf, Medium urbanization | 609±219 | 890±229 | 827±259 | 2.86±0.93 | 0.201±0.194 | 0.995±0.002 |
| Summer, Leaf, Low urbanization | 523±372 | 711±402 | 703±403 | 2.82±1.44 | 0.184±0.141 | 0.996±0.002 |
| Summer, Leaf, Huangshan | 381±199 | 650±303 | 558±296 | 2.44±0.86 | 0.259±0.157 | 0.996±0.002 |

### 3.3.5. Fungal community composition in water and leaf samples

The fungal specific ITS1 gene sequences obtained for water and leaf samples collected from different sampling locations in winter and summer of 2015 and 2016 were analysed at phylum, order and genus levels. In general, the fungal community in water samples was quite different from the community in leaf samples, and obvious variations with regards to seasons were found. In the leaf samples collected in winter 2016 (Figure 3.19), phylum Ascomycota was dominant (> 90%), and variations in the fungal community were observed in leaf samples collected in summer 2016. For the leaf samples collected in summer 2016, Ascomycota was represented at the highest percentage (60-95%) in samples collected from Huangshan, followed by low (10-95%), medium (5-65%) and high (8-38%) urbanization in Suzhou.

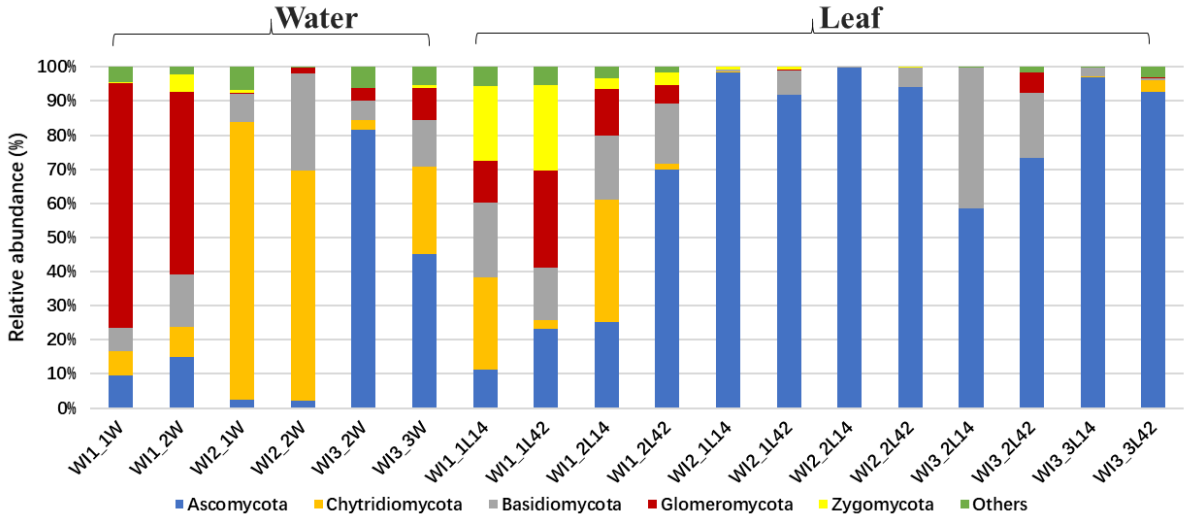
At order level (Figure 3.20), obvious differences between water and leaf samples and differences with regards to seasons were found. Variations in the fungal community in leaf samples collected in both winter and summer varied with urbanization. In winter 2016, Pleosporales was dominant (10-85%) for most leaf samples collected from locations with medium and low urbanization and Hypocreales was dominant (55-98%) for most leaf samples collected from locations with high urbanization. In summer 2016, Pleosporales was represented at the highest percentage (30-90%) in samples collected from Huangshan, followed by low (5-70%), medium (4-30%) and high (1-15%) urbanization in Suzhou.

At genus level (Figure 3.21), obvious differences between water and leaf samples and differences with regards to seasons were found. Variations in the fungal community with urbanization were observed only in leaf samples. *Trichothecium* was dominant (55-95%) for most leaf samples collected from locations with high urbanization in winter 2016, but it was represented at lower percentage in samples collected from locations with medium (1-50%) and

low (2-80%) urbanization. *Alternaria* was dominant (25-65%) for most leaf samples collected from Huangshan in summer 2016, but it was represented at lower percentage (< 50%) in samples collected from Suzhou, especially in location from high urbanization areas. Similar results were observed for fungal community analysis with samples collected in 2015 (Figures 3.16-18).

(A)

**Phylum level fungal community- Winter 2015**




(B)

**Phylum level fungal community- Summer 2015**

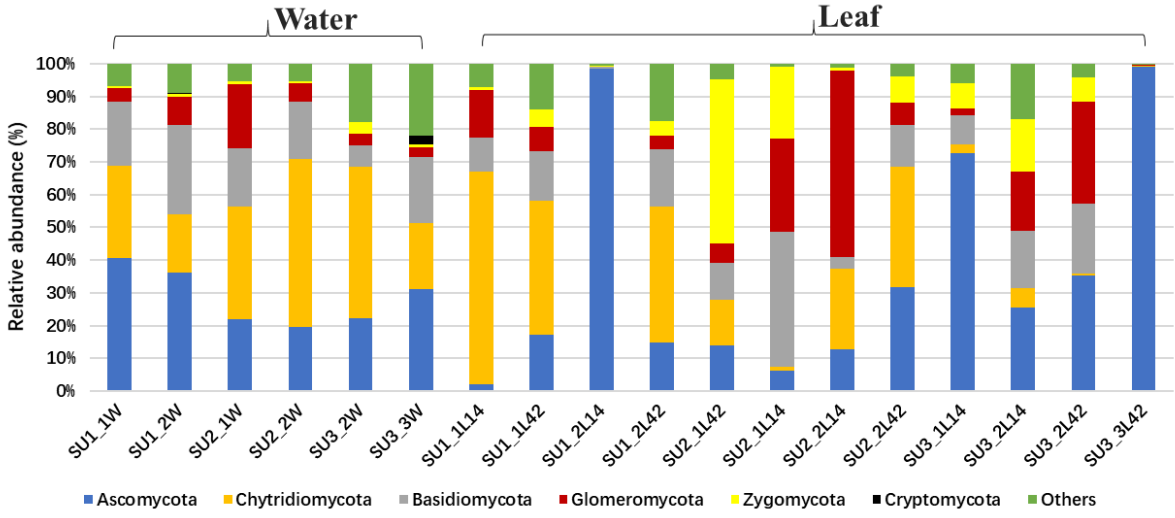



**Figure 3.16.** Relative abundance of fungal phyla in water and leaf samples collected from different locations in winter (A) and summer (B) 2015. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; W=Water samples; L14 and L42=Leaf samples collected on $14^{th}$ and $42^{nd}$ day, respectively.)

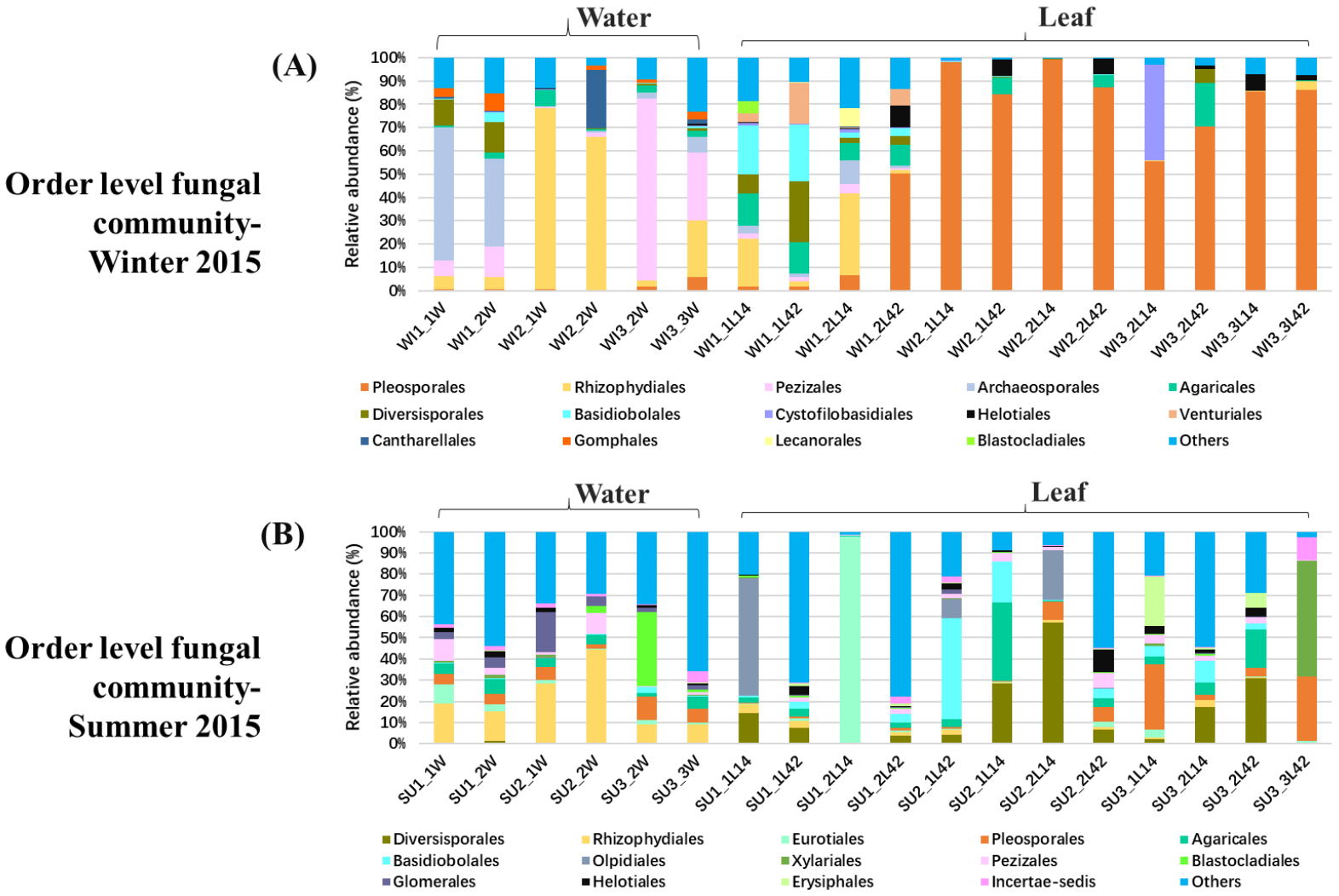
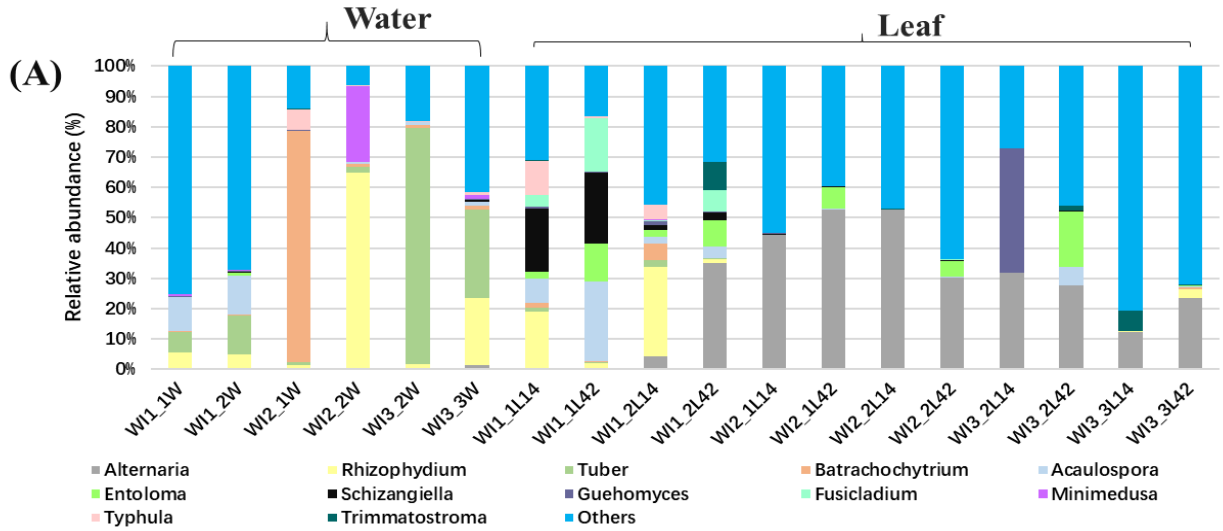



**Figure 3.17.** Relative abundance of fungal orders in water and leaf samples collected from different locations in winter (A) and summer (B) 2015. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; W=Water samples; L14 and L42=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively.)

Genus level fungal community- Winter 2015




Genus level fungal community- Summer 2015

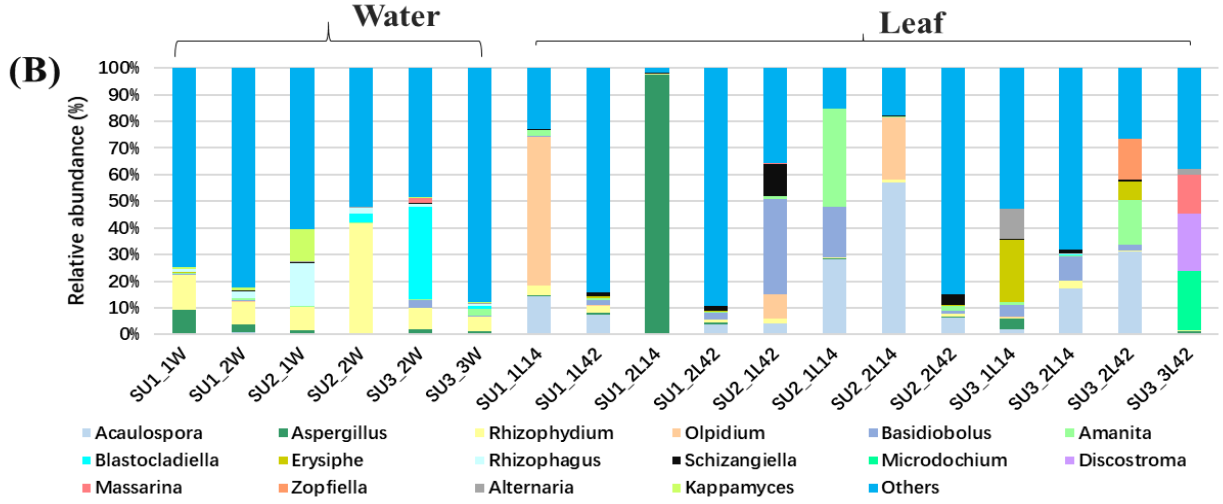



**Figure 3.18.** Relative abundance of fungal genera in water and leaf samples collected from different locations in winter (A) and summer (B) 2015. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; W=Water samples; L14 and L42=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively.)

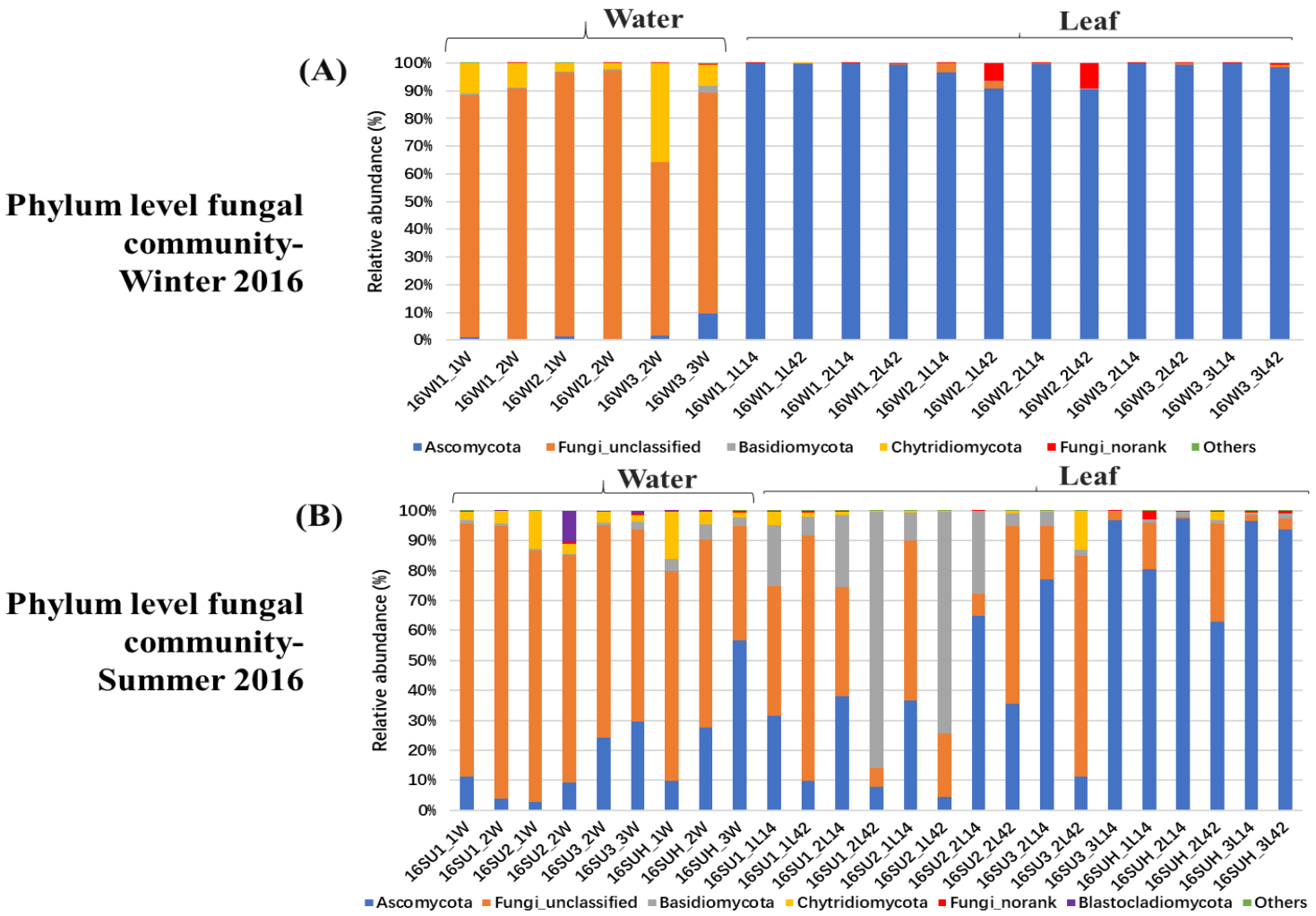



**Figure 3.19.** Relative abundance of fungal phyla in water and leaf samples collected from different locations in winter (A) and summer (B) 2016. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; H-1 to H-3=Control locations in Huangshan; L14 and L42=Leaf samples collected on $14^{th}$ and $42^{nd}$ day, respectively.)

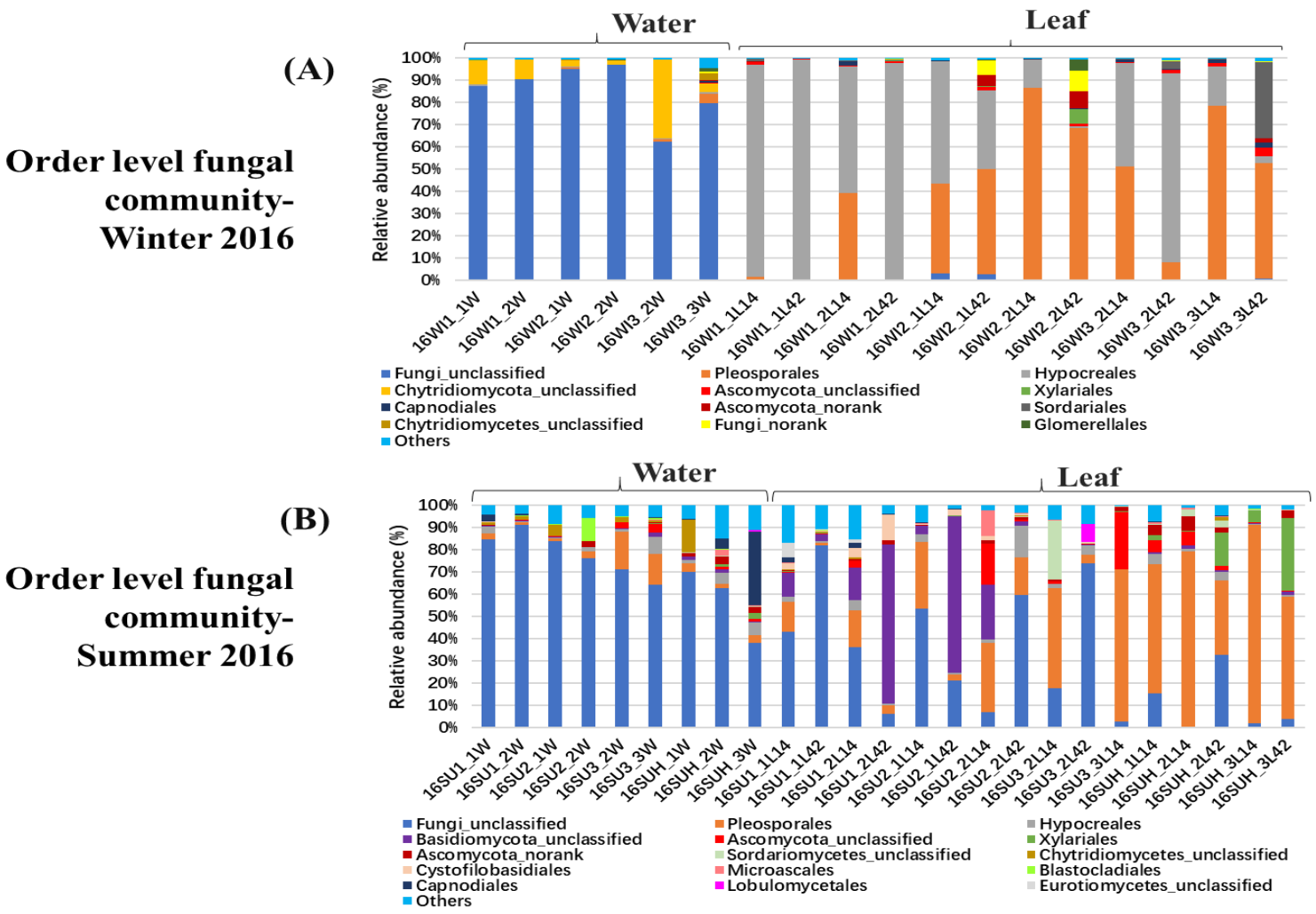



**Figure 3.20.** Relative abundance of fungal orders in water and leaf samples collected from different locations in winter (A) and summer (B) 2016. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; H-1 to H-3=Control locations in Huangshan; L14 and L42=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively.)

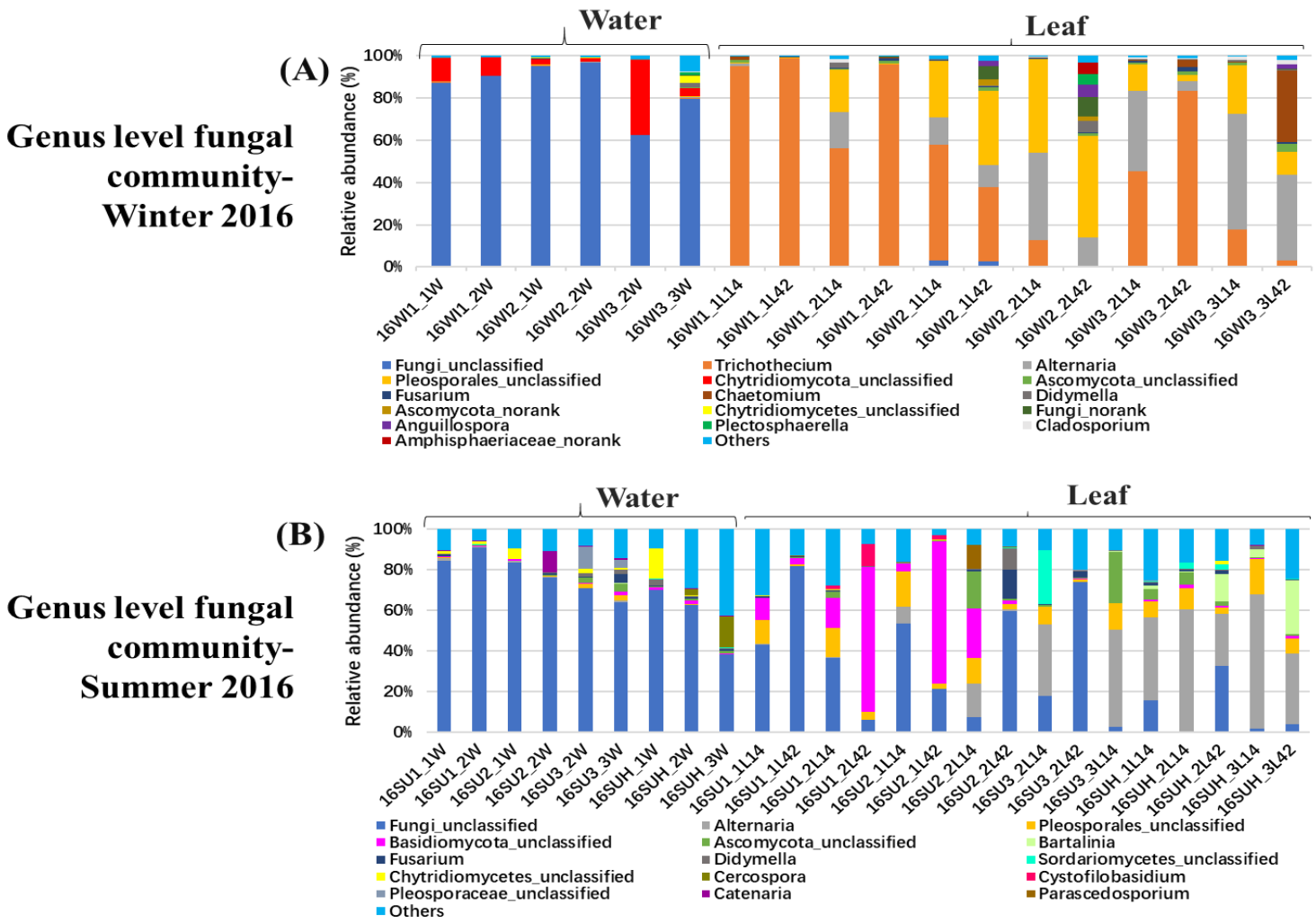



**Figure 3.21.** Relative abundance of fungal genera in water and leaf samples collected from different locations in winter (A) and summer (B) 2016. (WI=Winter; SU=Summer; 1-1 to 3-3=Sampling locations in high to low urban gradients, respectively; H-1 to H-3=Control locations in Huangshan; L14 and L42=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively.)

### 3.3.6. Comparison of fungal community in water and leaf samples collected from different sampling locations

*Venn diagram analysis:*

Venn diagram analysis was performed to find out the shared and unique OTUs present in water and leaf samples collected from different sampling locations in different seasons. When the water (W) and leaf (L) samples collected in winter (WI) and summer (SU) were analysed, the results showed that the number of unique OTUs were more in water samples (38-45%) as compared to leaf samples (22–25%) and the shared OTUs were in the range of 33-37% (Figures 3.22A and C). The comparison of OTUs between summer and winter samples revealed that the unique OTUs in summer samples were extremely high (64-77%) as compared to winter (10-13%) and the shared OTUs were 13-23% (Figures 3.22 B and D).

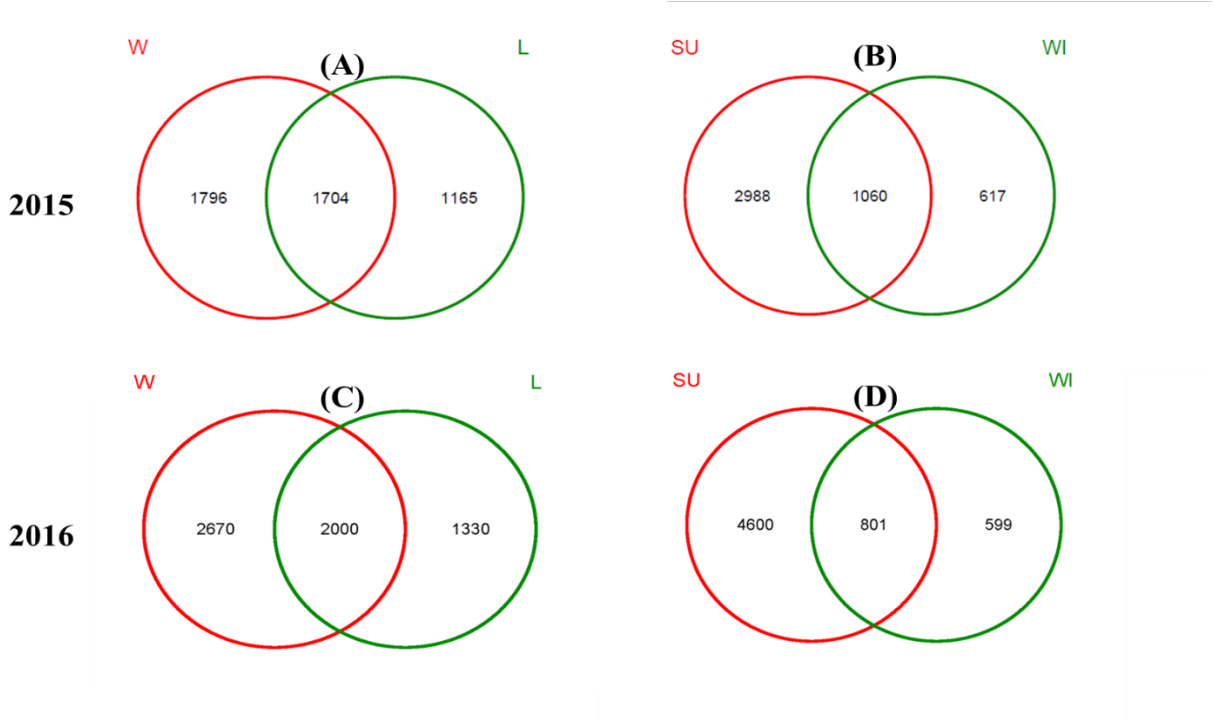



**Figure 3.22.** Venn diagram showing unique and shared fungal OTUs when the sequences were compared between water and leaf samples (A, C) and between winter and summer (B, D) 2015 and 2016. (W=Water; L= Leaf; SU=Summer and WI=Winter.)

Several comparisons were made to find out the unique and shared OTUs in the sequences obtained from water and leaf samples from different sampling locations (high, medium and low urbanization). In the samples collected in 2016 (Figure 3.24), for water samples in winter (Figure 3.24A), there were most unique OTUs in samples collected from locations with low (421) urbanization compared to high (87) and medium (158) urbanization. For leaf samples collected in winter 2016 (Figure 3.24B), there were more unique OTUs in samples collected from locations with medium (108) urbanization compared to high (34) and low (76) urbanization. For water samples in summer 2016 (Figure 3.24C), there were extremely high number of unique OTUs in samples collected from Huangshan (1577) than those from Suzhou (H: 293, M: 250, L: 456). For leaf samples collected in summer 2016 (Figure 3.24D), there were more unique OTUs in samples collected from locations with high (748) urbanization in Suzhou, followed by Huangshan (532), medium (291) and low (215) urbanization in Suzhou.

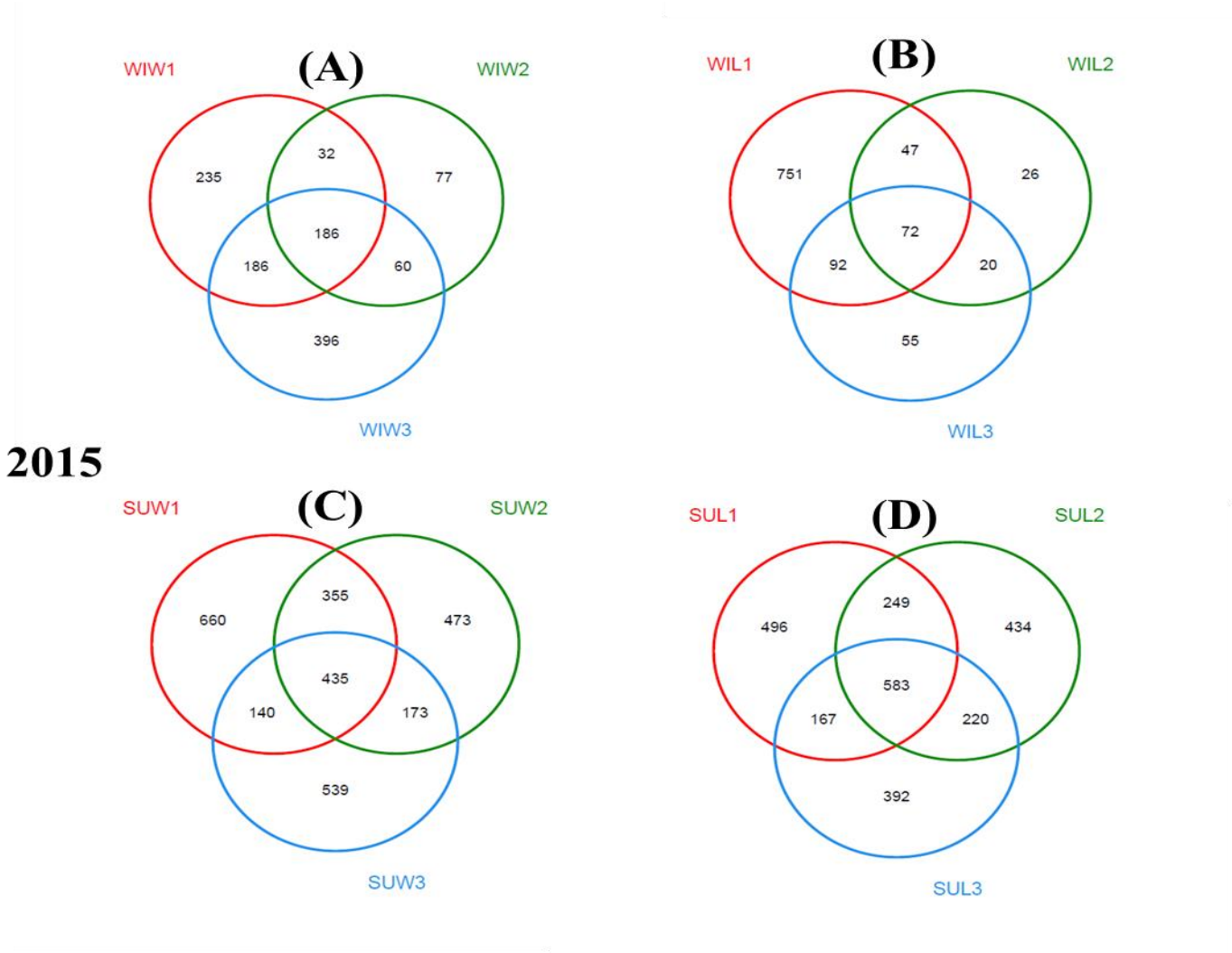



**Figure 3.23.** Venn diagram showing unique and shared fungal OTUs in the sequences compared between water and leaf samples collected from different sampling locations in winter (A, B) and summer (C, D) 2015. (WIW=Winter Water; WIL=Winter Leaf; SUW=Summer Water; SUL=Summer Leaf; 1,2,3=High, Medium and Low urbanization locations, respectively.)

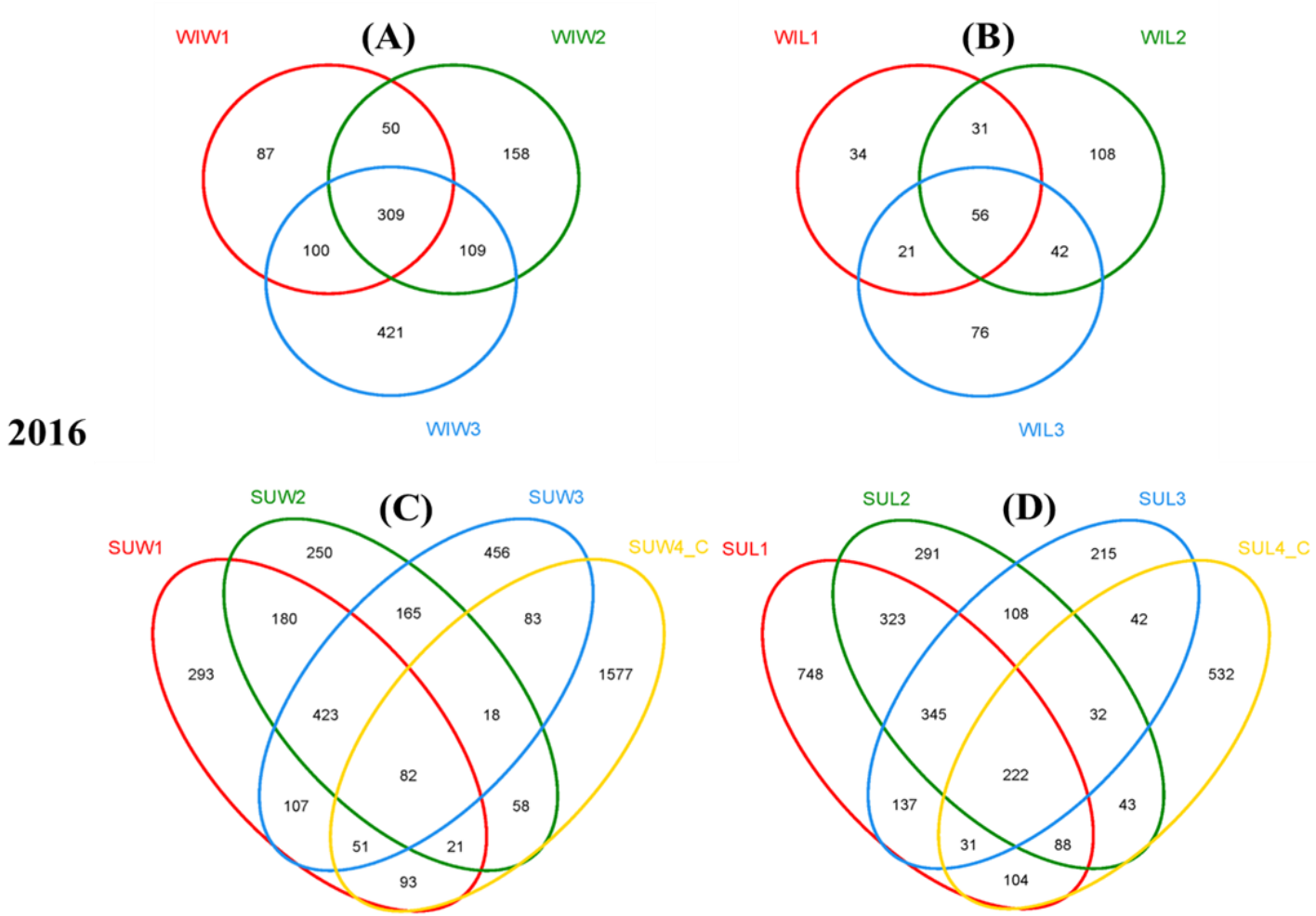
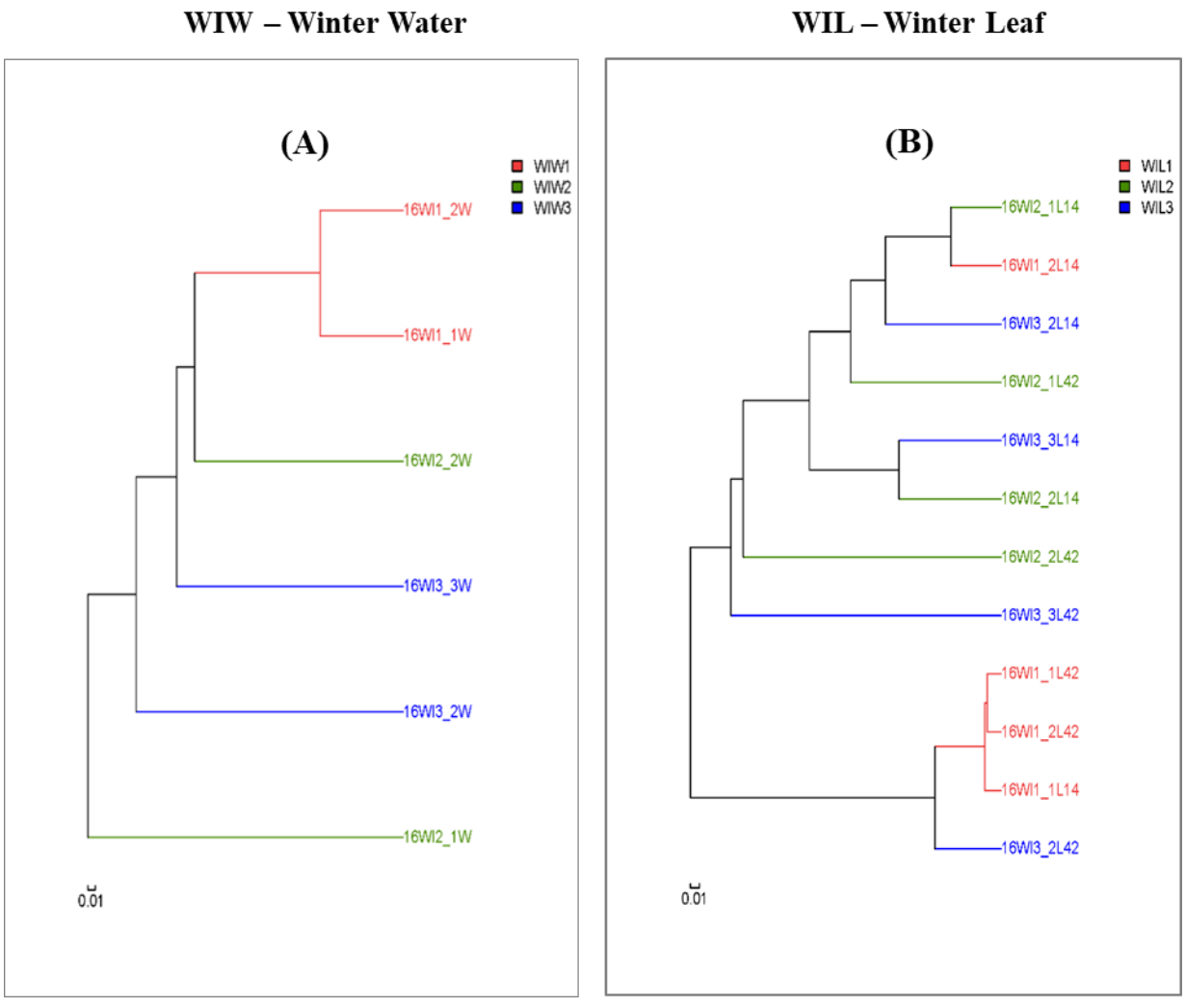



**Figure 3.24.** Venn diagram showing unique and shared fungal OTUs in the sequences compared between water and leaf samples collected from different sampling locations in winter (A, B) and summer (C, D) 2016. (WIW=Winter Water; WIL=Winter Leaf; SUW=Summer Water; SUL=Summer Leaf; 1,2,3=High, Medium and Low urbanization locations, respectively; SUW4_C and SUL4_C=Water and Leaf samples collected in control locations in Huangshan.)

*Beta diversity analysis (cluster analysis and NMDS diagrams):*

Cluster analysis and NMDS were used to find out the similarities between the fungal community in water and leaf samples collected from different sampling locations across the urbanization gradients. Distinct clusters with respect to sampling locations were observed for most samples. However, for leaf samples in summer, the distinct cluster with respect to sampling location was not as clear as water samples or leaf samples in winter (Figures 3.25 and 3.26). As similar pattern was observed in the samples collected in 2015, only the diagrams for samples collected in 2016 are shown.



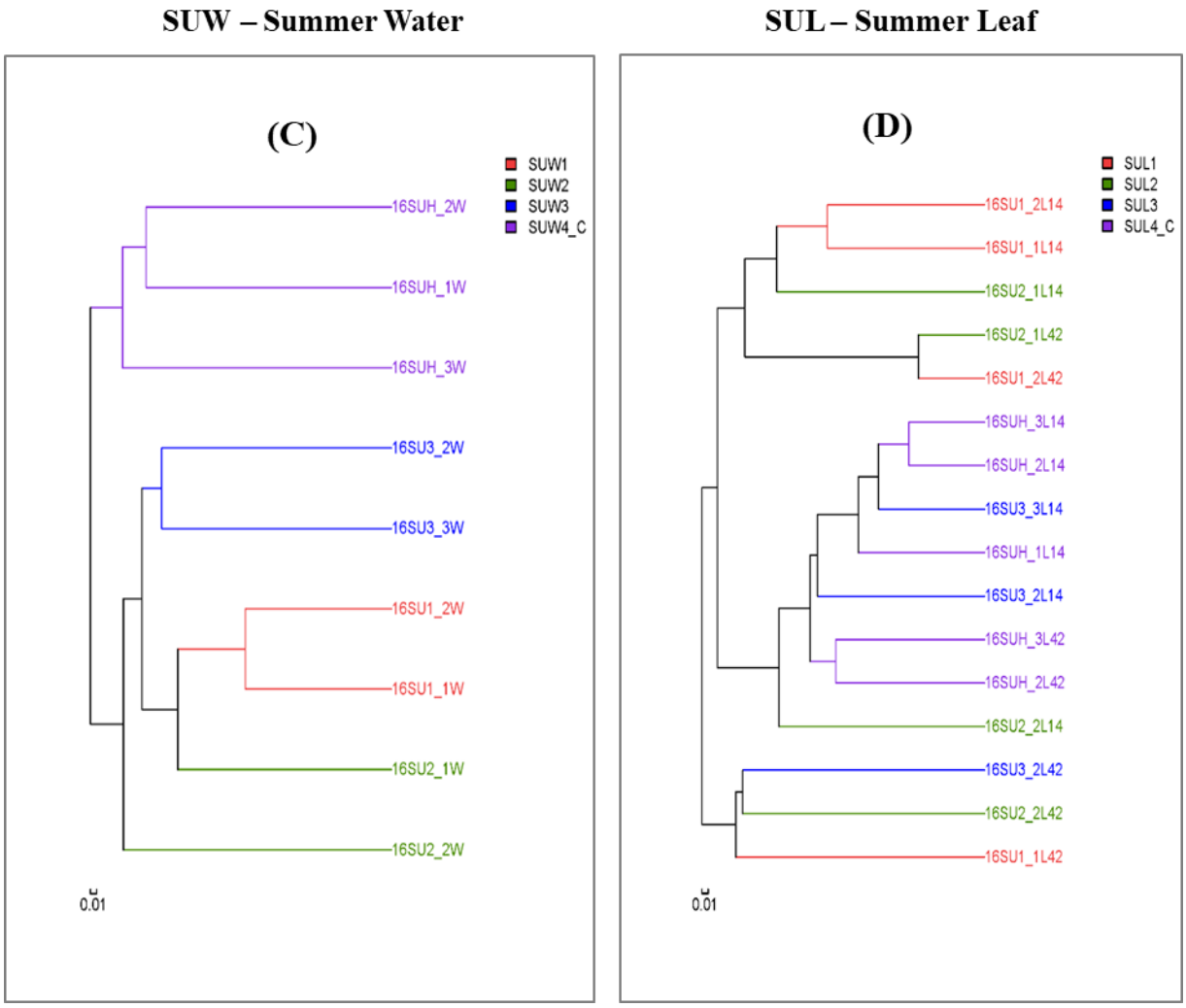
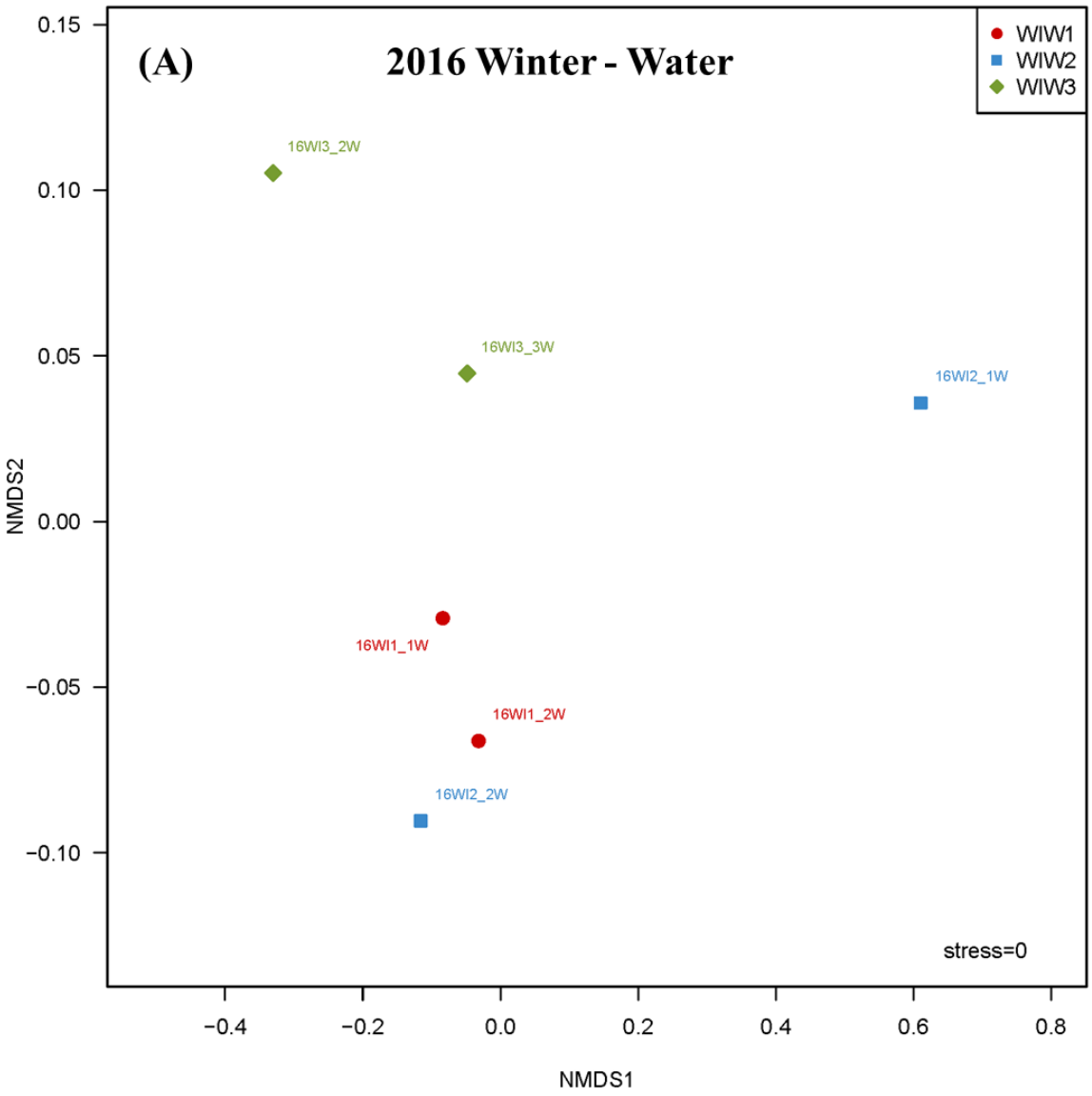
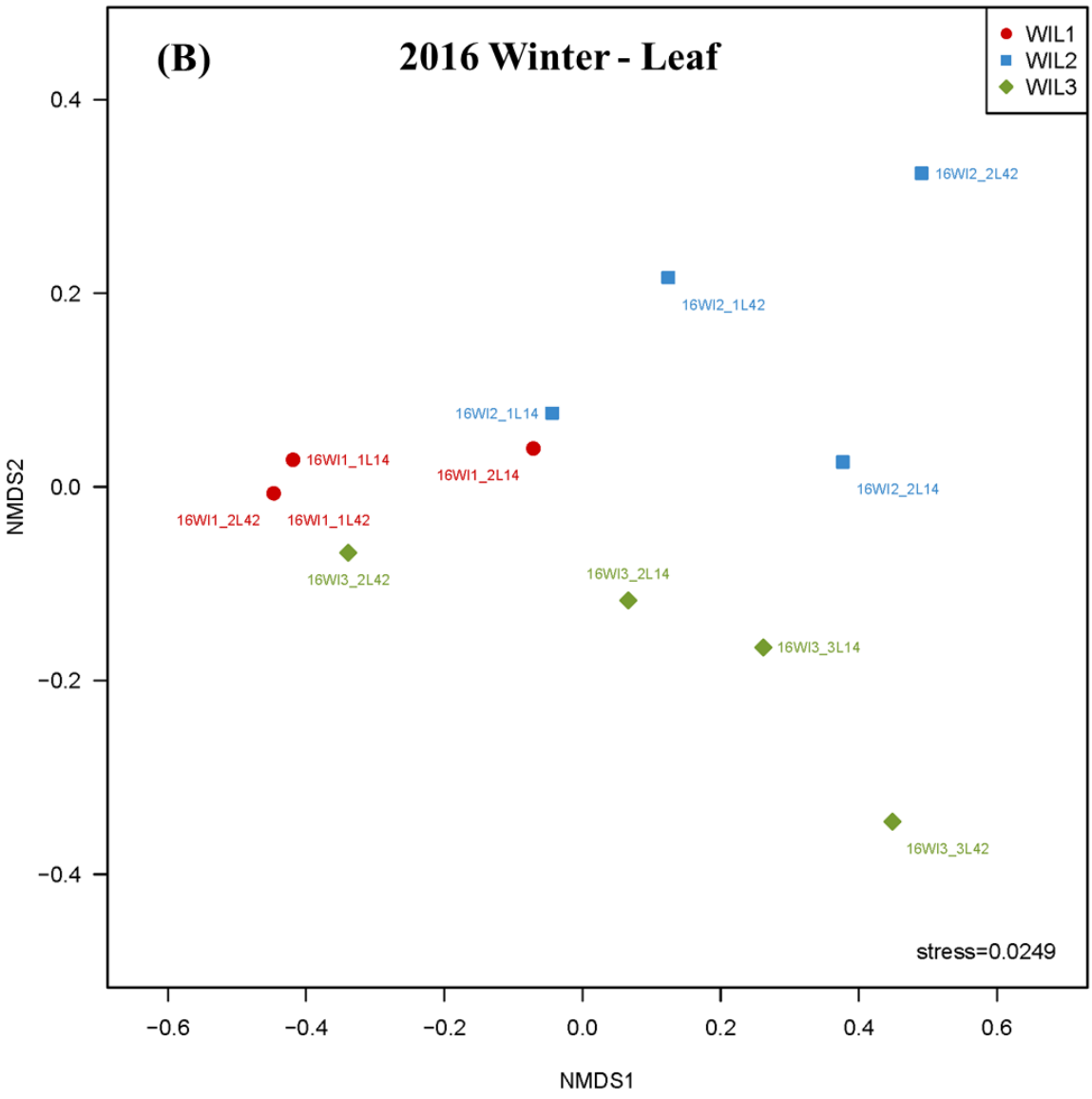
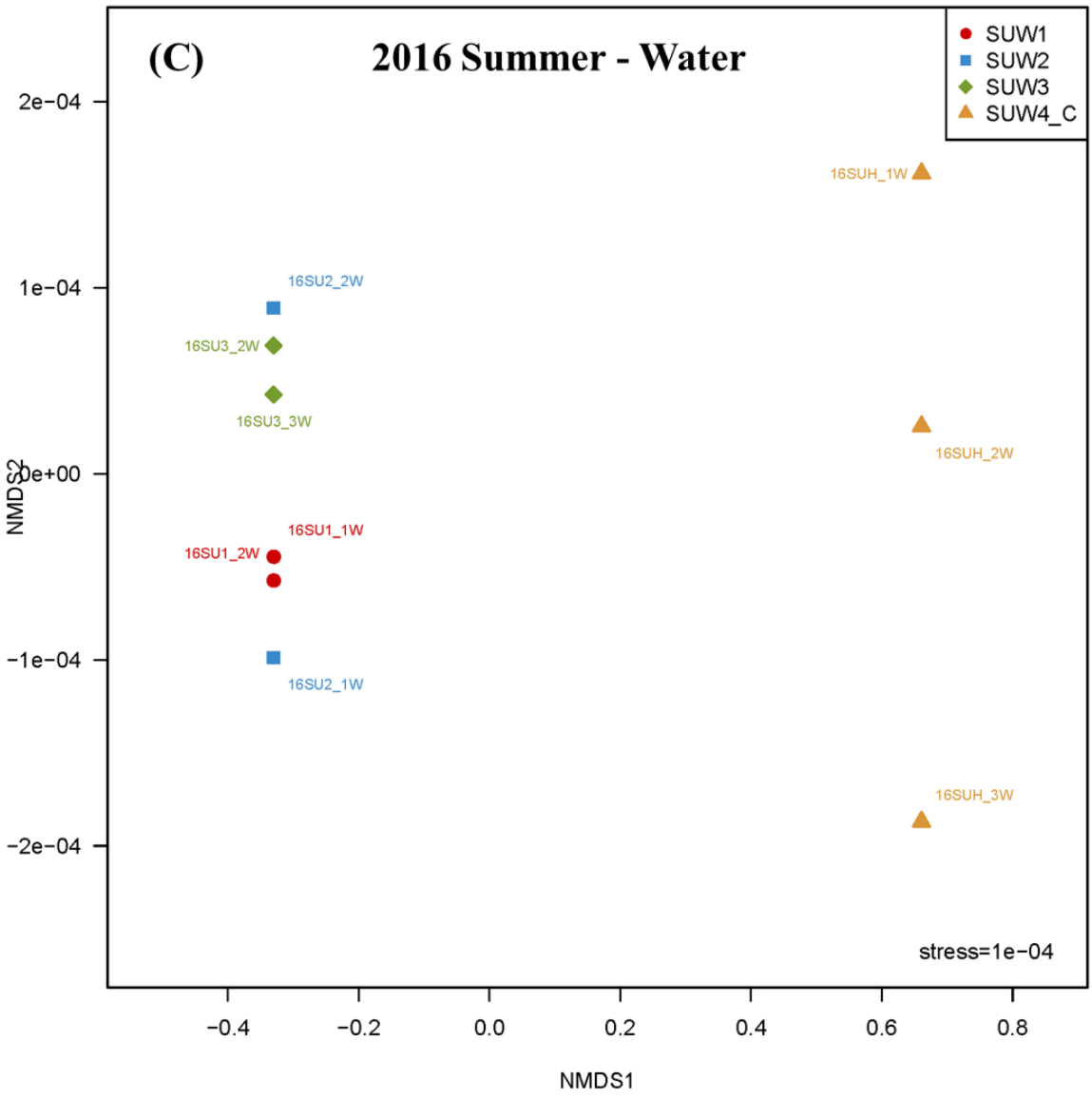



**Figure 3.25.** Cluster analysis showing the similarity in fungal diversity in water and leaf samples collected from sampling locations across urbanization gradient in winter and summer 2016. Comparison of fungal diversity in water (A, C) and leaf (B, D) samples collected from different locations across urbanization gradients in winter (A, B) and summer (C, D) in 2016. (WIW=Winter Water; WIL=Winter Leaf; SUW=Summer Water; SUL=Summer Leaf; 1,2,3=High, Medium and Low urbanization, respectively; SUW4_C and SUL4_C=Water and Leaf samples collected in control locations in Huangshan.)


(A)
2016 Winter - Water
WIW1
WIW2
WIW3
16WI3_2W
16WI3_3W
16WI2_1W
16WI1_1W
16WI1_2W
16WI2_2W
stress=0
NMDS1
NMDS2
0.15
0.10
0.05
0.00
−0.05
−0.10
−0.4
−0.2
0.0
0.2
0.4
0.6
0.8


(B)
2016 Winter - Leaf
WIL1
WIL2
WIL3
16WI2_2L42
16WI2_1L42
16WI2_1L14
16WI1_1L14
16WI1_2L14
16WI2_2L14
16WI1_2L42
16WI1_1L42
16WI3_2L42
16WI3_2L14
16WI3_3L14
16WI3_3L42
stress=0.0249
NMDS1
NMDS2
0.4
0.2
0.0
−0.2
−0.4
−0.6
−0.4
−0.2
0.0
0.2
0.4
0.6


(C)
2016 Summer - Water
SUW1
SUW2
SUW3
SUW4_C
16SUH_1W
16SU2_2W
16SU3_2W
16SU3_3W
16SUH_2W
16SU1_1W
16SU1_2W
16SU2_1W
16SUH_3W
stress=1e−04
2e−04
1e−04
0e+00
−1e−04
−2e−04
NMDS2
−0.4
−0.2
0.0
0.2
0.4
0.6
0.8
NMDS1

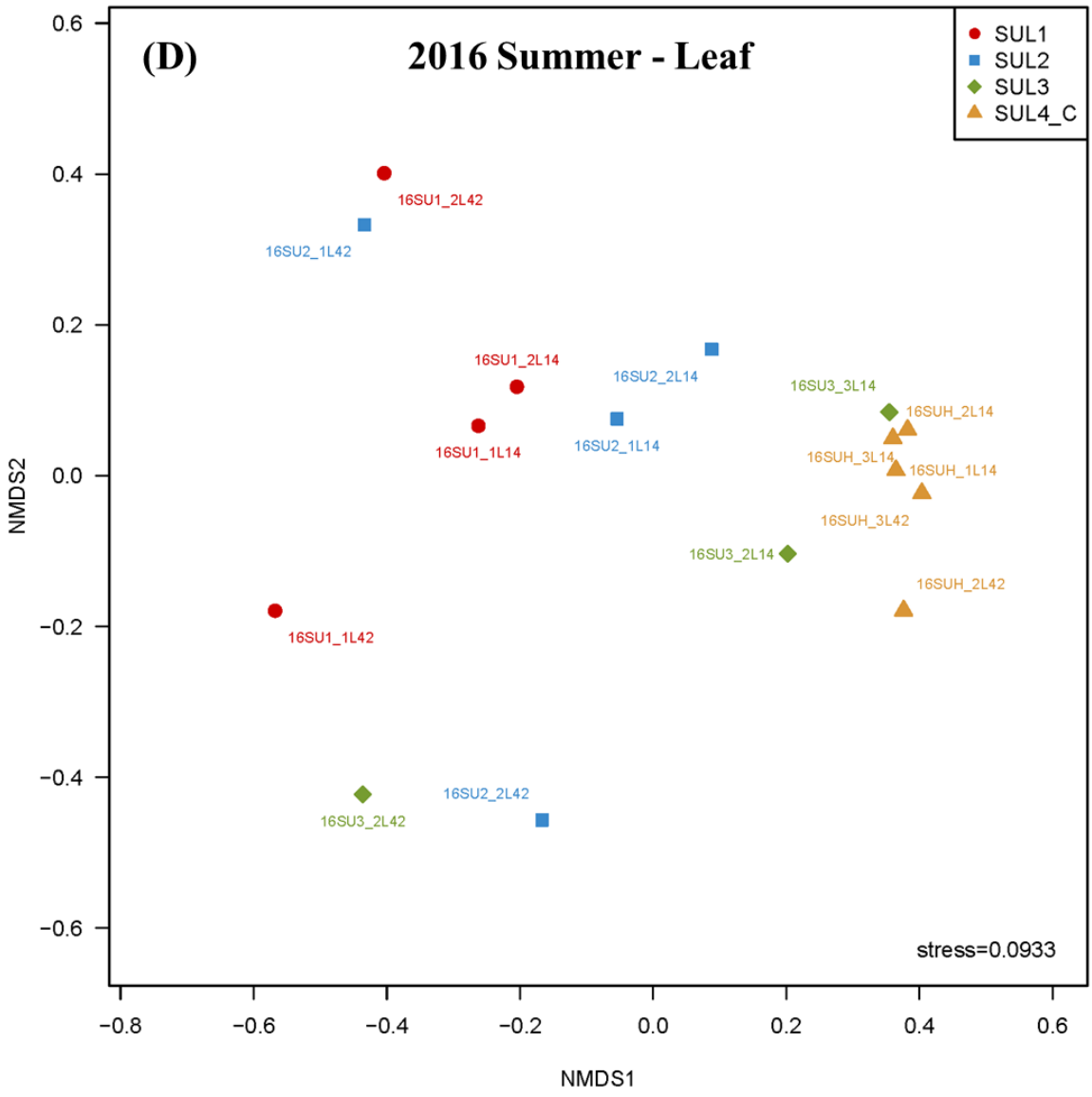


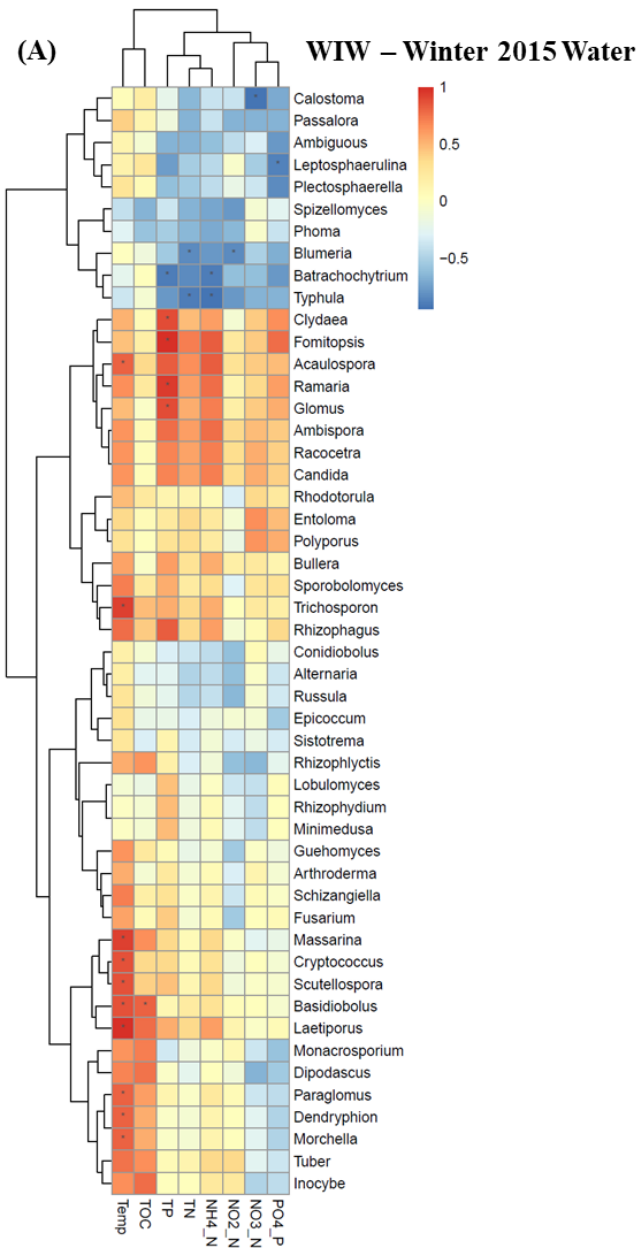

**Figure 3.26.** Visualization of fungal beta-diversity among water and leaf samples collected from different locations in winter and summer 2016 by non-metric multidimensional scaling (NMDS) analysis. (A and C - Water samples collected from different sampling locations in winter and summer 2016, respectively; B and D - Leaf samples collected from different sampling locations in winter and summer 2015, respectively; WIW=Winter Water; WIL=Winter Leaf; SUW=Summer Water; SUL=Summer Leaf; 1,2,3=High, Medium and Low urbanization locations, respectively; SUW4_C and SUL4_C=Water and Leaf samples collected from control locations in Huangshan.)

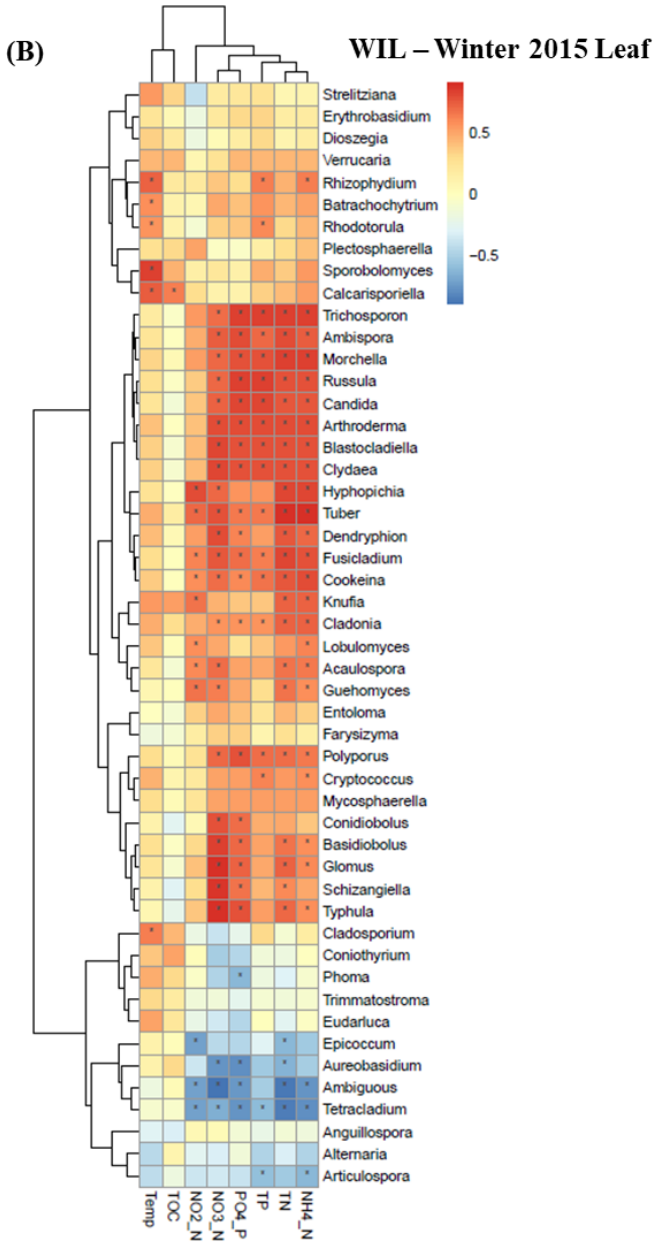
*Heat map and RDA / CCA for analysis the relationship between fungal community and environmental parameters:*

The relationship between fungal community and environmental parameters (temperature and nutrients) was analysed by both heat map and RDA / CCA, and the effect of nutrients (especially TN, TP and $NH_4$-N) on fungal community was found. For the samples collected in 2015, heat map of water samples collected in winter (Figure 3.27A) indicated temperature positively correlated with the genera *Acaulospora, Trichosporon, Massarina, Cryptococcus, Scutellospora, Bassidiobolus, Laetiporus, Paraglomus, Dendryphion* and *Morchella*; nutrients (TN, TP, $NH_4$-N, $PO_4$-P, $NO_3$-N and $NO_2$-N) contributed to the variations, particularly they positively correlated with *Clydaea, Fomitopsis, Ramaria* and *Glomus*, but negatively correlated with *Calostoma, Leptosphaerulina, Blumeria, Batrachochytrium* and *Trphula*. The heat map of leaf samples in winter 2015 (Figure 3.27B) indicated temperature positively correlated with *Cladosporium, Batrachochytrium, Rhizophydium, Rhodototorula, Sporobolomyces* and *Calcarisporiella*; nutrients positively correlated with the genera *Trichosporon, Morchella, Candida, Tuber* and *Fusicladium,* but negatively correlated with the genera *Aureobasidium* and *Tetracladium*. The heat map of water samples in summer 2015 (Figure 3.27C) indicated temperature positively correlated with *Fusarium*; nutrients positively correlated with the genera *Trichosporon, Entoloma* and *Tremella,* but negatively correlated with *Phoma*. The heat map of leaf samples in summer 2015 (Figure 3.27D) indicated temperature negatively correlated with *Hydnobolites*; nutrients positively correlated with the genera *Rhodotorula* and *Candida*, but negatively correlated with *Anguillospora*.

The relationship between environmental parameters (temperature and nutrients) and fungal genera was analysed by RDA / CCA for all the water and leaf samples collected in 2016 (Figure

3.28). The results showed that the parameters such as $NO_3$-N, $NH_4$-N, TN and TOC contributed to the most of variations observed in the high urbanization locations. The parameters such as $PO_4$-P, $NO_2$-N, TP and temperature contributed to the variations observed in the samples collected in Huangshan locations and low urbanization locations.


(A)
WIW – Winter 2015 Water
1
0.5
0
−0.5
Calostoma
Passalora
Ambiguous
Leptosphaerulina
Plectosphaerella
Spizellomyces
Phoma
Blumeria
Batrachochytrium
Typhula
Clydaea
Fomitopsis
Acaulospora
Ramaria
Glomus
Ambispora
Racocetra
Candida
Rhodotorula
Entoloma
Polyporus
Bullera
Sporobolomyces
Trichosporon
Rhizophagus
Conidiobolus
Alternaria
Russula
Epicoccum
Sistotrema
Rhizophlyctis
Lobulomyces
Rhizophydium
Minimedusa
Guehomyces
Arthroderma
Schizangiella
Fusarium
Massarina
Cryptococcus
Scutellospora
Basidiobolus
Laetiporus
Monacrosporium
Dipodascus
Paraglomus
Dendryphion
Morchella
Tuber
Inocybe
Temp
TOC
TP
TN
NH4_N
NO2_N
NO3_N
PO4_P


(B)
WIL – Winter 2015 Leaf
0.5
0
−0.5
Strelitziana
Erythrobasidium
Dioszegia
Verrucaria
Rhizophydium
Batrachochytrium
Rhodotorula
Plectosphaerella
Sporobolomyces
Calcarisporiella
Trichosporon
Ambispora
Morchella
Russula
Candida
Arthroderma
Blastocladiella
Clydaea
Hyphopichia
Tuber
Dendryphion
Fusicladium
Cookeina
Knufia
Cladonia
Lobulomyces
Acaulospora
Guehomyces
Entoloma
Farysizyma
Polyporus
Cryptococcus
Mycosphaerella
Conidiobolus
Basidiobolus
Glomus
Schizangiella
Typhula
Cladosporium
Coniothyrium
Phoma
Trimmatostroma
Eudarluca
Epicoccum
Aureobasidium
Ambiguous
Tetracladium
Anguillospora
Alternaria
Articulospora
Temp
TOC
NO2_N
NO3_N
PO4_P
TP
TN
NH4_N

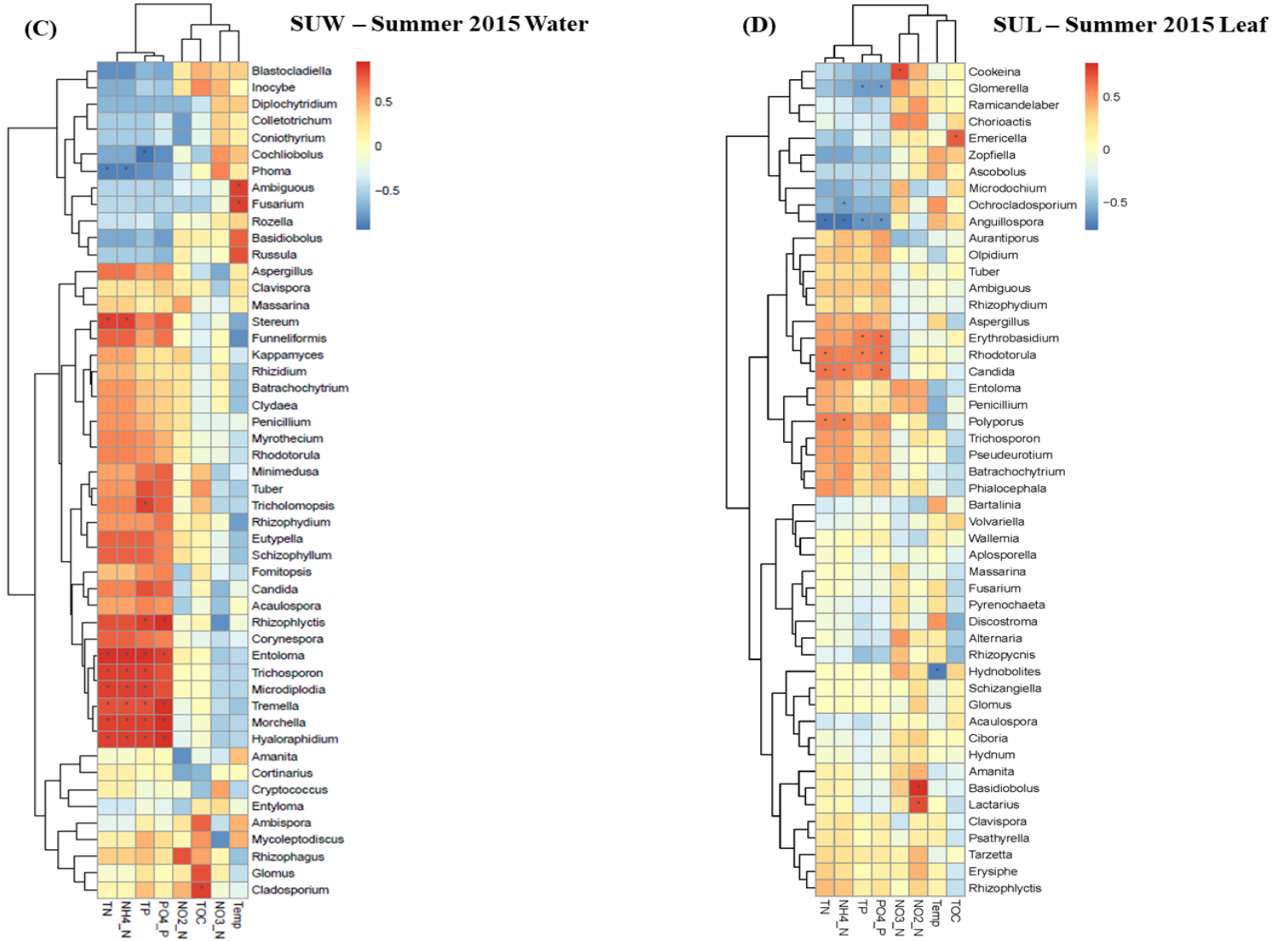



**Figure 3.27.** Clustered heat-map showing the relationship between different fungal genera and environmental parameters. A) Water samples B) Leaf samples collected in winter 2015; C) Water samples and D) Leaf samples collected in Summer 2015.

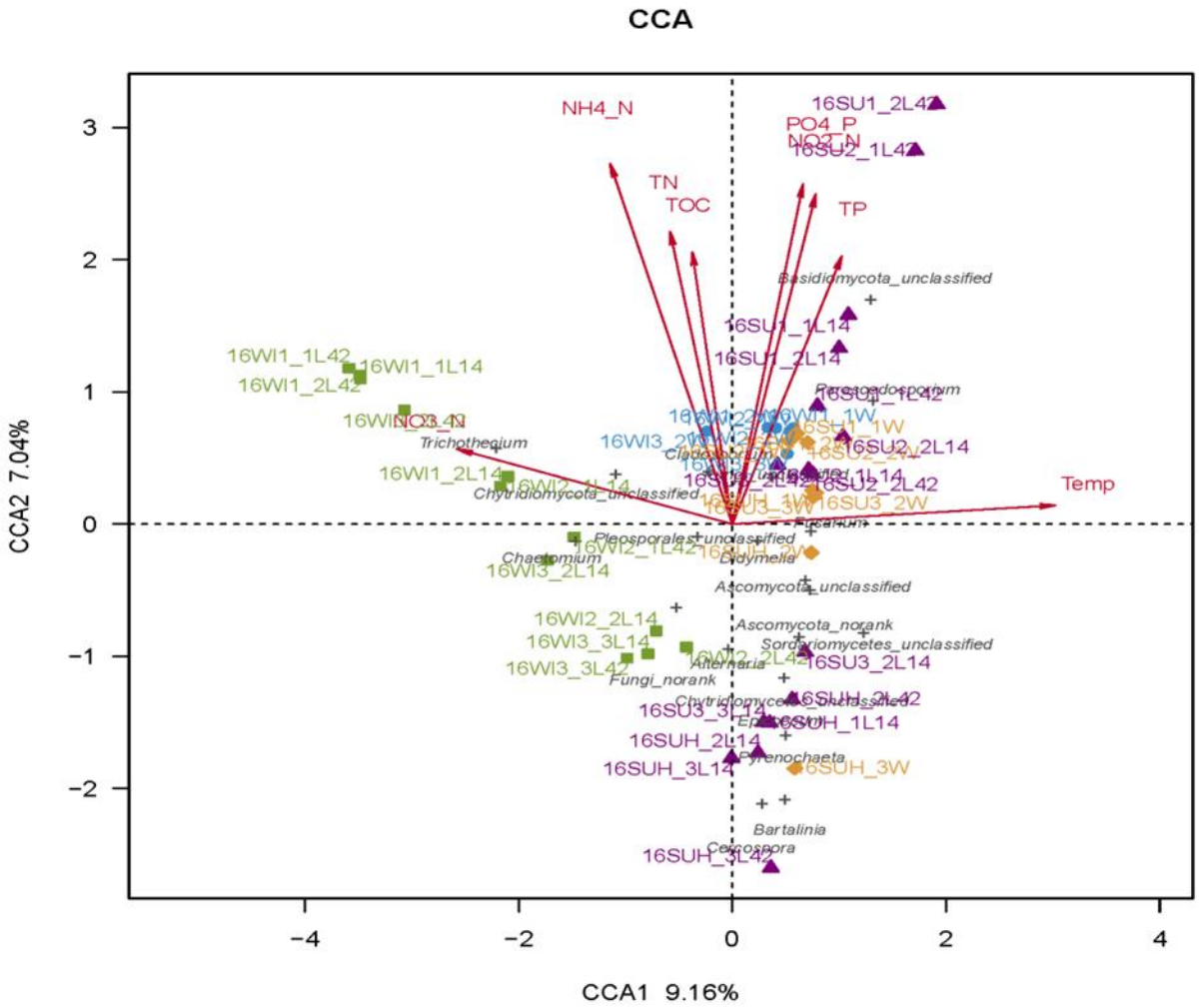



**Figure 3.28.** Canonical correspondence analysis (CCA) showing correlation of physico-chemical parameters with fungal genera in water and leaf samples collected from different sampling locations in Suzhou and Huangshan in winter and summer 2016.

### 3.3.7. Organic matter breakdown rate (ecosystem function)

Leaf litter breakdown (an indication of organic matter breakdown) was analyzed from the triplicate leaf bags collected from each location. Results of ash-free dry weight (AFDW) loss rate measured in samples collected on Day 14 and 42 during winter and summer in 2015 and 2016 are shown in Figure 3.29. The one-way ANOVA and unpaired t test analysis results (Table 3.5A) indicate that AFDW loss rate was significantly ($P = 0.000$) different between seasons whereas, no significant difference was observed among urban intensifications (Table 3.5B), no matter in short-term (14-day) or long-term (42-day). Average water temperatures calculated with data collected from waterproof temperature loggers in nine sampling locations in Suzhou in winter and summer 2015 and 2016 are showed in Table 3.6. According to average water temperatures from each location in each season, correlation between AFDW loss rate and temperature was confirmed by Pearson's correlation analysis for both short-term (14-day) (Table 3.7a) ($P < 0.0001$, $R^2 = 0.6407$) and long-term (42-day) (Table 3.7b) ($P < 0.0001$, $R^2 = 0.7078$).

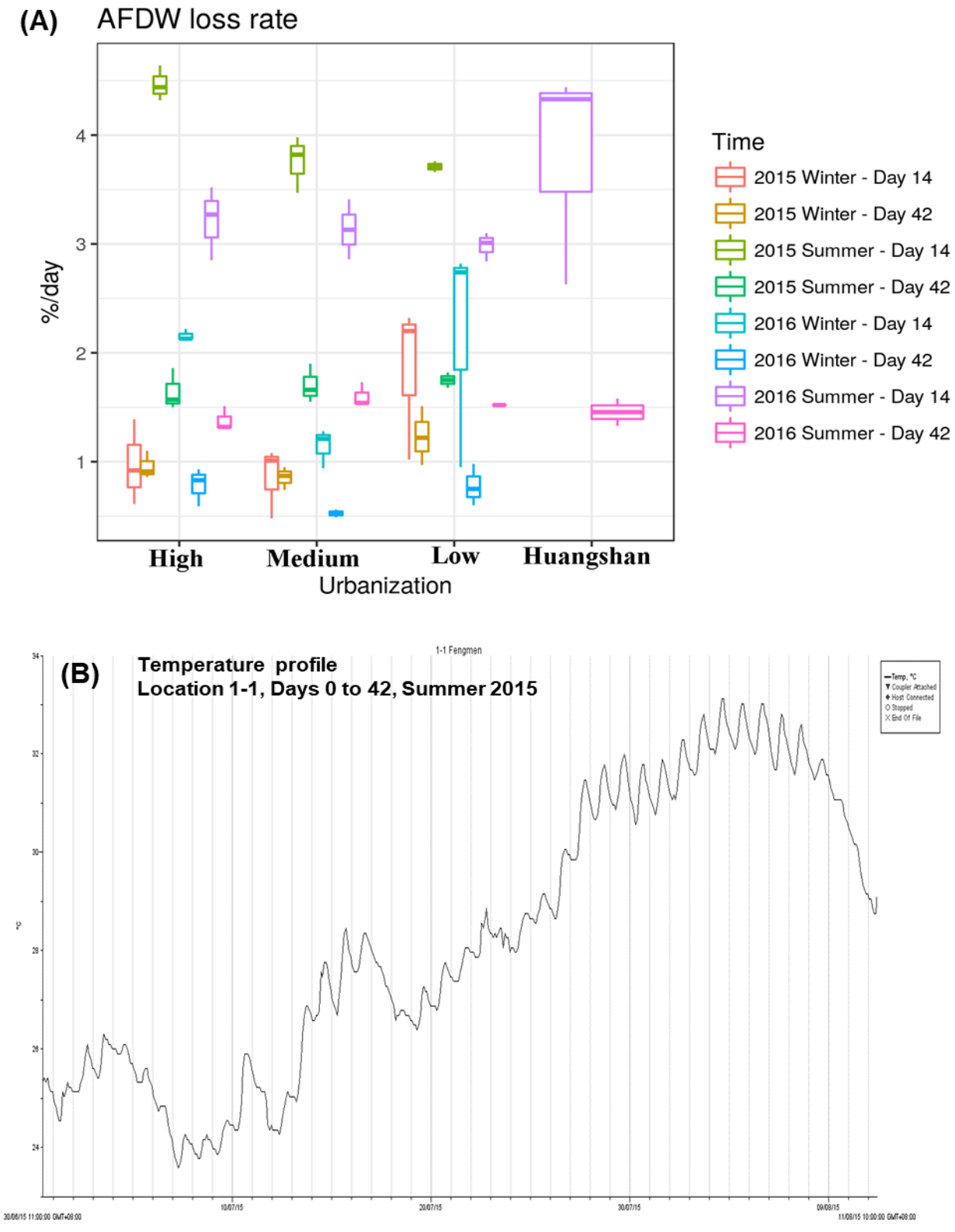



**Figure 3.29.** The organic matter breakdown (ash free dry weight loss, %) measurement to study the ecosystem function in Suzhou canals during winter and summer 2015 and 2016. A) The leaf samples were retrieved on 14$^{th}$ and 42$^{nd}$ day in each season. B) Temperature profile obtained for each sampling locations during the study period. The measurements were carried out using temperature logger and the readings were obtained for every 2 hours from day 0 to day 42.

**Table 3.5A.** Two-way ANOVA for variations of AFDW loss rate for both short- (14-day) and long- (42-day) term effect.

| Source | df | SS | MS | F | P |
|---|---|---|---|---|---|
| **Short-term (14-day)** | | | | | |
| A. Urbanization† | 2 | 0.106 | 0.053 | 2.018 | 0.154 |
| B. Season | 1 | 1.140 | 1.140 | 43.235 | **0.000** |
| A×B | 2 | 0.106 | 0.053 | 2.014 | 0.155 |
| **Long-term (42-day)** | | | | | |
| A. Urbanization | 2 | 0.015 | 0.075 | 0.830 | 0.448 |
| B. Season | 1 | 0.511 | 0.511 | 56.683 | **0.000** |
| A×B | 2 | 0.032 | 0.016 | 1.779 | 0.190 |

† Urbanization: High vs. Medium vs. Low;

df: degree of freedom; SS: Sum Square; MS: Mean Square

**Table 3.5B.** AFDW loss rate with varying urbanization.

| Urbanization | Mean Difference | Std. Error | Sig. (P) | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower Bound | Upper Bound |
| **Short-term (14-day)** | | | | | |
| High vs. Medium | 0.116 | 0.068 | 0.099 | -0.023 | 0.256 |
| High vs. Low | -0.023 | 0.081 | 0.783 | -0.190 | 0.145 |
| Medium vs. Low | -0.139 | 0.083 | 0.105 | -0.309 | 0.031 |
| **Long-term (42-day)** | | | | | |
| High vs. Medium | 0.024 | 0.040 | 0.556 | -0.058 | 0.105 |
| High vs. Low | -0.038 | 0.047 | 0.426 | -0.136 | 0.059 |
| Medium vs. Low | -0.062 | 0.048 | 0.210 | -0.162 | 0.037 |

**Table 3.6.** Average water temperature collected from waterproof temperature loggers in nine sampling locations across three urban intensifications in Suzhou in winter and summer 2015 and 2016.

| Location | 2015 Winter 0-14 day | 2015 Winter 0-42 day | 2015 Summer 0-14 day | 2015 Summer 0-42 day | 2016 Winter 0-14 day | 2016 Winter 0-42 day | 2016 Summer 0-14 day | 2016 Summer 0-42 day |
|---|---|---|---|---|---|---|---|---|
| 1-1 | 7.80 °C | 7.82 °C | 25.09 °C | 28.28 °C | 10.23 °C | 9.51 °C | 28.70 °C | 30.97 °C |
| 1-2 | 8.10 °C | 8.10 °C | 25.16 °C | 28.43 °C | 10.37 °C | 9.66 °C | 28.44 °C | 30.84 °C |
| 1-3 | 7.84 °C | 7.95 °C | 25.18 °C | 28.43 °C | 10.20 °C | 9.51 °C | 28.44 °C | 30.79 °C |
| 2-1 | 9.82 °C | 10.56 °C | 24.86 °C | 28.22 °C | 9.59 °C | 8.90 °C | 28.54 °C | 30.92 °C |
| 2-2 | 9.69 °C | 10.42 °C | 25.13 °C | 28.23 °C | 9.47 °C | 8.72 °C | 29.00 °C | 31.22 °C |
| 2-3 | 7.78 °C | 7.86 °C | 25.55 °C | 28.57 °C | 9.56 °C | 9.50 °C | 29.12 °C | 31.19 °C |
| 3-1 | 7.42 °C | 7.67 °C | * | * | 9.40 °C | 8.79 °C | * | * |
| 3-2 | 10.28 °C | 11.08 °C | 25.81 °C | 29.00 °C | 9.72 °C | 9.40 °C | 29.65 °C | 31.70 °C |
| 3-3 | 10.21 °C | 10.93 °C | 25.69 °C | 29.89 °C | 9.05 °C | 8.76 °C | 29.31 °C | 30.9 °C |

*Temperature loggers missed

**Table 3.7.** Pearson's correlation analysis of AFDW loss rate and temperature in short-term (14-day) (a) and long-term (42-day) (b).

(a)

| | |
|---|---|
| Number of XY Pairs | 33 |
| Pearson r | 0.8004 |
| 95% confidence interval | 0.6303 to 0.8972 |
| P value (two-tailed) | < **0.0001** |
| P value summary | **** |
| Is the correlation significant? (alpha=0.05) | **Yes** |
| R square | 0.6407 |

(b)

| | |
|---|---|
| Number of XY Pairs | 32 |
| Pearson r | 0.8413 |
| 95% confidence interval | 0.6971 to 0.9201 |
| P value (two-tailed) | < **0.0001** |
| P value summary | **** |
| Is the correlation significant? (alpha=0.05) | **Yes** |
| R square | 0.7078 |

### 3.4. Discussion

The microbial community results particularly the OTU results showed obvious variation in the bacterial and fungal composition were observed in Suzhou canals with respect to season and also water and leaf samples, which indicated temperature is a key factor that affect bacterial and fungal composition. The results were similar with a field research in Greece, a river which runs through agricultural, natural protected and urban sewage polluted area, but seasonal variations in the microbial community and function diversity were higher as compared to spatial variations (Meziti et al., 2016). Temporal heterogeneity in stream microbial diversity was previously observed (Zeglin, 2015) and correlation between the temperature and bacterial community changes were observed in a pond ecosystem (Zhong et al., 2018). Samad and Bertilsson also observed seasonal variation in diversity, abundance and community composition of bacterial methanotrophs in five temperate lakes (Samad and Bertilsson, 2017). Higher number of unique bacterial and fungal OTUs was observed in both water and leaf samples collected from Huangshan than Suzhou, which indicated Huangshan, as a control area in the absence any influence from urbanization and other anthropogenic activities, showed good water quality with high microbial diversity. However, the variation in bacterial and fungal community composition in water and leaf collected from different sampling locations in Suzhou did not show much variations. Within the bacterial community, Proteobacteria, as a major phylum of Gram-negative bacteria, was dominant in almost all the water and leaf samples collected in 2015 and 2016. Proteobacteria includes a wide range of bacteria involved in bio-geo chemical cycles, free living bacteria and potential pathogens (Woese, 1987). Burkholderiales was dominant in most of the samples at order level. At genus level, *Flavobacterium*, *Pseudomonas* and members of Comamonadaceae were represented at higher percentages in both water and leaf samples in winter than in summer. *Flavobacterium* was dominant (3-47%) in most water and leaf samples in winter 2015. Considering

temperature in Suzhou is much lower in winter as compared to summer, *Flavobacterium* may prefer to grow better at lower temperature. Members of *Flavobacteria* are found to be high in fresh water and several species may cause diseases of freshwater fish (Bernardet et al., 1996). For example, *F. psychrophilum* causes the bacterial cold water disease and the rainbow trout fry disease; *F. columnare* causes the cotton-wool disease on freshwater fishes; *F. branchiophilum* causes the bacterial gill disease (Bitinaite et al., 1998). *Milikia*, *Arcobacter* and *Polynucleobacter* were represented at higher percentages in water samples than in leaf samples, whereas *Acinetobacter* was represented at higher percentage in leaf samples than in water samples. Some bacterial genera (*Arcobacter*, *Massilia* and *Acinetobacter*) typically found in wastewater or associated with human / animal microbiomes, were represented at high percentages in high and medium urban intensification areas. *Arcobacter* was represented at 41-50% in water samples collected in 1-2 (high urban intensification) in winter 2015, which is in consistent with water samples in 1-2 were polluted by extremely high level of nutrients in winter 2015. Species of the genus *Arcobacter* are found in both animal and environmental sources (Donachie et al., 2005), some species can be human and animal pathogens (Miller et al., 2007). *Massilia* was represented at 31% in leaf samples collected in the location 2-1 on 14$^{th}$ day in winter 2015, two species were found associated to human health: *Massilia timonae* was isolated from blood of an immunocompromised patient (La Scola et al., 1998, Lindquist et al., 2003), and *Massilia consociata* was isolated from a human clinical specimen (Kampfer et al., 2011). *Acinetobacter* was represented at 49% in leaf samples collected in 2-1 on 14$^{th}$ day in summer 2015. *Acinetobacter* is widely distributed in nature particularly in water, soil and hospital environments and members of this genus has been reported to grow in wide range of temperature (Doughari et al., 2011). Some species are key sources of infection in debilitated patients in the hospitals (Dent et al., 2010), and *Acinetobacter* infection could cause an emerging threat to human health (Visca et al., 2011).

An earlier study showed that urban-influenced waterways harboured significantly greater bacterial abundance as compared to the bacterial diversity in Lake Michigan which is an oligotrophic lake (Newton and McLellan, 2015). Another study in Malaysia which was focused on a tropical stream reported that the urbanization had severe impact on bacterial diversity related leaf-litter decomposition in Ampang River (Yule et al., 2015). In an another study, Hosen et al. reported that urbanization not only strongly influenced headwater stream chemistry and hydrology, but also significantly impact the bacterial community composition (Hosen et al., 2017). Wang et al. also reported that watershed urbanization caused variation in the total and denitrifying bacterial community composition, and the changes were strongly associated with the gradient of urbanization (Wang et al., 2011b).

Within the fungal community, Ascomycota was dominant in most of the water and leaf samples. Ascomycota is a largest phylum in fungi which consists of over 64,000 species (Cavalier-Smith, 1998). Ascomycota are important decomposers and can breakdown organic matter, such as dead leaves. Along with other fungi, ascomycetes can breakdown large molecules, such as cellulose or lignin. It has been reported that members of Ascomycota and Basidiomycota play key roles in leaf litter breakdown in rivers and creeks (Ittner et al., 2018). At order level, Pleosporales was dominant (10-99%) in most leaf samples collected from locations with medium and low urbanization in winter and collected from Huangshan in summer 2016. The major species of Pleosporales are saprobes on decaying plant material in fresh water (Shearer et al., 2009), this was the reason that Pleosporales was represented at high percentage in most leaf samples. At genus level, *Trichothecium* was dominant (55-95%) for most leaf samples collected from locations with high urbanization in winter 2016. Members of *Trichothecium* are capable of growing in a variety of habitats, such as leaf litter. One of the species, *Trichothecium roseum*, is a plant pathogen and show a significant impact on human health (Batt and Tortorello, 2014). *Alternaria* was represented at higher

percentages in most leaf samples collected from Huangshan locations and low urbanization locations in Suzhou. *Alternaria* is ubiquitous in the environment and is a natural part of fungal flora can be found almost everywhere, such as soil and water (Kirk et al., 2008). *Alternaria* is normal agent of decay and decomposition (Nowicki et al., 2012), this was the reason that it represented at a high percentage in leaf samples collected from Huangshan where the waterways are protected. Based on beta diversity analysis (NMDS and clustering analysis), more obvious in bacterial and fungal community with respect to urbanization was observed in both water and leaf samples. The relationship between bacterial / fungal community and temperature / nutrient parameters was confirmed by heat maps and RDA / CCA, and nutrient parameters found to affect bacterial / fungal composition. These results are consistent with previous findings. Gardestrom et al. reported that fungicide and nutrients with enriched concentrations were stressors that affect microbial diversity in steams and also litter decomposition (Gardeström et al., 2016), and nutrient enrichment altered bacterial and fungal contributions to litter breakdown (Tant et al., 2015). Nutrient stoichiometry was an important factor in shaping microbial community structure in freshwater ecosystems (Lee et al., 2017). Nutrient availability together with other factors, such as hydrology, metal contamination, and land-use, were found to cause heterogeneity in bacterial diversity (Zeglin, 2015). Bacterial community composition in freshwater reservoirs was found to be strongly influenced by pH, alkalinity and organic carbon content (Lliros et al., 2014). The dynamic of aquatic fungal communities in a heavily-contaminated tropical river was mainly driven by seasonality and multiple physico-chemical parameters, such as pH, dissolved oxygen and multiple nutrients (Ortiz-Vera et al., 2018). In streams, higher level of nutrients and xenobiotics were observed in urbanized watersheds than in forested watersheds. However, fungal biomass in leaves was lower in urbanized watersheds than in forested watersheds, due to pesticide toxicity (Rossi et al., 2019). Bai reported that fungal community in the aquatic system can act as bioindicator as

their study found that the level of anthropogenic activity was strongly correlated with the water quality and plankontic fungal community (Bai et al., 2018). In our study, the changes in the bacterial and fungal diversity were observed with respect to type of samples (water or leaf) and seasons (winter or summer); some variations across the urban gradients were also observed, as the nutrient levels were highly different between water samples from high and low urbanization locations. It appears from this study that (and also from our microcosm study, reported in Chapter 4) the bacterial community was less affected by the nutrients as compared to the seasons or the sampling dates. Perhaps, studies focusing on functional microbial communities may provide better pictures on the impact of urbanization in Suzhou canals.

The results of leaf litter breakdown were consistent with the observation in the field study by Martinez et al. that the positive relationship between temperature and leaf breakdown rate was found (Martinez et al., 2014), this field study carried out in forest streams in Spain by using leaf bag experiments to find out the effect of temperature on the headwater stream functioning. Batista et al. also reported that increasing temperature stimulated leaf decomposition by microbes (Batista et al., 2012). Temperature was found to be the most important environmental factor for microbial breakdown (Mora-Gómez et al., 2015).

### 3.5. Conclusions

The results obtained from this study showed that the bacterial and fungal community composition varied with respect to samples (water vs. leaf samples) and seasons, whereas fewer variations were observed with respect to urbanization except in few locations. However, the microbial communities observed in Suzhou sampling locations were very different from the community observed in the locations in Huangshan. The bacterial phylum,

Proteobacteria was dominant in almost all the water and leaf samples, followed by either Bacteroidetes in winter or Actinobacteria during summer in water samples. In leaf samples, the Proteobacteria were dominant followed by dominance of Bacteroidetes during winter and Firmicutes and/or Chloroflexi during summer. At genus level, *Flavobacterium*, *Pseudomonas* and members of Comamonadaceae were represented at higher percentages in both water and leaf samples in winter than in summer. *Malikia*, *Arcobacter* and *Polynucleobacter* were represented at higher percentages in water samples than in leaf samples, whereas *Acinetobacter* was represented at higher percentage in leaf samples than in water samples. For fungal community at phylum level, Ascomycota was dominant in most samples, especially in leaf samples. Some fungal genera (such as *Trichothecium*), associated with pathogens/microbiomes and human health, were represented at high percentages in high urban intensification areas; whereas natural fungal flora (such as *Alternaria*), associated with decomposition / ecosystem function, were represented at high percentages in low urban intensification areas and natural reserve areas. These findings indicated ecosystem functions and services were well maintained in freshwater systems with less human eutrophic activities. Beta diversity (NMDS and cluster) analysis showed more obvious variation of urbanization in Suzhou on bacterial and fungal community, as compared to OTU and community analysis. Heat maps and RDA / CCA confirmed the effects of both temperature and nutrient parameters on shifting composition of microbial (bacterial / fungal) community. Ecosystem function (AFDW loss rate) was significantly affected by season rather than urbanization; correlation between AFDW loss rate and temperature was also confirmed, and high temperature accelerate AFDW loss rate. In summary, the results showed that microbial diversity and ecosystem function in Suzhou canals were affected by both season and urbanization, but season had a greater influence than urbanization.

# Chapter 4 - Assessing the Influence of Key Environmental Parameters on the Bacterial Diversity and Ecosystem Function in Freshwater Ecosystems by Laboratory Microcosm

***Abstract***

With rapid urbanization in China, surface water bodies in urban areas are polluted by multiple contaminants, which cause serious issues to water quality, as well as flow-on effects on freshwater biodiversity and ecosystem function. Microbial communities are fundamental components in freshwater ecosystems and indicate shifts in ecosystem structure due to changing environmental conditions. Therefore, this study was aimed at investigating the influence of key environmental parameters, such as temperature, nutrients and heavy metals, on microbial diversity and ecosystem function by laboratory microcosm studies. In the microcosm, four water baths were set at three different temperatures (5, 20 and 35 °C) to study the impact of varying temperatures on the bacterial diversity and ecosystem function. One water bath was set at 20 °C and water samples collected from three different urban gradients (low, medium and high) with and without addition of nutrients (ammonium and phosphate) were used to study the impact of nutrients on bacteial diversity and ecosystem function. To study the effect of heavy metals, three different concentrations (0.001 mM, 0.01 mM and 0.1 mM) of $CuCl_2$ and $ZnCl_2$ were added separately to the 2$^{nd}$ microcosm which was set at 20 °C. Capsules filled with polycaprolactonediol 2000 (PCP 2000) (1g in each capsule) were immersed in the water samples and they were collected on 14$^{th}$ (short-term) and 42$^{nd}$ (long-term) day to assess the organic matter (OM) decomposition rate. Leaf bags were deployed in every microcosm and the bags were retrieved on the same day as PCP 2000 and used for DNA extraction to study the bacterial community by NGS of 16S rRNA gene amplicons. The results indicated that higher temperature and nutrients accelerated PCP 2000 breakdown rate and high concentration (100 μM) of Cu reduced PCP 2000 breakdown rate in the short-term. Based on OTU, alpha and beta diversity analyses, it was observed that temperature was found to affect the composition of bacterial community and the bacterial diversity increased at high temperature (35 °C) with more OTUs as compared to medium

(20 °C) or low (5 °C) temperatures, whereas nutrients had fewer effects on the composition of bacterial community. In contrast, heavy metals had potential effects on the bacterial community with reduced OTUs. The results from the present study indicate that temperature is a key factor that affects bacterial diversity and ecosystem function, and high temperature increased bacterial diversity and accelerated OM breakdown rate. Heavy metals are also important factor particularly Cu, which in high concentration (100 μM) decreased bacterial diversity and reduced OM breakdown rate.

### 4.1. Introduction

Rapid economic development and high speed of urbanization in China in the last decades had positive impact on various aspects of society development. However, urbanization also had negative impact on surface water bodies. The water quality has been affected by excess nutrients such as endocrine disrupting chemicals, antibiotics, steroid hormones, pharmaceuticals and personal care products (PPCPs) (Chang et al., 2009, Du et al., 2010, Dai et al., 2016, Sun et al., 2016b, Xu et al., 2018). Many pollutants that impact waterways have also had flow-on effects on freshwater biodiversity, shifts in activity and diversity of the functional groups (Zhang et al., 2015). Loss of biodiversity and ecosystem function were identified as major issues resulting from declining water quality in waterways (Qu and Fan, 2010). Loss of lateral hydrological connectivity (LHC) was a key cause of decline in biodiversity and functional richness in Yangtze River (Liu and Wang, 2018). Ecosystems contain multiple functions, which requires great biodiversity; however, different ecosystem functions are influenced significantly by different sets of species (Hector and Bagchi, 2007) and species loss could accelerate shifts in ecosystem processes such as decomposition and productivity (Hooper et al., 2012). To investigate the relationship between microbial biodiversity and ecosystem functions, the key is to understand how differences in

the structure and performance of microbial communities translate into differences in function (Naeem et al., 2009). The link between microbial diversity and ecosystem processes is a fundamental research goal for microbial ecology (Bernhard and Kelly, 2016). Microbial communities are the fundamental components of freshwater ecosystems which can indicate shifts in ecosystem structure as microbes play major roles, such as primary production, decomposition and degradation of pollutants. All these roles are important for nutrients cycling and ecosystem services (Paerl et al., 2003, Naeem et al., 2009, Kim et al., 2014). The recent advances in molecular techniques, particularly 'omics', enable researchers to study microbial diversity at high resolution and its relevance to the metabolic activity or specific function/processes (Morales and Holben, 2011). Metagenomics and Geo-Chip have also been applied to characterize microbial communities to determine microbial diversity and potential functional structures (Bertilsson et al., 2012, Wang et al., 2014, Llorens-Mares et al., 2015, Gourmelon et al., 2016). Microbial communities in the ecosystem play other functions such as removal of nutrients or contaminants. Ecosystems with more species are more efficient in removing nutrients from water than ecosystems with fewer species, so that conservation of biodiversity could be a useful tool for controlling nutrient levels in watersheds (Cardinale et al., 2012). Therefore, it is important to find out how changes in key environmental parameters, such as temperature, pH, nutrients, heavy metals and antibiotics affect microbial diversity and ecosystem function.

In recent years, there has been a push to not only assess the structure of freshwater communities but also the function of these communities and also to study the relationship between microbial diversity and ecosystem functioning (Duarte et al., 2010, Delgado-Baquerizo et al., 2016, Daam et al., 2019, Kumar et al., 2019). Combining eco-physiological studies with bio-molecular techniques in biodiversity-ecosystem functioning (BEF) research reinforces the ability to link microbial diversity to ecosystem processes (Dudgeon and Gao,

2010, Krause et al., 2014, Bernhard and Kelly, 2016). One of the most regularly used measures for river ecosystem function is leaf breakdown (organic matter decomposition) rates. Leaf breakdown is a key process that drives the flow of nutrients and energy in freshwater ecosystem (Clapcott et al., 2012, Collier et al., 2013, Thompson et al., 2016). Organic matter decomposition was affected by shifts of land-use types in varying ways (Young and Collier, 2009, Gardeström et al., 2016). Besides leaf bag experiments, a new method, using synthetic organic material – polymers, has been developed. Therefore breakdown rate of synthetic polymers provided an improved method for field research, which could complement traditional leaf bag experiments to assess water ecosystem function (Rivas et al., 2016). Although this new method has still not been widely used for assessing ecosystem function, some studies have been carried out, which indicated the potential of using polymers for ecosystem function assessment and research on environmental issues (Pauli et al., 2017, Dey and Tribedi, 2018).

An earlier study showed that eutrophic waterways in an urbanized estuary harboured significantly greater bacterial diversity than an oligotrophic lake (Newton and McLellan, 2015). Eco-genomics studies revealed that metals pollution caused by land-use pressures are major drivers of microbial communities and ecosystem functions in the waterways of megacities (Saxena et al., 2015, Emilson et al., 2016, Hosen et al., 2017). However, detailed effects of specific changes in environmental parameters caused by urbanization, land use shift and other factors on microbial diversity – ecosystem function relationships were not studied well. The main scope of the research is to investigate the influence of key environmental parameters (temperature, nutrients and heavy metals) on microbial diversity and ecosystem function in freshwater ecosystem through microcosm studies. The lab microcosm studies were planned based on two years of field work in Suzhou canals.

## 4.2. Materials and Methods

### 4.2.1. Microcosm set up and experiments

The influence of key environmental parameters (temperature, nutrients and heavy metals) on the microbial diversity and ecosystem function (in terms of decomposition of organic matter) relationship was assessed by microcosm experiments. The microcosm experiments were set up as shown in the schematic diagram (Figure 4.1). Four water baths were set up at three different temperatures (5, 20 and 35 °C; 2 baths were set at 20 °C) to study the influence of varying temperatures on the microbial diversity and ecosystem function. One of the water baths set at 20 °C was used for nutrients group. The water samples for this experiment were collected from Suzhou canals representing three different urban gradients (Location 1-2, 2-1 and 3-3 showed in Chapter 2 were selected to represent High, Medium and Low urbanization, respectively) based on the two years of field study. The water samples for nutrients group were amended with $KH_2PO_4$ and $NH_4Cl$ (1 μM and 50 μM, respectively) as suggested previously to study the effect of ammonium and phosphate (Rivas et al., 2016). To study the effect of heavy metals, three different concentrations (1 μM, 10 μM and 100 μM for low, medium and high concentration respectively) of heavy metal salts ($CuCl_2$ and $ZnCl_2$) were added separately to the 2$^{nd}$ microcosm which was set at 20 °C. To control the background values of heavy metals' concentrations, only water samples collected from Medium urbanization (Location 2-1) were used in heavy metals group, as water samples collected from medium urbanization indicated the average water quality status in Suzhou. Microcosm with water samples collected from Medium urbanization (Location 2-1) without addition of nutrients in the first water bath set at 20 °C, served as control. The water samples were changed every two weeks until the end of the experiments. Capsules filled with polycaprolactonediol 2000 (PCP 2000) (SIGMA-ALDRICH, Co., USA) (1g in each) were immersed in the water samples and they were collected on 14$^{th}$ and 42$^{nd}$ day to assess the OM

decomposition rate as reported previously (Rattanapan et al., 2016, Rivas et al., 2016). Three grams of dried willow (*Salix* sp.) leaves were weighed by using a digital balance and placed in ready-made nylon bags (15 x 10 cm dimension with 0.5-1.0 mm mesh) along with a number tag. Leaf bags were deployed in each microcosm and retrieved on the same dates as PCP 2000 ($14^{th}$ and $42^{nd}$ day). Leaf bags were used for studying the bacterial communities.

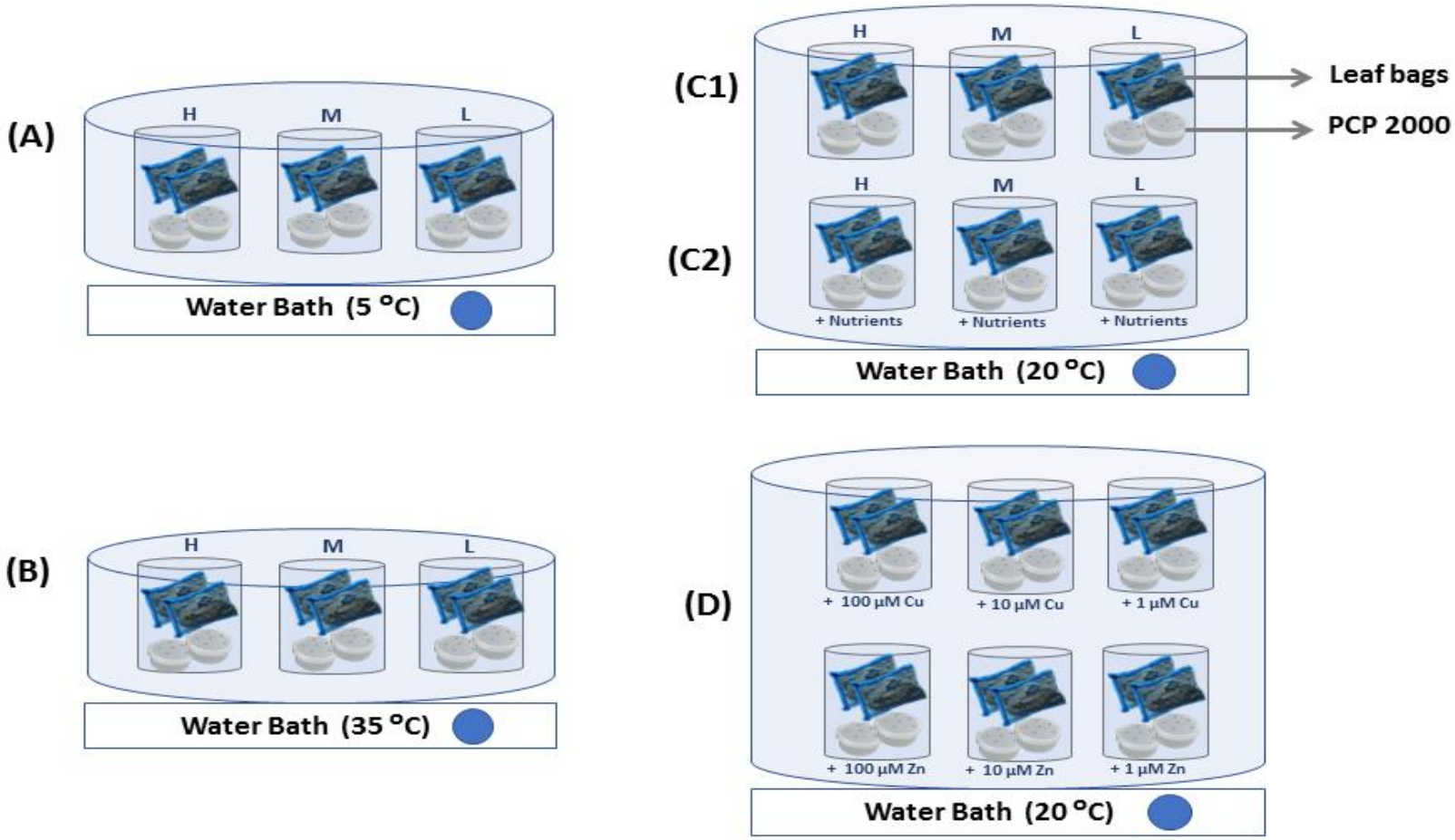



**Figure 4.1.** Schematic diagram of microcosm experimental setup to study the influence of temperature (A, B and C1), nutrients (C2) and heavy metals (D) on the microbial diversity and ecosystem function. The water samples used in (D) were collected from Medium urbanization location. Microcosm with water samples collected from Medium urbanization location without addition of nutrients and heavy metals served as control.

### 4.2.2. Collection of water samples for microcosm experiments and physico-chemical and microbiological analysis

The water samples were collected from the Suzhou canals representing three urbanization gradients (1-High, 2-Medium and 3-Low). One 5-liter of water sample was collected from each sampling location and used for the microcosm experiments particularly to study the influence of water temperature and nutrients on microbial diversity and ecosystem function (Figure 4.1). The samples were also used to characterize the physico-chemical and microbiological parameters including temporal water temperature (WT), pH, electrical conductivity (EC), dissolved oxygen (DO), total dissolved solids (TDS), total nitrogen (TN), total phosphorous (TP), nitrate-N ($NO_3$-N), nitrite-N ($NO_2$-N), ammonium nitrogen ($NH_4$-N), phosphate ($PO_4$-P), total organic carbon (TOC), chlorophyll *a* (Chl *a*) and total coliforms (TC) (Figure 4.2).

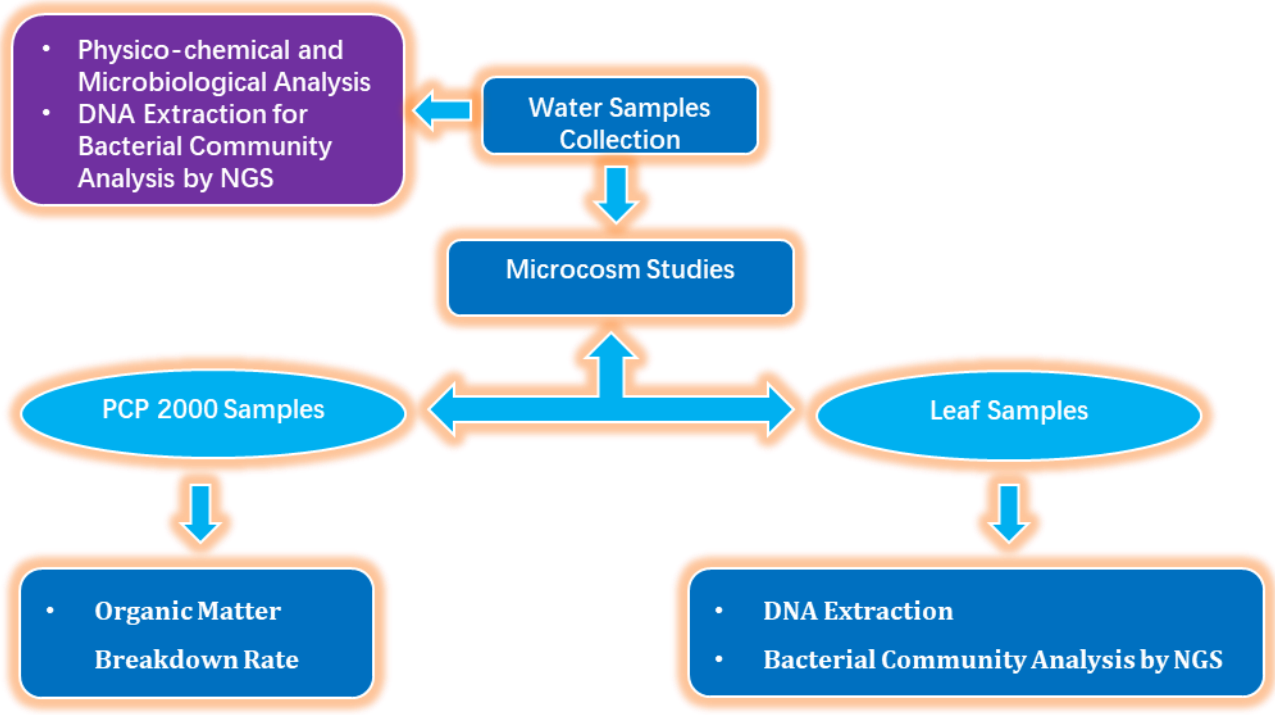



**Figure 4.2.** Schematic diagram showing various analyses carried out in this study.

### 4.2.3. DNA extraction and Bacterial community analysis

*DNA extraction from water and leaf samples:*

Water samples (500 mL for each) were filtered through 0.22 µm 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 grinded using liquid nitrogen and the extracted DNA was quantified using a Nanodrop, verified by gel electrophoresis and stored at -20 °C until further processing.

*Bacterial community analysis:*

The bacterial communities in water and leaf samples were studied by next generation sequencing. Primer sequences which target V3 and V4 regions (CCTACGGRRBGCASCAGKVRVGAAT and GGACTACNVGGGTWTCTAATCC) of the 16S rRNA gene were used to study bacterial diversity by using the MiSeq250 platform. Illumina sequencing applied clonal array assays for rapid and accurate large-scale sequencing, which has been widely used for microbial community analysis (Xie et al., 2015, Wang et al., 2018, Wang et al., 2019).

The PCR reactions were performed in triplicate. The PCR mixture (20 µL) contained 2 µL of 10 × Taq buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.2 µL of Takara Taq Polymerase, 0.2 µL of BSA and 10 ng of template DNA. The PCR cycling conditions used were: 95 °C for 3 mins, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 10 mins. The PCR amplified products were extracted and purified using AxyPrep DNA extraction kit and sequenced using Illumina MiSeq platform according to the standard protocols. The sequencing and the preliminary data

analyses were carried by the GENEWIZ, Inc. Suzhou, China. The sequencing results were analyzed by using VSEARCH (1.9.6) (Rognes et al., 2016, Jin et al., 2019) for sequence clustering and operational taxonomic units (OTUs) were clustered with 97% similarity cut-off. VSEARCH, as a versatile open source tool for preparing and processing metagenomics and genomics nucleotide sequence data, includes many commands for analyzing nucleotide sequences, such as clustering by similarity (Rognes et al., 2016). Silva 128 (Pruesse et al., 2007, Quast et al., 2013, Pfeiffer et al., 2014) was used as the reference database for 16S rRNA analysis; RDP (Ribosomal Database Program) (Wang et al., 2007b, Cole et al., 2014) classifier Bayesian algorithm was used for OTU analysis.

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.

### 4.2.4. Organic matter breakdown rate

The organic matter breakdown rate is simply the percent of PCP 2000 weight loss over the period when it was immersed in microcosm. Prior to microcosm experiment, approximately 1 g of dry PCP 2000 samples were weighed by using a digital balance and the exact readings were noted down. Then, along with a number tag, the PCP 2000 samples were filled in a capsule (size: 4 cm - top diameter, 3.1 cm - bottom diameter, 3.3 cm - height) with 0.2mm-diameter holes on the top. Placed the capsule in microcosm and water samples could flow into the capsule smoothly through the holes, which ensured the PCP 2000 samples were immersed in water and also avoided PCP 2000 overflowing the capsule. After 14 or 42 days, the PCP 2000 samples were collected and dried to a constant weight during 5-7 days, then weighed the final dry weight to compare with the initial weight and calculated PCP 2000 weight loss rate.

### 4.2.5. Statistical 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 (Cu and Zn) were analyzed by ANOVA.

Based on OTU analysis results, 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). The variations in the diversity between samples (beta diversity) were studied by Nonmetric Multidimensional Scaling (NMDS) analysis, Principal Coordinate Analysis (PCoA) and cluster analysis.

## 4.3. Results

### 4.3.1. Physico-chemical and microbiological properties

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 4.1. The results reflect actual water quality gradient in Suzhou canals and consistent with the water quality results obtained during the field study (Chapter 2). Therefore, the water samples used for the microcosm experiments are highly representative for the study.

**Table 4.1.** Physico-chemical and microbiological parameters of water samples collected from three sampling locations across three urban intensifications in Suzhou for microcosm studies.

| **Location** | WT (°C) | pH | EC (µS/cm) | DO (mg/L) | TDS (mg/L) | TN (mg/L) | TP (mg/L) | $NO_3$-N (mg/L) | $NO_2$-N (mg/L) | $PO_4$-P (µg/L) | $NH_4$-N (mg/L) | TOC (PPM) | Chl *a* (µg/L) | TC (cfu/mL) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **H (1-2)** | 32.4 | 7.91 | 569 | 9.21 | 373 | 2.77 | 0.159 | 0.596 | 0.096 | 174.3 | 0.763 | 2.89 | 16.04 | 867 |
| **M (2-1)** | 32.9 | 8.1 | 561 | 6.63 | 660 | 1.66 | 0.065 | 0.680 | 0.125 | 49.43 | 0.774 | 3.36 | 67.05 | 260 |
| **L (3-3)** | 32.9 | 7.99 | 340 | 10.39 | 674 | 1.74 | 0.088 | 0.900 | 0.025 | 40.92 | 0.091 | 9.26 | 9.65 | 593 |

### 4.3.2. Organic matter (OM) breakdown rate

PCP 2000 breakdown rate (OM breakdown rate) was measured for each group of the experiments tested (e.g. the influence of temperature, nutrients and heavy metals). The degradation rate significantly varied between the temperatures tested; the higher temperature (35 ºC) accelerated PCP 2000 breakdown rate, no matter for short-term or long-term (Figure 4.3; Table 4.2 A and B).

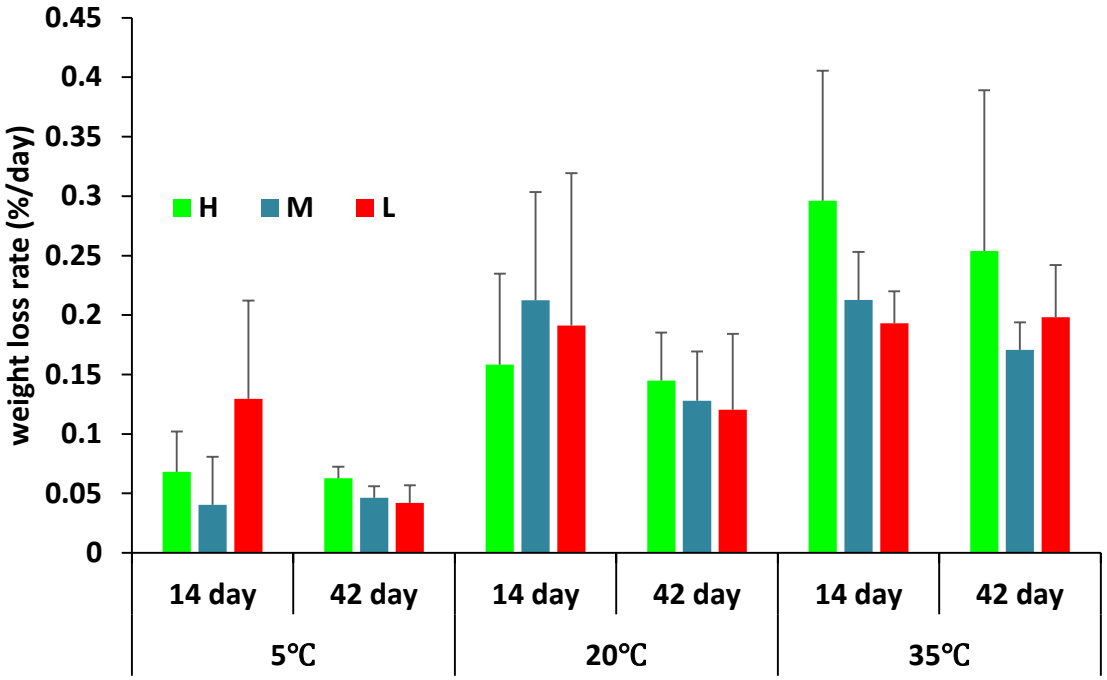



**Figure 4.3.** The effect of temperature on the organic matter (PCP 2000) breakdown rate. (PCP 2000 samples in quadruplicate were used for the study)

**Table 4.2A.** Results of two-way ANOVA showing the influence of varying temperatures and urbanization gradients on the organic matter (PCP 2000 breakdown rate) in short-term and long-term.

| Source | df | SS | MS | F | P |
|---|---|---|---|---|---|
| **Short-term (14-day)** | | | | | |
| A. Urbanization† | 2 | 0.003 | 0.001 | 0.210 | 0.812 |
| B. Temperature‡ | 2 | 0.151 | 0.075 | 12.486 | **0.000** |
| A×B | 4 | 0.044 | 0.011 | 1.823 | 0.154 |
| **Long-term (42-day)** | | | | | |
| A. Urbanization | 2 | 0.011 | 0.005 | 1.629 | 0.203 |
| B. Temperature | 2 | 0.148 | 0.074 | 23.459 | **0.000** |
| A×B | 4 | 0.006 | 0.001 | 0.466 | 0.760 |

† Urbanization: High vs. Medium vs. Low; ‡ Temperature: 35 vs. 20 vs. 5;
df: degree of freedom; SS: Sum Square; MS: Mean Square.

**Table 4.2B.** Comparison between different temperatures on the PCP 2000 breakdown rate in short-term and long-term.

| Temperature | Mean Difference | Std. Error | Sig. (P) | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower Bound | Upper Bound |
| **Short-term (14-day)** | | | | | |
| 35 ºC vs. 20 ºC | 0.047 | 0.032 | 0.153 | -0.019 | 0.112 |
| 35 ºC vs. 5 ºC | 0.155 | 0.032 | **0.000** | 0.089 | 0.220 |
| 20 ºC vs. 5 ºC | 0.108 | 0.032 | **0.002** | 0.043 | 0.173 |
| **Long-term (42-day)** | | | | | |
| 35 ºC vs. 20 ºC | 0.077 | 0.023 | **0.002** | 0.029 | 0.124 |
| 35 ºC vs. 5 ºC | 0.157 | 0.023 | **0.000** | 0.110 | 0.204 |
| 20 ºC vs. 5 ºC | 0.081 | 0.023 | **0.002** | 0.034 | 0.128 |

The OM breakdown rate in the nutrients group was compared with control group. $KH_2PO_4$ and $NH_4Cl$ (final concentration addition: 1 μM and 50 μM) were amended in the water for nutrients group, whereas originally collected water samples without any nutrient materials amended were used for control group. Higher concentrations of nutrients accelerated PCP 2000 breakdown rate, particularly in the short-term (14-day) (Figure 4.4). However, no variation between control and nutrient group was observed based on ANOVA analysis (Table 4.3).

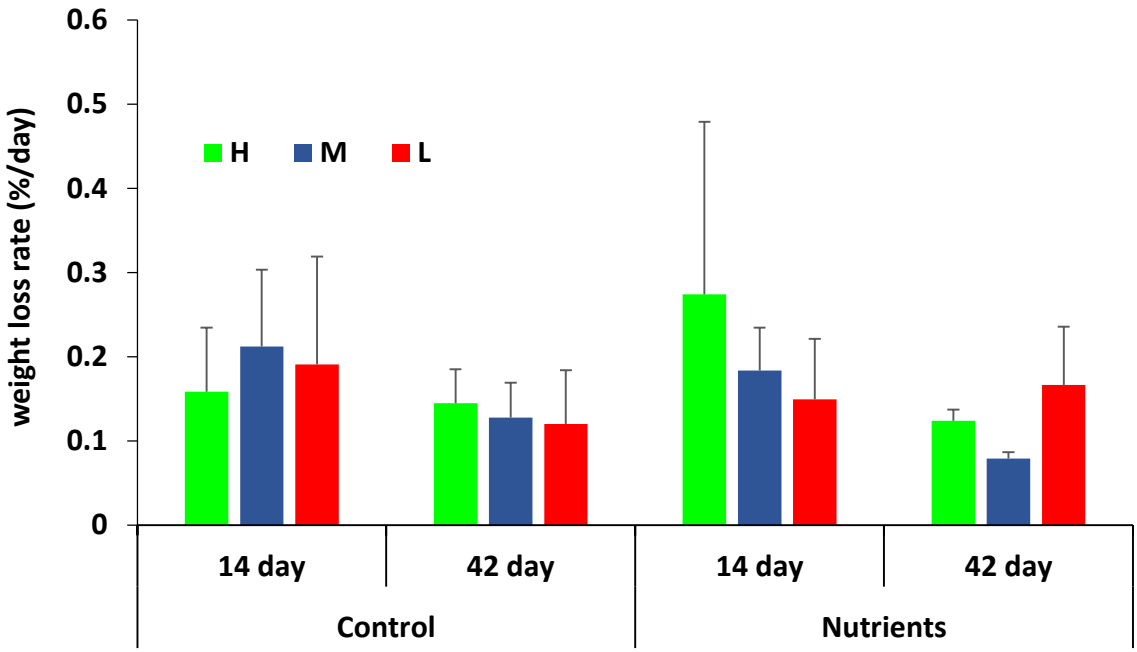



**Figure 4.4.** The organic matter (PCP 2000) breakdown in the nutrients group and control group. (PCP 2000 samples in quadruplicate were used for the study)

**Table 4.3.** Results of two-way ANOVA showing the influence of varying nutrients and urbanization gradients on the organic matter (PCP 2000 breakdown rate) in short-term and long-term.

| Source | df | SS | MS | F | P |
|---|---|---|---|---|---|
| **Short-term (14-day)** | | | | | |
| A. Urbanization† | 2 | 0.009 | 0.004 | 0.320 | 0.730 |
| B. Nutrient‡ | 1 | 0.001 | 0.001 | 0.102 | 0.753 |
| A×B | 2 | 0.030 | 0.015 | 1.138 | 0.343 |
| **Long-term (42-day)** | | | | | |
| A. Urbanization | 2 | 0.007 | 0.004 | 1.705 | 0.210 |
| B. Nutrient | 1 | 0.000 | 0.000 | 0.178 | 0.678 |
| A×B | 2 | 0.010 | 0.005 | 2.309 | 0.128 |

† Urbanization: High vs. Medium vs. Low; ‡ Nutrient: Control vs. Nutrient;

df: degree of freedom; SS: Sum Square; MS: Mean Square

The influence of heavy metals (Cu and Zn) at varying concentrations on the PCP 2000 breakdown rate was compared with control group. High concentration (100 μM) of Cu reduced PCP 2000 breakdown rate in short-term (14-day). However, no effect of Zn on breakdown rate was observed (Figure 4.5 and Table 4.4 A and B). The results of ANOVA showed that in short-term, variation between High vs. Control, High vs. Low and High vs. Medium were observed for the effect of Cu on the PCP 2000 breakdown rate.

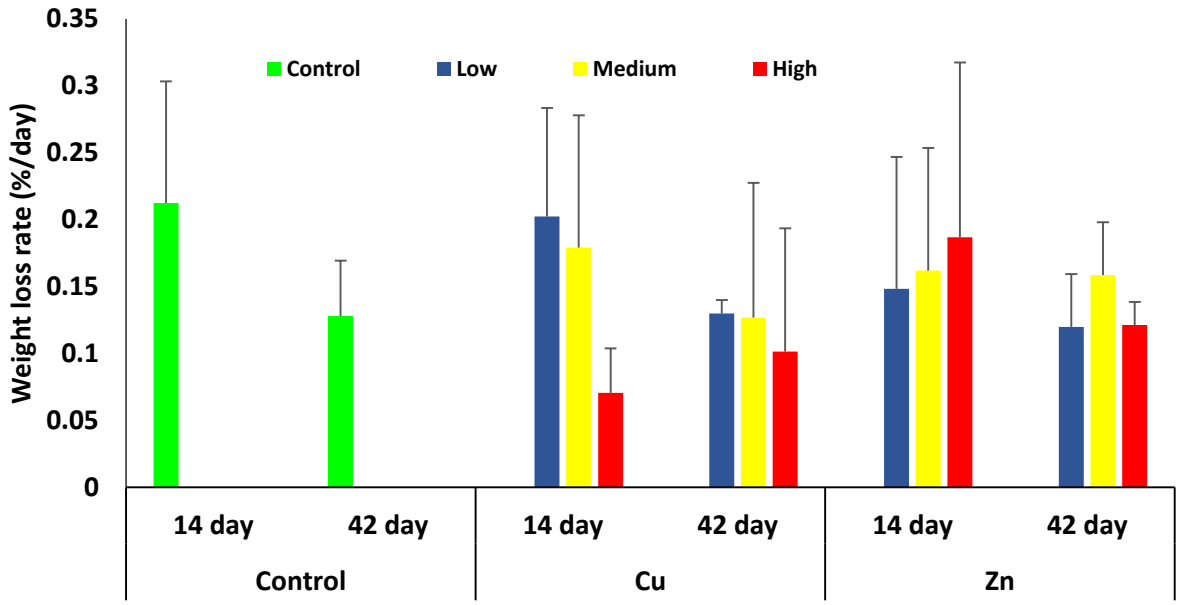



**Figure 4.5.** The effect of heavy metals (Cu and Zn in three different concentrations) on the PCP 2000 breakdown rate. (PCP 2000 samples in quadruplicate were used for the study) The water samples used for this study were collected from medium urbanization location. The microcosm with water samples collected from medium urbanization location without addition of heavy metals served as control.

**Table 4.4A** Results of ANOVA showing the influence of heavy metals (Cu and Zn) on the organic matter (PCP 2000) breakdown rate in short-term and long-term.

| Source | df | SS | MS | F | P |
|---|---|---|---|---|---|
| **Short-term (14-day)** | | | | | |
| Cu† | 3 | 0.607 | 0.202 | 4.646 | **0.022** |
| **Long-term (42-day)** | | | | | |
| Cu | 3 | 0.116 | 0.039 | 0.689 | 0.576 |
| **Short-term (14-day)** | | | | | |
| Zn‡ | 3 | 0.087 | 0.029 | 0.475 | 0.705 |
| **Long-term (42-day)** | | | | | |
| Zn | 3 | 0.041 | 0.014 | 0.945 | 0.450 |

† Cu: High vs. Medium vs. Low vs. Control; ‡ Zn: High vs. Medium vs. Low vs. Control;

df: degree of freedom; SS: Sum Square; MS: Mean Square.

**Table 4.4B** Comparison between heavy metals (Cu and Zn) in different concentrations (Low, Medium and High) on the PCP 2000 breakdown rate in short-term and long-term.

| Heavy metals | Mean Difference | Std. Error | Sig. (P) | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower Bound | Upper Bound |
| **Cu _ Short-term (14-day)** | | | | | |
| Control vs. High | 0.482 | 0.148 | **0.007** | 0.161 | 0.804 |
| Control vs. Medium | 0.087 | 0.148 | 0.565 | -0.234 | 0.409 |
| Control vs Low | 0.027 | 0.148 | 0.856 | -0.294 | 0.349 |
| High vs. Medium | -0.395 | 0.148 | **0.020** | -0.716 | -0.073 |
| High vs. Low | -0.455 | 0.148 | **0.010** | -0.776 | -0.133 |
| Medium vs. Low | -0.060 | 0.148 | 0.691 | -0.382 | 0.262 |
| **Cu _ Long-term (42-day)** | | | | | |
| Control vs. High | 0.196 | 0.168 | 0.264 | -0.169 | 0.562 |
| Control vs. Medium | 0.072 | 0.168 | 0.675 | -0.293 | 0.437 |
| Control vs Low | -0.022 | 0.168 | 0.898 | -0.387 | 0.343 |
| High vs. Medium | -0.124 | 0.168 | 0.473 | -0.489 | 0.241 |
| High vs. Low | -0.218 | 0.168 | 0.217 | -0.583 | 0.147 |
| Medium vs. Low | -0.094 | 0.168 | 0.585 | -0.459 | 0.271 |
| **Zn _ Short-term (14-day)** | | | | | |
| Control vs. High | 0.091 | 0.174 | 0.612 | -0.289 | 0.471 |
| Control vs. Medium | 0.129 | 0.174 | 0.473 | -0.251 | 0.509 |
| Control vs Low | 0.204 | 0.174 | 0.264 | -0.176 | 0.584 |
| High vs. Medium | 0.038 | 0.174 | 0.830 | -0.342 | 0.418 |
| High vs. Low | 0.114 | 0.174 | 0.527 | -0.267 | 0.494 |
| Medium vs. Low | 0.075 | 0.174 | 0.674 | -0.305 | 0.455 |
| **Zn _ Long-term (42-day)** | | | | | |
| Control vs. High | 0.010 | 0.085 | 0.907 | -0.175 | 0.195 |
| Control vs. Medium | -0.100 | 0.085 | 0.261 | -0.285 | 0.085 |
| Control vs Low | 0.031 | 0.085 | 0.722 | -0.154 | 0.216 |
| High vs. Medium | -0.110 | 0.085 | 0.218 | -0.295 | 0.075 |
| High vs. Low | 0.021 | 0.085 | 0.811 | -0.164 | 0.206 |
| Medium vs. Low | 0.131 | 0.085 | 0.149 | -0.054 | 0.316 |

### 4.3.3. Bacterial community analysis by NGS

The bacterial community in the water and leaf samples collected from microcosm experiments were investigated by next-generation sequencing (NGS) of 16S rRNA gene amplicons. The rarefaction curves showed saturated trend in the observed OTUs (Figure 4.6) and high Good's coverage (0.995-1) (Table 4.6) 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. These sequences were classified into OTUs with 97% similarity cut-off. As shown in Tables 4.5 and 4.6, the samples were grouped in to different categories for further analyses.

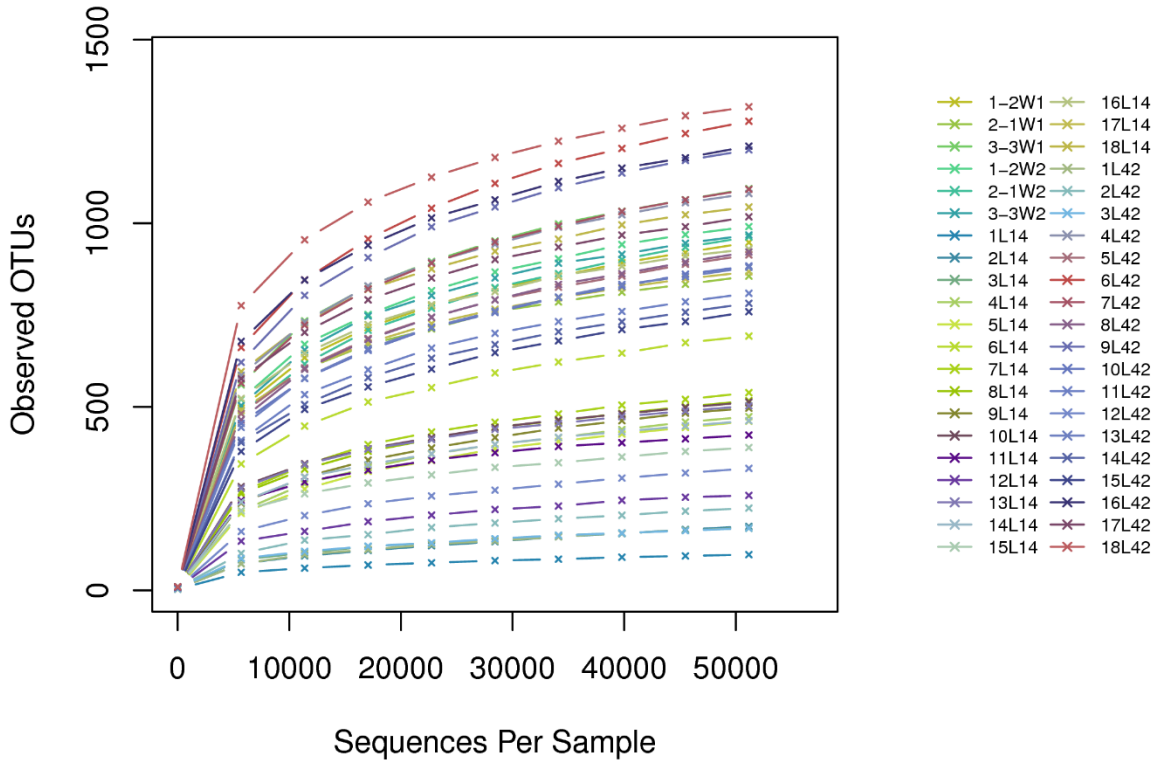



**Figure 4.6.** Rarefaction curves for observed OTUs in all the water and leaf samples. The sample names with 'W' are water samples and 'L' are leaf samples.

*Alpha Diversity analysis:*

Alpha diversity measures revealed that bacterial communities in leaf samples were more diverse with higher indices (ACE, Chao1, Shannon and Simpson) (Table 4.6) at higher temperature than lower temperature. Nutrient had less effects on bacterial diversity in leaf samples. Heavy metals decreased bacterial diversity with lower indices in leaf samples, especially at high concentration (100 μM) of Cu (12L14 and 12L42 in the table).

**Table 4.5.** The details of the samples within each group used for further analyses.

| Groups | Details of the samples within each group | Number of samples |
|---|---|---|
| W | Water samples | 6 |
| A1 | Leaf (14 day) (Low temperature - 5 °C) | 3 |
| B1 | Leaf (14 day) (Medium temperature - 20 °C) | 3 |
| D1 | Leaf (14 day) (Medium temperature - 20 °C) (Nutrients) | 3 |
| E1 | Leaf (14 day) (Medium temperature - 20 °C) (Heavy metal – Cu (Low, Medium, High)) | 3 |
| F1 | Leaf (14 day) (Medium temperature - 20 °C) (Heavy metal – Zn (Low, Medium, High)) | 3 |
| C1 | Leaf (14 day) (High temperature - 35 °C) | 3 |
| A2 | Leaf (42 day) (Low temperature - 5 °C) | 3 |
| B2 | Leaf (42 day) (Medium temperature - 20 °C) | 3 |
| D2 | Leaf (42 day) (Medium temperature - 20 °C) (Nutrients) | 3 |
| E2 | Leaf (42 day) (Medium temperature - 20 °C) (Heavy metal – Cu (Low, Medium, High)) | 3 |
| F2 | Leaf (42 day) (Medium temperature - 20 °C) (Heavy metal – Zn (Low, Medium, High)) | 3 |
| C2 | Leaf (42 day) (High temperature - 35 °C) | 3 |

**Table 4.6.** Similarity-based OTUs and species richness and diversity estimates of all the water and leaf samples used for sequencing.

| Group | Sample ID | Reads | ACE | Chao1 | Shannon index | Simpson | Good's Coverage |
|---|---|---|---|---|---|---|---|
| W | 1-2W1 | 51166 | 1186.009 | 1228.654 | 6.741 | 0.97 | 0.996 |
| | 2-1W1 | 51166 | 1052.431 | 1122.577 | 6.789 | 0.971 | 0.997 |
| | 3-3W1 | 51166 | 1301.528 | 1299.922 | 6.696 | 0.961 | 0.996 |
| | 1-2W2 | 51166 | 1197.949 | 1242.267 | 6.672 | 0.965 | 0.996 |
| | 2-1W2 | 51166 | 1186.694 | 1161.224 | 6.52 | 0.962 | 0.996 |
| | 3-3W2 | 51166 | 1141.414 | 1201.702 | 6.557 | 0.96 | 0.996 |
| A1 | 1L14 | 51166 | 131.954 | 133.364 | 2.075 | 0.636 | 1 |
| | 2L14 | 51166 | 363.146 | 365.474 | 3.003 | 0.787 | 0.999 |
| | 3L14 | 51166 | 319.05 | 358.462 | 3.08 | 0.782 | 0.999 |
| B1 | 4L14 | 51166 | 638.554 | 661.085 | 3.436 | 0.689 | 0.997 |
| | 5L14 | 51166 | 642.503 | 653.517 | 3.445 | 0.691 | 0.997 |
| | 6L14 | 51166 | 866.798 | 902.123 | 5.06 | 0.859 | 0.997 |
| D1 | 7L14 | 51166 | 686.799 | 676.766 | 4.097 | 0.751 | 0.998 |
| | 8L14 | 51166 | 653.538 | 657.147 | 4.666 | 0.856 | 0.998 |
| | 9L14 | 51166 | 665.873 | 682.148 | 3.374 | 0.66 | 0.997 |
| E1 | 10L14 | 51166 | 643.529 | 635.444 | 5.643 | 0.956 | 0.998 |
| | 11L14 | 51166 | 507.502 | 517.068 | 5.48 | 0.942 | 0.998 |
| | 12L14 | 51166 | 362.086 | 350.278 | 4.496 | 0.902 | 0.999 |
| F1 | 13L14 | 51166 | 625.069 | 648.188 | 5.349 | 0.933 | 0.998 |
| | 14L14 | 51166 | 587.619 | 597.519 | 5.117 | 0.912 | 0.998 |
| | 15L14 | 51166 | 513.639 | 506.279 | 5.313 | 0.946 | 0.998 |
| C1 | 16L14 | 51166 | 1113.042 | 1137.011 | 7.278 | 0.982 | 0.997 |
| | 17L14 | 51166 | 1038.342 | 1079.11 | 7.257 | 0.984 | 0.997 |
| | 18L14 | 51166 | 1221.46 | 1278.319 | 7.683 | 0.988 | 0.997 |
| A2 | 1L42 | 51166 | 262.482 | 248.333 | 2.721 | 0.731 | 0.999 |
| | 2L42 | 51166 | 354.419 | 360.731 | 4.085 | 0.903 | 0.999 |
| | 3L42 | 51166 | 256.138 | 260.556 | 3.194 | 0.747 | 0.999 |
| B2 | 4L42 | 51166 | 1309.966 | 1341.05 | 7.296 | 0.975 | 0.996 |
| | 5L42 | 51166 | 1148.716 | 1211.387 | 6.483 | 0.954 | 0.996 |
| | 6L42 | 51166 | 1556.586 | 1592.102 | 7.254 | 0.972 | 0.995 |
| D2 | 7L42 | 51166 | 1359.416 | 1390.165 | 7.173 | 0.972 | 0.995 |
| | 8L42 | 51166 | 1137.862 | 1195.229 | 6.767 | 0.97 | 0.996 |
| | 9L42 | 51166 | 1436.184 | 1448.075 | 6.904 | 0.954 | 0.996 |
| E2 | 10L42 | 51166 | 1124.057 | 1187.396 | 6.602 | 0.972 | 0.996 |
| | 11L42 | 51166 | 997.936 | 1021 | 6.329 | 0.96 | 0.997 |
| | 12L42 | 51166 | 460.305 | 446.319 | 3.824 | 0.808 | 0.998 |
| F2 | 13L42 | 51166 | 1090.939 | 1149.413 | 6.582 | 0.97 | 0.996 |
| | 14L42 | 51166 | 972.555 | 977.772 | 6.04 | 0.944 | 0.997 |
| | 15L42 | 51166 | 971.5 | 1013.118 | 6.041 | 0.955 | 0.996 |
| C2 | 16L42 | 51166 | 1438.597 | 1462.938 | 8.014 | 0.991 | 0.996 |
| | 17L42 | 51166 | 1198.273 | 1194.697 | 7.537 | 0.986 | 0.997 |
| | 18L42 | 51166 | 1517.397 | 1553.875 | 8.235 | 0.991 | 0.996 |

*OTU Taxa analysis:*

Operational taxonomic unit (OTU) analysis results for bacterial community data obtained for water and leaf samples collected from microcosm experiments indicated that there were more unique OTUs in water samples (507) than in leaf samples (about 200) (Figure 4.7). For leaf samples, more OTUs were observed on 42$^{nd}$ day (4-152) than on 14$^{th}$ day (0-19); 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 reduced OTUs in long-term (42-day) but nutrients had fewer effects on OTUs.

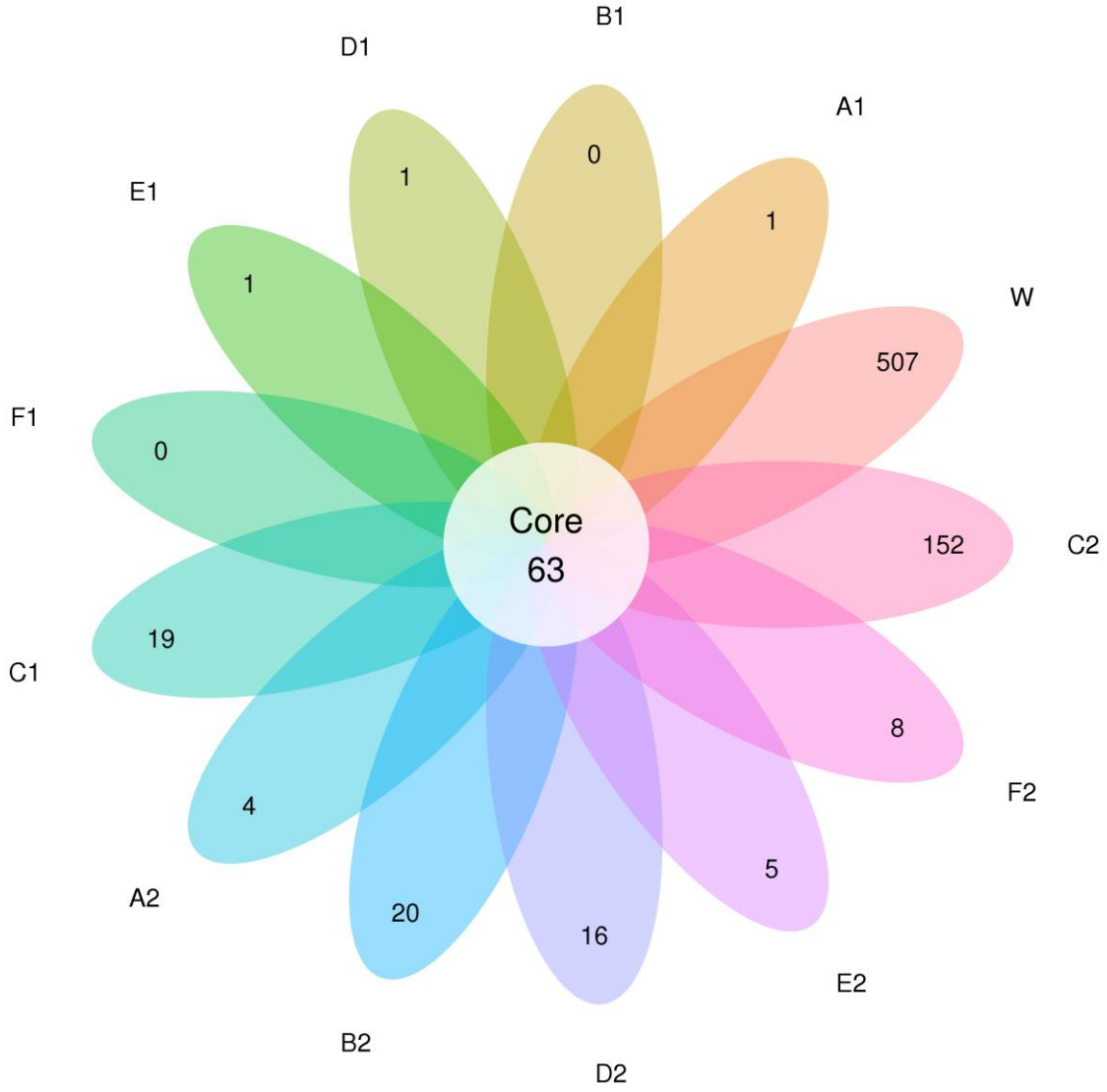



**Figure 4.7.** The group flower diagram showing the OTU Taxa analysis results for bacterial community in water and leaf samples collected during microcosm studies. (W=Water samples; A1, B1, C1=Leaf samples collected on 14$^{th}$ day and A2, B2 and C2=Leaf samples collected on 42$^{nd}$ day from microcosm experiments set at 5 °C, 20 °C and 35 °C, respectively; D1 and D2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from nutrient group set at 20 °C; E1 and E2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Cu) (which includes samples from three different concentrations tested) set at 20 °C; F1 and F2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Zn) (which includes samples from three different concentrations tested) set at 20 °C.) Refer to Table 4.5 for more details.

*Bacterial community composition:*

The relative abundance of the sequences obtained from each group of samples at the phylum level is shown in Figure 4.8. The bacterial community in water samples is entirely 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%), whereas leaf samples were dominated by Proteobacteria (20-85%) followed by Bacteroidetes (10-70%) and Firmicutes (5-20%). Proteobacteria was represented at extremely high level in leaf samples with low temperature (5 °C) (70-80%) (Group: A1 and A2), and Bacteroidetes was represented at high level in leaf samples with medium temperature (20 °C) (30-65%) (Group: B1, D1, E1, F1 and B2, D2, E2, F2) as compared to other temperature, which indicated the effect of temperature on shifts of bacterial community. In short-term (14-day), Proteobacteria was represented at higher level in leaf samples treated by Cu (Group E1: 60%) as compared to normal samples (Group B1: 20%) at 20 °C, whereas Bacteroidetes was represented at lower level in leaf samples treated by (Group E1: 30%) Cu than in normal samples (Group B1: 70%), which indicated the obvious effect of Cu on changing composition of bacterial community in short-term.

At the order level (Figure 4.9A), Burkholderiales (20%) and Frankiales (30%) were dominant in water samples (Group: W), whereas they were represented at very low level in leaf samples (<5%). Enterobacteriales was represented at extremely high level in leaf samples with low temperature (5 °C) (50%) (Group: A1 and A2), and Bacteroidales was represented at high level in leaf samples with medium temperature (20 °C) (20-65%) (Group: B1, D1, E1, F1 and B2, D2, E2, F2), which was similar to the results at the phylum level and indicated the effect of temperature on changing bacterial composition. In short-term (14-day), Enterobacteriales was represented at higher level in leaf samples treated by Cu (Group E1: 30%) as compared to normal samples (Group B1: 15%) at 20 °C, whereas Bacteroidales was

represented at lower level in leaf samples treated by Cu (Group E1: 20%) than in normal samples (Group B1: 67%), which was also indicated the obvious effect of Cu on changing composition of bacterial community in short-term.

At the genus level (Figure 4.9B), *Pseudomonas* represented at high level in leaf samples with low temperature (5 °C) (15-25%) (Group: A1 and A2); *Macellibacteroides* was represented at high level in leaf samples with medium temperature (20 °C) (10-25%) (Group: B1, D1, E1, F1 and B2, D2, E2, F2) and *Prevotella_9* was represented at extremely high level in leaf samples with medium temperature (20 °C) (45-50%) on $14^{th}$ day in control group (B1) and nutrient group (D1).

The relative abundance of the sequences for individual samples at the phylum, order and genus level are shown in supplementary Figures S4.1 to S4.3. For most groups, few internal differences were observed, except for Group E1 (10L14, 11L14 and 12L14) of leaf samples treated by Cu with different concentrations in short-term (14-day). This indicated that higher concentrations of Cu changed more obviously on the composition of bacterial community in leaf samples on short-term.

In summary, bacterial community composition results indicated: 1) Variation in bacterial community between water and leaf samples (W vs. A-F); 2) The effect of temperature on shifts in bacterial community in leaf samples in both short-term (A1 vs. B1 vs. C1) and long-term (A2 vs. B2 vs. C2); 3) The effect of Cu on changing the composition of bacterial community in leaf samples in short-term (B1 vs. E1), especially with high concentration and 4) No obvious effect of nutrients on shifts in bacterial community in leaf samples in both short-term (B1 vs. D1) and long-term (B2 vs. D2).

*Heatmaps of clustered bacterial community:*

Heatmaps of clustered bacterial community at phylum, order and genus levels are shown in Figure 4.10. At phylum level, Group W (water samples) clustered individually, which indicated difference in bacterial community between water and leaf samples. Proteobacteria, Actinobacteria and Planctomycetes were dominant in water samples. For leaf samples, Group A1 and A2 (5 °C) clustered together. Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Cyanobacteria were represented at high percentages in A1 and A2 as compared to other groups. These results indicated the effect of temperature on bacterial community in leaf samples. Group B1 and D1 (20 °C, 14$^{th}$ day: control and nutrients group) were clustered together, Bacteroidetes, Proteobacteria, Firmicutes and Cyanobacteria were dominant in these two groups; Group B2 and D2 (20 °C, 42$^{nd}$ day: control and nutrients group) were also clustered together, Bacteroidetes, Proteobacteria, Firmicutes, Cyanobacteria, Gracilibacteria, Spirochaetae, Chlorobi, Verrucomicrobia, Acidobacteria and Armatimonadetes were dominant in these two groups. These results indicated no effect of nutrients on bacterial community in both short-term and long-term. Group E2 and F2 (20 °C, 42$^{nd}$ day: Cu and Zn) were clustered together, but no clustering with Group B2 was observed, which indicated the effect of heavy metals on bacterial community in leaf samples.

At order level, Group W (water samples) clustered individually which indicated the difference in bacterial community between water and leaf samples; Frankiales and Burkholderiales were dominant in water samples. For leaf samples, Group A1 and A2 (5 °C) were clustered together, Enterobacteriales and Pseudomonadales were represented at higher percentage in A1 and A2 as compared to other groups, which indicated the effect of temperature on bacterial community in leaf samples. Group B1 and D1 (20 °C, 14$^{th}$ day: control and nutrients group) were clustered together, Bacteroidales were dominant in these two groups; Group B2 and D2 (20 °C, 42$^{nd}$ day: control and nutrients group) were also

clustered together, Bacteroidales, Burkholderiales, Enterobacteriales, Pseudomonadales, Clostridiales, Selenomonadales, Desulfovibrionales and Spirochaetales were dominant in these two groups. These results indicated that no effect of nutrients on bacterial community in both short-term and long-term. Group E2 and F2 (20 °C, 42$^{nd}$ day: Cu and Zn) were clustered together, but they were not clustered with Group B2 (control), which indicated the effect of heavy metals on bacterial community in leaf samples.

At genus level, Group W (water samples) was clustered individually which indicated the difference in bacterial community between water and leaf samples, *Polynucleobacter, Candidatus_Methylopumilus* and *Planktothrix* were dominant in water samples, but they were not observed in leaf samples. For leaf samples, Group A1 and A2 (5 °C) were clustered together, *Pseudomonas* and *Aeromonas* were represented at higher percentage in A1 and A2 as compared to other groups, which indicated the effect of temperature on bacterial community in leaf samples. Group B1 and D1 (20 °C, 14$^{th}$ day: control and nutrients group) were clustered together, *Prevotella_9, Bacteroides* and *Aeromonas* were dominant in these two groups; Group B2 and D2 (20 °C, 42$^{nd}$ day: control and nutrients group) were also clustered together, *Bacteroides, Pseudomonas, Desulfovibrio* and *Macellibacteroides* were dominant in these two groups. These results indicted no effect of nutrients on bacterial community. Group E1 and E2 (20 °C, Cu: 14$^{th}$ and 42$^{nd}$ day) were clustered together, and Group F1 and F2 (20 °C, Zn: 14$^{th}$ and 42$^{nd}$ day) were also clustered together, but they were not clustered together with Group B1 and B2 (control), which indicated the effect of heavy metals on bacterial community in leaf samples.

All the heatmaps of clustered bacterial community taxonomy were very similar at phylum, order and genus levels: 1) Variation of bacterial community between water and leaf samples; 2) Temperature was a key factor that affected the composition of bacterial community in leaf samples; 3) Nutrients had no effect on the bacterial composition in leaf

samples and 4) Heavy metals (Cu and Zn) also affected the bacterial composition in leaf samples.

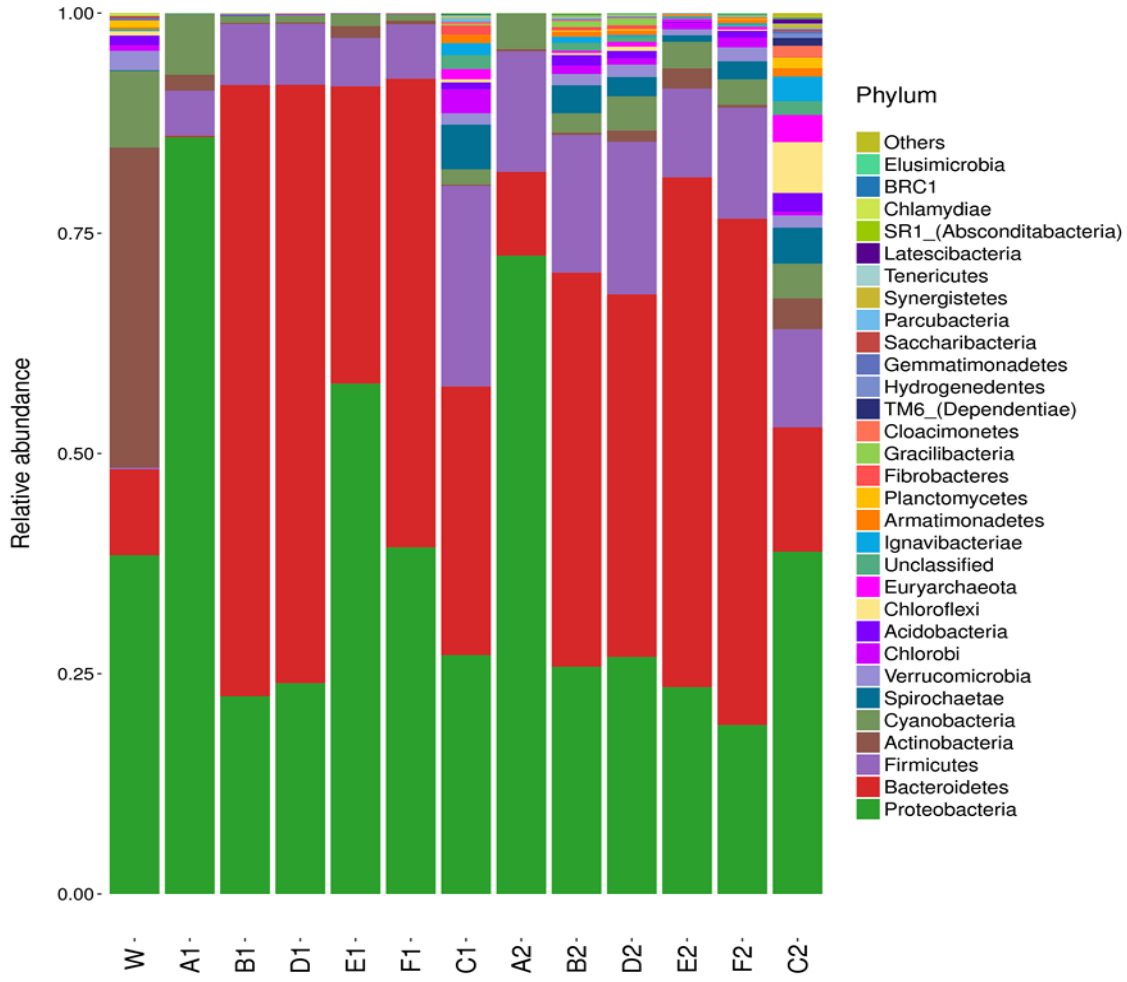



**Figure 4.8.** Bacterial community composition (phylum level) in water and leaf samples collected from different experimental groups in the microcosm study. (W=Water samples; A1, B1, C1=Leaf samples collected on 14$^{th}$ day and A2, B2 and C2=Leaf samples collected on 42$^{nd}$ day from microcosm experiments set at 5 °C, 20 °C and 35 °C, respectively; D1 and D2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from nutrient group set at 20 °C; E1 and E2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Cu) (which includes samples from three different concentrations tested) set at 20 °C. F1 and F2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Zn) (which includes samples from three different concentrations tested) set at 20 °C.) (Group A-F represents temperatures (5 °C-A, 20 °C-BDEF, 35 °C-C) and sampling days (14 day-1, 42 day-2) on the figure.) Refer to Table 4.5 for more details.

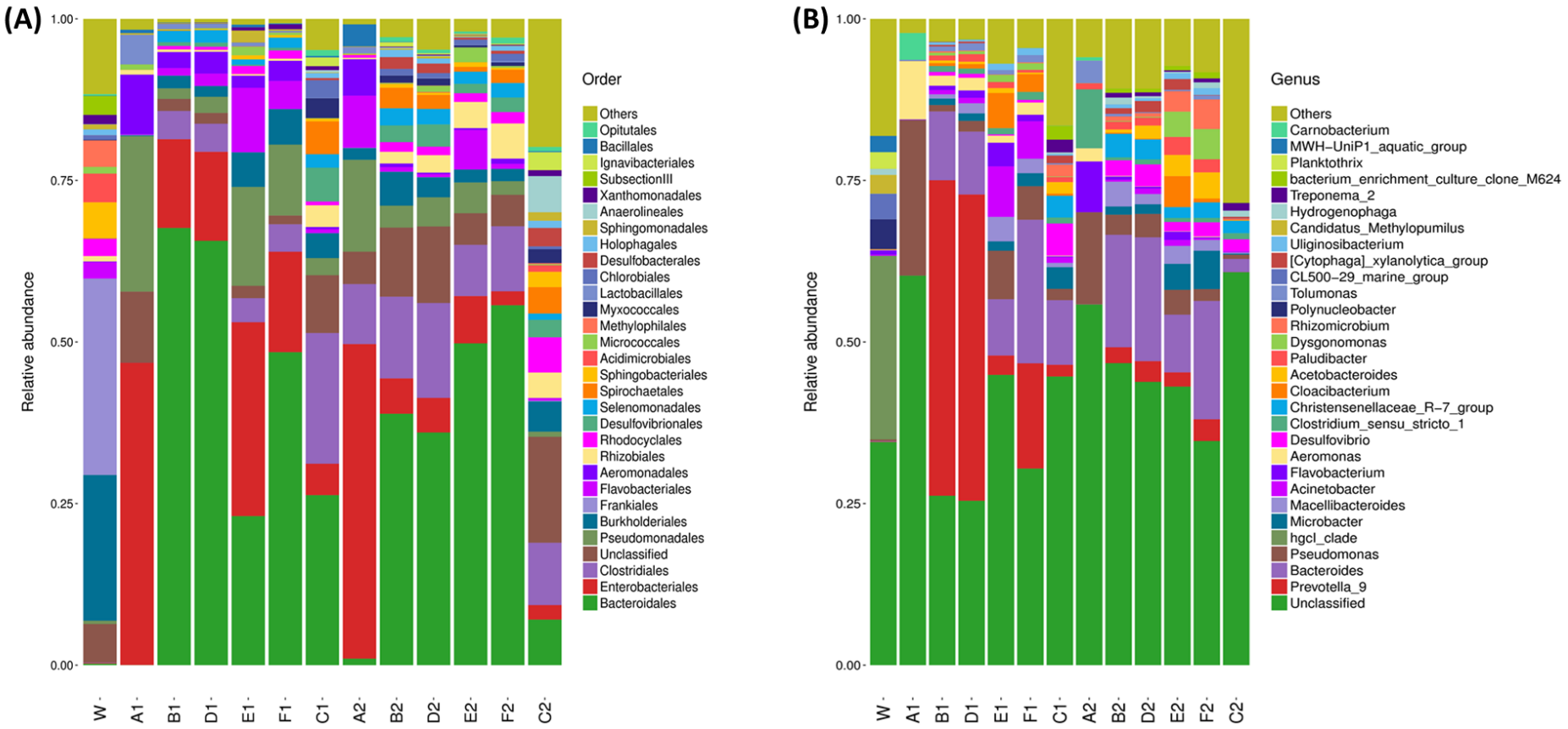



**Figure 4.9.** Bacterial community composition at order (A) and genus (B) level in water and leaf samples collected from different experimental groups in the microcosm study. (W=Water samples; A1, B1, C1=Leaf samples collected on 14$^{th}$ day and A2, B2 and C2=Leaf samples collected on 42$^{nd}$ day from microcosm experiments set at 5 °C, 20 °C and 35 °C, respectively; D1 and D2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from nutrient group set at 20 °C; E1 and E2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Cu) (which includes samples from three different concentrations tested) set at 20 °C. F1 and F2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Zn) (which includes samples from three different concentrations tested) set at 20 °C.) (Group A-F represents temperatures (5 °C-A, 20 °C-BDEF, 35 °C-C) and sampling days (14 day-1, 42 day-2) on the figure.) Refer to Table 4.5 for more details.

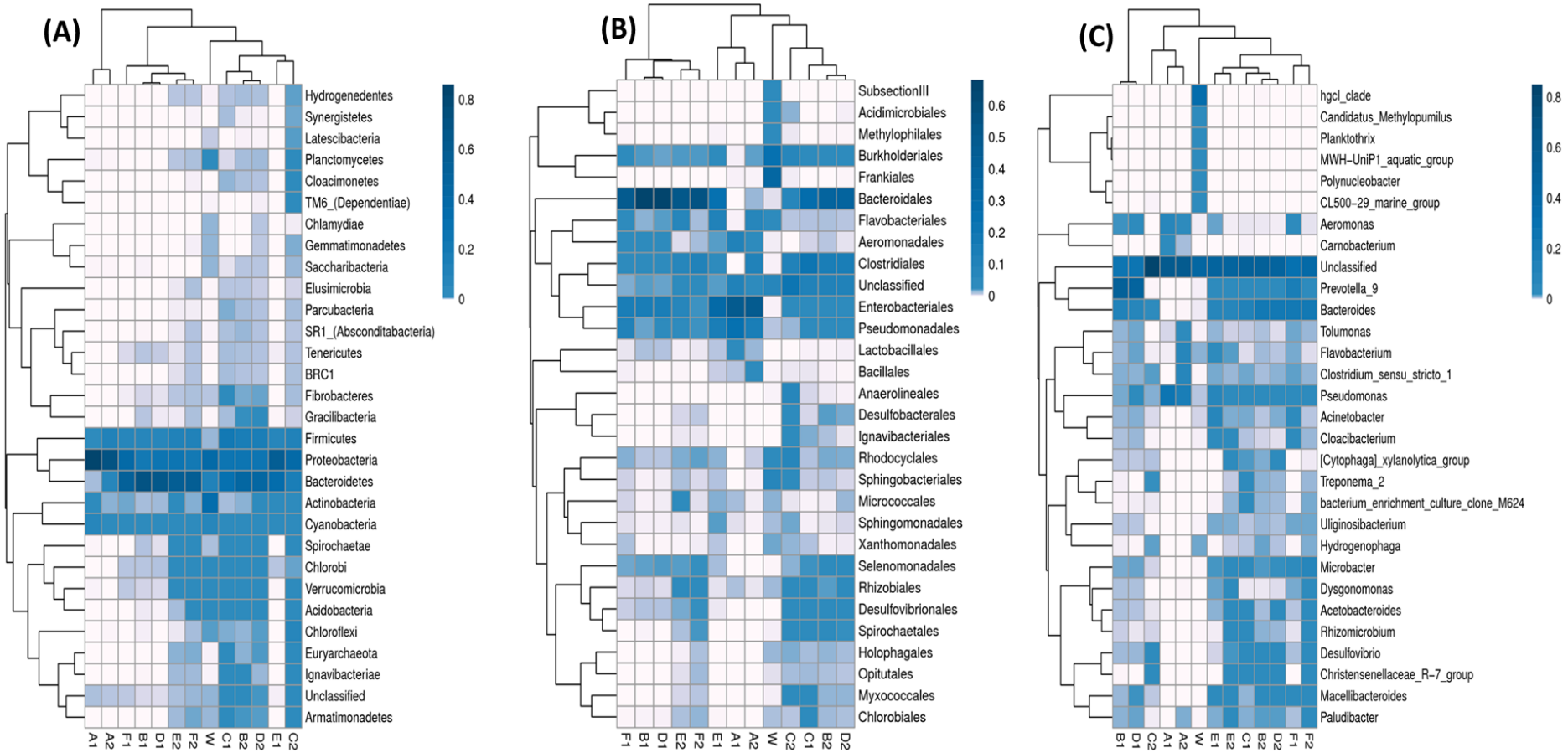



**Figure 4.10.** Heatmap of clustered bacterial community at phylum (A), order (B) and genus (C) levels in water and leaf samples collected from different experimental groups in the microcosm study. (W=Water samples; A1, B1, C1=Leaf samples collected on 14$^{th}$ day and A2, B2 and C2=Leaf samples collected on 42$^{nd}$ day from microcosm experiments set at 5 °C, 20 °C and 35 °C, respectively; D1 and D2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from nutrient group set at 20 °C; E1 and E2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Cu) (which includes samples from three different concentrations tested) set at 20 °C. F1 and F2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Zn) (which includes samples from three different concentrations tested) set at 20 °C.) Refer to Table 4.5 for more details.

*Changes in beta diversity:*

NMDS diagram was used to show the difference in bacterial communities between different samples in a two-axis matrix; the samples are represented in different points / symbols and the distances between the points (symbols) indicates the dissimilarities of the samples (Laforest-Lapointe et al., 2017, Byloos et al., 2018, Sanchez-Soto Jimenez et al., 2018). NMDS diagram (Figure 4.11) indicated the high difference in bacterial communities between water (W) and leaf (A-F). The NMDS1 value for water samples (W: < -0.5) was smaller than leaf samples (A-F: > -0.5). For leaf samples, they could be classified separately into 4 large groups as A1+A2 (5 °C: $14^{th}$ and $42^{nd}$ day), B1+D1+E1+F1 (20 °C: $14^{th}$ day), B2+D2+E2+F2+C1 (20 °C: $42^{nd}$ day and 35 °C: $14^{th}$ day) and C2 (35 °C: $42^{nd}$ day). The NMDS2 values for these 4 groups of leaf samples expanded from small to large with higher temperature and longer term, which indicated the effect of temperature and duration (sampling day) on shifts in bacterial composition in leaf samples. However, no obvious effect of nutrients (B vs. D) and heavy metals (B vs. E and F) on shifts in bacterial community in leaf samples was observed (Figure 4.11).

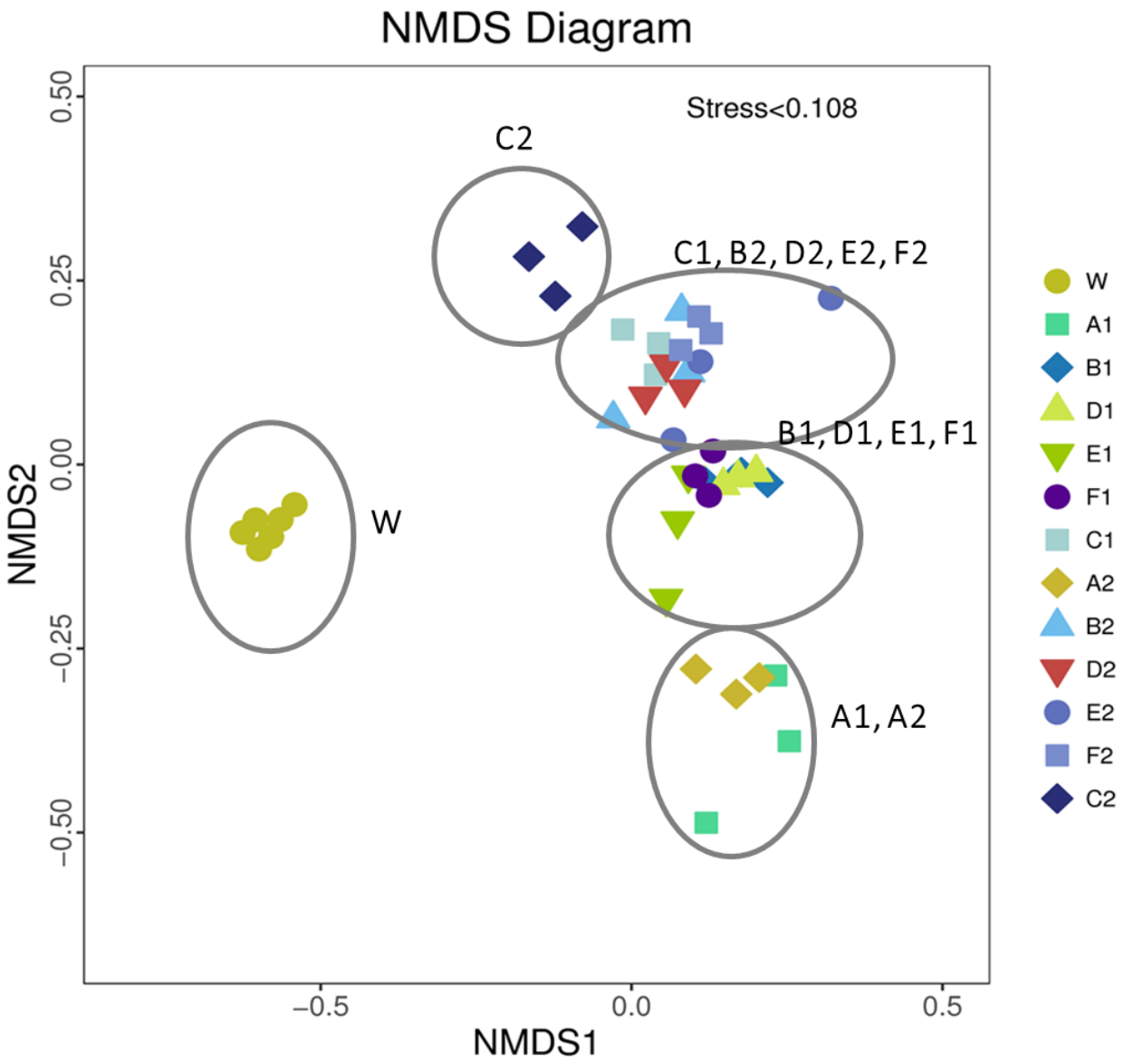



**Figure 4.11.** Nonmetric multidimensional scaling (NMDS) analysis of the sequences obtained in the microcosm study. (W=Water samples; A1, B1, C1=Leaf samples collected on 14$^{th}$ day and A2, B2 and C2=Leaf samples collected on 42$^{nd}$ day from microcosm experiments set at 5 °C, 20 °C and 35 °C, respectively; D1 and D2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from nutrient group set at 20 °C; E1 and E2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Cu) set at 20 °C. F1 and F2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Zn) set at 20 °C.) Refer to Table 4.5 for more details.

The Principal Coordinate Analysis (PCoA) was applied to identify factors that explains the differences among bacterial communities (Lozupone et al., 2011) (Figure 4.12). Components (PC1, PC2 and PC3) accounted for 25.71%, 17.32% and 13.26% of total variation, respectively. PC1 mainly showed variation between water (W) and leaf (A-F) samples, as PC1 values for water samples were smaller than -0.25, whereas PC1 values for leaf samples were larger than -0.25. For leaf samples, PC2 mainly showed variation due to the temperature and the sampling date, as PC2 values expanded from small to large with higher temperature and longer term. Potential variation in heavy metals (Cu) could also been observed, as samples from Group E1 and E2 (20 °C, Cu: $14^{th}$ and $42^{nd}$ day) were usually separated individually, but this variation was hardly explained by any component (PC1, PC2 and PC3). Similar to PC2, the PC3 also indicated variation in bacterial diversity due to temperature and duration (sampling day). Among varying temperatures, the PC3 mainly showed the difference between medium temperature (20 °C) and low and high temperature (5 °C and 35 °C). However, few variations between low (5 °C) and high (35°C) temperature were observed, Group A1, A2 and C2 were circled together in Figure 4.12b, all of them have higher PC3 (> 0.2) values than other groups. No obvious variation of nutrients (B vs. D) was observed again.

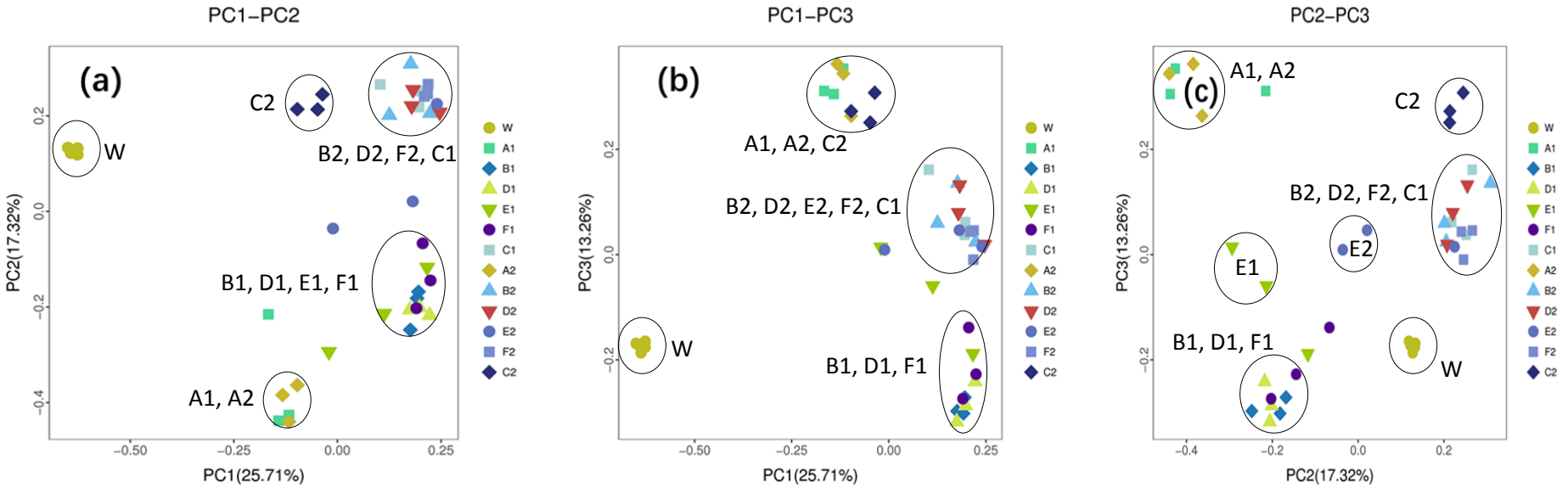



**Figure 4.12.** Principal Coordinate Analysis (PCoA) bacterial community in water and leaf samples collected during microcosm studies. (W=Water samples; A1, B1, C1=Leaf samples collected on 14$^{th}$ day and A2, B2 and C2=Leaf samples collected on 42$^{nd}$ day from microcosm experiments set at 5 °C, 20 °C and 35 °C, respectively; D1 and D2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from nutrient group set at 20 °C; E1 and E2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Cu) set at 20 °C. F1 and F2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Zn) set at 20 °C.) Refer to Table 4.5 for more details.

UPGMA (Unweighted pair group method with arithmetic mean) tree analysis results of bacterial community data indicated that water samples were clustered separately as compared to leaf samples (Figure 4.13). In leaf samples, bacterial community clustering was mainly based on temperature (5, 20 and 35 °C), sampling days (14$^{th}$ and 42$^{nd}$ day) and heavy metals (Cu and Zn), but not nutrients. The leaf samples with high concentration (0.1 mM) of Cu clustered individually (12L14 and 12L42, Refer to Tables 4.5 and 4.6).

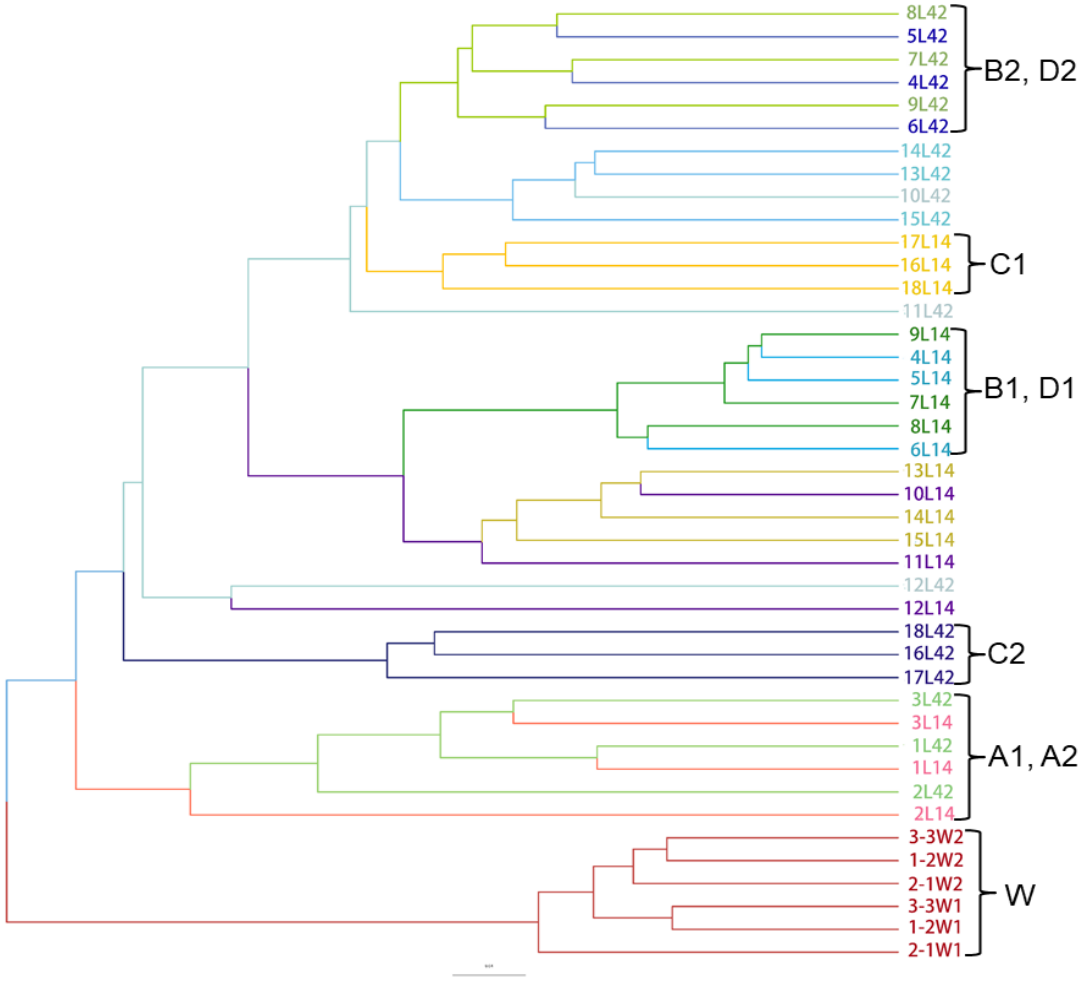



**Figure 4.13.** UPGMA Tree for bacterial community analysis in water and leaf samples collected during microcosm studies. (W=Water samples; A1, B1, C1=Leaf samples collected on 14$^{th}$ day and A2, B2 and C2=Leaf samples collected on 42$^{nd}$ day from microcosm experiments set at 5 °C, 20 °C and 35 °C, respectively; D1 and D2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from nutrient group set at 20 °C; E1 and E2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Cu) set at 20 °C. F1 and F2=Leaf samples collected on 14$^{th}$ and 42$^{nd}$ day, respectively from heavy metal group (Zn) set at 20 °C.) Refer to Table 4.5 for more details.

## 4.4. Discussion

Microcosms are artificial and simplified ecosystems that are used to mimic and predict natural ecosystems under controlled conditions, which provide an experimental method for ecologists to study natural ecological processes (Roeselers et al., 2006, Jiang and Patel, 2008). Microcosm study could be a useful tool to study the effects of environmental parameters on microbial diversity and ecosystem function in freshwater ecosystems, which is a good complement to field study. Therefore, the aim of this project was to study the effects of temperature, nutrients and heavy metals (Cu and Zn) on bacterial diversity and organic matter breakdown rate in microcosms based on previous two-year field studies in Suzhou canals.

The field and lab studies carried out by Martinez et al. confirmed the positive relationship between temperature and leaf breakdown rate (Martinez et al., 2014). They have carried out the field study in low-order forest streams in Spain by using leaf bag experiments to investigate the effect of temperature on headwater stream functioning; in the lab microcosm study, three different temperatures (5, 10 and 15 °C) were set up. Both their field and microcosm studies indicated that temperature affected leaf litter decomposition. Batista et al. reported that increasing temperature stimulated leaf decomposition by microbes (Batista et al., 2012). Temperature was found to be the most important environmental factor for microbial breakdown (Mora-Gómez et al., 2015). Polycaprolactonediol 2000 (PCP 2000) is an organic compound and it has been previously used to study the biodegradability (Rattanapan et al., 2016), as well as for assessment of river ecosystem function (Rivas et al., 2016). The results from our studies showed that the PCP 2000 (organic matter) breakdown rate was highly influenced by temperature, and higher temperature accelerated the breakdown of organic matter, which is consistent with the results from leaf litter decomposition studies carried out by Martinez and colleagues as mentioned previously in Chapter 3.

Recent studies carried by Tant et al. and Gardestrom et al. also showed that nutrient enrichment contributed to microbial-mediated leaf litter breakdown (Tant et al., 2015, Gardeström et al., 2016). Gardestrom et al. studied the effects of both fungicide and nutrients on microbial diversity and litter decomposition in Sweden by selected three streams: a forest stream, a mixed land-use stream subject to nutrient enrichment but little pesticide, and an agricultural stream subject to both nutrients and pesticides. The results indicated that pesticides, in interaction with nutrients, could impact microbial diversity and ecosystem processes. Tant and colleagues studied the effect of nutrient enrichment on the contribution of both decomposers (fungi and bacteria) and detritivores (shredders) on litter breakdown rate in streams in USA. Their results showed that nutrient enrichment accelerated decomposer contributions on litter breakdown. In our study, the effect of different gradients of nutrients (from low, medium and highly urbanized areas) with amendments of phosphate and ammonium did not show significant variation in OM breakdown rate between control and nutrient groups. A recent study reported that combined effect of temperature and nutrients played a synergistic and an important role in organic matter decomposition (Manning et al., 2018) , but such an effect was not observed in our microcosms.

Martinez et al. observed that Cu pollution could negatively affect OM decomposition in soil ecosystem through a microcosm study (Martinez et al., 2016), this result is similar to the observation of my present study that high concentration of Cu reduced PCP 2000 breakdown rate. Ferreira et al. proved that heavy metal contamination significantly inhibited litter decomposition, and the effect was usually stronger for laboratory microcosm than for field studies (Ferreira et al., 2016). However, negative effects of mixtures with high Zn concentration (about 0.15 mM) and phosphate on stream ecosystem function was also observed (Fernandes et al., 2009). The results collected from Fernandes et al. supported the results obtained in my experiment that no effect of Zn (0.001 mM, 0.01 mM and 0.1 mM) on

PCP 2000 breakdown was observed. It can be interpreted that low concentrations (≤ 0.1mM) of Zn or other related factors may have no effect on OM breakdown rate. Although the concentrations of heavy metals in Suzhou canals were not measured in this study, heavy metals concentrations in water and sediment samples of the Grand Canal were measured by Yu et al. and Zhuang et al., respectively (Yu et al., 2012, Zhuang et al., 2016b). Wu et al. also measured the concentrations of heavy metals in sediment of lakes along the middle and lower reaches of the Yangtze River (Wu et al., 2012). Cu in water samples collected from most urban sections of the Grand Canal met the Criterion Continuous Concentration (CCC) (US EPA, 2007) of 9 μg/L, except water samples collected from a small size cite in the Yangtze River Delta, with Cu concentration ranging from 14-71 μg/L. Zn in water samples collected from 3 towns and a large size city in the Yangtze River Delta were not acceptable by the CCC, whereas others met the CCC of 120 μg/L (Yu et al., 2012). In Zaozhuang, a city near the Grand Canal, the contents of both Cu and Zn in surface sediments collected from most positions in the canal were higher than the background values (13 and 40 μg/g, respectively) (Zhuang et al., 2016b). The background concentrations of Cu and Zn in sediment of lakes in Jiangsu Province were 22.3 mg/kg and 62.6 mg/kg respectively. The concentration of Cu in Yangcheng Lake, Jinji Lake, Dushu Lake and Taihu Lake (all in Suzhou, Jiangsu) were 38 mg/kg, 370 mg/kg, 45 mg/kg and 26 mg/kg respectively. The concentration of Zn in Yangcheng Lake, Jinji Lake, Dushu Lake and Taihu Lake were 138 mg/kg, 431 mg/kg, 208 mg/kg and 85 mg/kg respectively. Taihu Lake is the freshwater source for Suzhou with less pollution, which was the reason why similar concentration of heavy metals as compared to background values was observed in sediment samples collected from Taihu Lake. Land use patterns near Yangcheng Lake, Jinji Lake and Dushu Lake were aquaculture, business center and education area, respectively. Multiple pollutants caused high levels of heavy metals in these three lakes, especially Jinji Lake near the city center, where

the concentration of heavy metals was extremely high (Wu et al., 2012). All these reports highlighted the issues of heavy metal pollution in the freshwater ecosystem, and the concentration levels (64 μg/L, 640 μg/L, 6.4 mg/L for Cu and 65 μg/L, 650 μg/L, 6.5 mg/L for Zn) used in this microcosm study were representative of typical pollution levels in Chinese urban waterways. Heavy metals across trophic levels result in bioaccumulation, which triggers biodiversity loss through 'biological death' (Hewitt et al., 2009), and in turn leads to depletion of ecosystem functions like decomposition (Faupel and Traunspurger, 2012). Cu in particular has negative impacts on natural ecosystems (Martinez et al., 2016).

For bacterial community analysis, multiple statistical tools were applied, such as OTU taxa analysis, bacterial taxonomy on phylum, order and genus level, heatmap analysis, alpha (diversity and dominance indices) and beta diversity analysis (NMDS diagram, PCoA and UPGMA Tree analysis). Besides the variation between water and leaf samples, temperature was found to be a key factor that affected the composition of bacterial community. Bacterial diversity increased at high temperature (35 °C) with more OTUs as compared to medium (20 °C) or low (5 °C) temperature. However, fewer effect of nutrients (ammonium and phosphate) on the composition of bacterial community was observed. The potential effects of heavy metals on the shifts in bacterial community was found, especially high concentration (100μM) of Cu reduced OTUs. These bacterial community results were correlated to PCP 2000 breakdown experiment. At a suitable higher temperature (35 °C), bacterial diversity increased with more OTUs and accelerated microbial-mediated organic matter decomposition rate. High concentration of Cu could change composition of bacterial community in freshwater ecosystem and reduce biodegradation function, as Cu is a natural antimicrobial material (Dollwet and Sorenson, 1985). Although Zn also shows an antimicrobial effect, this heavy metal is an essential cofactor supporting cellular growth for all living organisms. The level of Zn is tightly regulated in microorganisms and is maintained well below the suitable

range, but extremely high concentrations of Zn have been found to be cytotoxic (Costello et al., 1997). It is likely that the concentrations of Zn used in this study were not high enough to cause toxicity, therefore minimal effects on bacterial community changes and biodegradation function were observed in this study. The bacterial community results observed for temperature groups were consistent with a recent research at Kalamas River in Northwest Greece (Meziti et al., 2016). This river runs through an agricultural area, nature protected area and urban sewage polluted area. Final results showed temporal (seasonal) variations of bacterial taxonomic and functional diversity were more pronounced than spatial variations (Meziti et al., 2016). It was also reported that both variations of temperature and sites were significantly related to the variation of the bacterial community in a pond, and redundancy analysis (RDA) indicated that water temperature and total dissolved solids had the largest impact on bacterial populations (Zhong et al., 2018). Besides total bacterial community, seasonal variation or variation of temperature on specific bacterial communities (e.g. ammonia oxidizing bacterial community and methanotrophs) (Samad and Bertilsson, 2017, Awolusi et al., 2018, Kong et al., 2018) or biodegradation genes (Fang et al., 2018) in water ecosystem were also confirmed through multiple diversity analysis (e.g. network analysis, principal coordinates analysis, heatmaps, NMDS and partial least squares (PLS) regression). All these reported results further proved the effect of temperature on shifts of composition of specific bacterial communities, which could have flow-on effects on ecosystem function. However, the results of nutrients group had some differences with results published recently. For example, urban-influenced waterways harbored significantly greater bacterial abundance and diversity in Lake Michigan-an oligotrophic lake (Newton and McLellan, 2015). Urbanization had severe impacts on bacterial diversity related leaf-litter decomposition in Ampang River-a tropical stream in Malaysia (Yule et al., 2015). Proliferation of cyanobacteria was influenced by both nitrogen and phosphorus concentrations, which

confirmed the relationship between nutrients and specific bacteria associated to bloom events (Wilhelm et al., 2011). Since the distance between all the sampling locations in Suzhou maybe not far enough, no significant differences with urbanization on bacterial diversity and ecosystem function were observed in microcosm studies as the water samples collected from Suzhou canals were used in this study. At the same time, only one level concentration of nutrients was amended in the nutrients group in this project, which caused no variation between control and nutrients treatment group. A higher concentration of nutrients may significantly change the bacterial community and increase PCP 2000 breakdown rate in microcosms. The effects of heavy metal pollution on bacterial activity and functional diversity in soil ecosystem were observed because of the toxicity of heavy metals to microorganisms (Klimek et al., 2016) and zinc concentration was reported to cause the changes in microbial functional genes structure and abundance (Navarrete et al., 2017). These proved the results obtained in this project that high concentration of Cu shifted the bacterial composition in leaf samples with reduced OTUs and lowered PCP 2000 breakdown rate.

## 4.5. Conclusions

In laboratory microcosm studies, the influence of some key environmental parameters such as temperature, nutrients and heavy metals on the relationship of microbial diversity and ecosystem function were studied. Based on OM breakdown rates and bacterial community results, temperature was found to be a key factor that affects the composition of bacterial community. Bacterial diversity increased at high temperature (35 °C) with more OTUs compared to medium (20 °C) or low (5 °C) temperature and OM decomposition rate was also accelerated at higher temperature. Whereas, nutrients had fewer effects on the composition of bacterial community and nutrients accelerated only the OM breakdown rate in short-term. The heavy metals had potential effects on the shifts in bacterial community, a reduced number of OTUs was observed in the presence of Cu in high concentration (0.1 mM) and OM biodegradation rate was also reduced.

# Chapter 5 - Impact of Urbanization on the Presence and Abundances of Fecal Markers and Bacterial Pathogens in Suzhou Canals

***Abstract***

The aim of this study was to assess the impact of urbanization on fecal and bacterial pathogen contaminations in Suzhou canals. Selected fecal markers (BacUni: total *Bacteroidales*; HF183: human-associated *Bacteroidales*; GFD: avian-associated fecal marker) and bacterial pathogens (*Campylobacter jejuni*, *Shigella* sp., Shiga toxin-producing *Escherichia coli (E. coli)* (STEC), *Arcobacter butzleri*, *Salmonella* spp. and *Enterococcus* spp.) were quantified by qPCR in water samples collected during winter and summer 2015 and 2016 from Suzhou canals with varying urban intensifications (High, Medium and Low). The samples collected from the control locations in Huangshan during summer 2016 were also included in the analysis. The impact of urbanization was evident in the abundances of fecal markers across the urban grandients. Higher levels of BacUni and HF183 markers were observed in water samples collected from locations with high urbanization than medium and low urbanized locations in Suzhou and also in the control locations in Huangshan. GFD markers were detected in few samples at extremely low concentrations compared to BacUni and HF183 markers. The concentrations were in the range of 5.52 to 9.63-$\log_{10}$ gene copies/100 mL for BacUni, 2.15 to 7.65-$\log_{10}$ gene copies/100 mL for HF183 and 2.64 to 2.89-$\log_{10}$ gene copies/100 mL for GFD. Among bacterial pathogens tested, *Enterococcus* spp. were the most frequently detected pathogens in all the water samples (100%), followed by *Arcobacter butzleri* (74%), Shiga toxin-producing *E. coli* (STEC) (41%), *Shigella* sp. (36%), whereas *Campylobacter jejuni* and *Salmonella* spp. were least frequently detected (10%). The impact of urbanization was observed for *Enterococcus* spp. and *Arcobacter butzleri*, which were detected in high concentrations (3.67 to 7.76-$\log_{10}$ gene copies/100 mL and 4.79 to 6.21-$\log_{10}$ gene copies/100 mL, respectively) in water samples from highly urbanized locations as compared to medium and low urbanized locations in Suzhou and in control control locations (Huangshan). *Shigella* sp., *Campylobacter jejuni*, *Salmonella* spp.

and STEC were detected in few samples mainly collected from locations with high urbanization in Suzhou with very low concentrations (2.31 to 3.65-$\log_{10}$ gene copies/100 mL) as compared to *Enterococcus* spp. and *Arcobacter butzleri*. In conclusion, high urbanization significantly increased the abundances of fecal and bacterial pathogen contaminations in Suzhou canals, particuarly with higher levels of total *Bacteroidales*, human-associated *Bacteroidales* and bacterial pathogens (mainly *Enterococcus* spp. and *Arcobacter butzleri*) in locations with high urbanization as campared to medium and low urbanization.

## 5.1. Introduction

Although urbanization improves living standards, it can also create many issues for freshwater environments due to the discharge of excess nutrients, toxic chemicals, antibiotics, steroid hormones, and sewage (Paul and Meyer, 2001, Shen et al., 2005, Chang et al., 2009, Du et al., 2010, Litton et al., 2010, Newton et al., 2013, Medeiros et al., 2014, Prasad et al., 2014, Xu et al., 2018). Sewage is considered as one of the important sources of many pathogens, causing microbial water quality impairment in an urbanized watershed (Kirs et al., 2017). Therefore, proper monitoring of urban waters is required to address the issue with the presence of sewage associated pathogens and to implement remedial actions. Traditionally, culture-based methods such as fecal indicator bacteria (FIB) enumeration were commonly used for monitoring of fecal pollution in environmental waters to address the associated human health risk (Griffith et al., 2009). However, FIB enumeration to monitor the microbial quality of environmental waters has several limitations. For instance, it was reported that these bacteria can persist and multiply outside of the host gastrointestinal tract, leading to the difficulty in predicting recent fecal contamination in surface waters (Byappanahalli et al., 2003, Jamieson et al., 2005). Moreover, the correlation between FIB and pathogen presence is poor (McQuaig et al., 2012, Ahmed et al., 2013). The main limitation with FIB is that it

cannot identify the origin or source of fecal contamination (Field and Samadpour, 2007), which is confining to depict the human health risk and implement remedial actions (Santo Domingo et al., 2007). In this regard, microbial source tracking (MST) techniques have been developed in the recent decade to identify the origin of fecal sources (Bohrerova et al., 2017, Balleste et al., 2018, Haramoto and Osada, 2018).

Although library-dependent (LD-MST) and library-independent MST (LI-MST) methods were developed for predicting the origin of fecal sources, LI-MST methods have shown promising results in properly identifying the host-specific fecal sources (Bernhard and Field, 2000a, Layton et al., 2006). Quantitative PCR (qPCR) based LI-MST methods have demonstrated their extensive applicability in environmental waters as they can accurately quantify the host-specific MST target sequences to study fecal contamination (Layton et al., 2006, Kildare et al., 2007, Reischer et al., 2007). Most of the LI-MST methods targeted bacteria that belongs to order "*Bacteroidales*", due to their obligate anaerobic nature and are found at a higher concentration in the human and animal gut than facultatively anaerobic *E. coli* (Bernhard and Field, 2000b). Several host-associated *Bacteroidales* 16S rRNA gene markers have been developed to differentiate human feces from other animal feces detected in the environmental samples (Bernhard and Field, 2000a). Furthermore, host-associated *Bacteroidales* markers to identify human (Kildare et al., 2007, Green et al., 2014), ruminant (Raith et al., 2013), cow (Shanks et al., 2008), and swine (Mieszkin et al., 2009) fecal sources were also developed in recent years (Haramoto and Osada, 2018, Merino-Mascorro et al., 2018). However, it was reported that avian fecal source could be distinguished from other fecal sources by targeting bacterial taxonomic groups such as *Helicobacter* spp. (Green et al., 2012).

Though MST methods could inform potential sources of fecal pollution, they cannot confirm the presence of pathogenic microorganisms and the associated human health risk.

Lakes, rivers, and canals in a high population density urban area provide as a site for recreational activities to citizens. Therefore, human exposure to such water bodies may pose a significant public health risk due to microbial pathogens found in these water (Hlavsa et al., 2015). Furthermore, evaluating the occurrence of bacterial pathogens in waterbodies at high urbanization areas is vital to assess the impact of urbanization (Medeiros et al., 2014). However, related studies have not previously been carried out in Suzhou canals and the results generated here will be very insightful to assess the impact of urbanization. Therefore, this study aims at quantifying the abundance of fecal and bacterial pathogen markers in Suzhou canals across a gradient of urban intensification (High, Medium and Low). Based on literature review (Oster et al., 2014), suitable waterborne enteric bacterial pathogens were selected for this study. Moreover, the genus level bacterial community observed in different sampling locations through 2 years filed study indicated presence and abundance of some of the pathogens (reported in Chapter 3). For example, the genus *Arcobactor* was represented at extremely high percentage (50%) in water samples collected from location 1-2 in winter 2015, therefore *Arcobacter butzleri* was selected as one of the target pathogens in this study.

## 5.2. Materials and Methods

### 5.2.1. Sample collection and processing

As mentioned in earlier Chapter(s), nine sampling locations with three urban gradients (High, Medium and Low) in Suzhou were selected for this study. Water samples were collected in sterile 5-liter polypropylene containers from these locations on four occasions (winter and summer in 2015 and 2016). Three locations from Huangshan area were also included in the analysis as a control group and water sampling was conducted from those locations in summer 2016. After the transportation of the samples to lab on ice, water

samples (500 mL) were filtered immediately onto 0.22 µm polycarbonate membrane filters (Millipore, UK) in triplicates and stored at -20 °C until the DNA extraction.

### 5.2.2. Quantification of Fecal Markers and Bacterial Pathogens by qPCR Assays

#### 5.2.2.1. DNA extraction

As mentioned in Chapter 3, DNA was extracted from membrane filters (water samples) using PowerSoil DNA Isolation Kit (Mo Bio, USA). The membrane filters were cut into pieces and placed into the PowerBead tubes aseptically for DNA extraction according to the manufacturer's instructions. The quality and quantity of DNA were analyzed using NanoDrop ND 2000C spectrophotometer (Thermo Scientific, USA). The DNA extracts were stored at -20 °C until further analysis.

#### 5.2.2.2. Plasmid DNA standards for qPCR assays

Plasmid DNA standards were constructed for all the qPCR assays targeting fecal markers and genes of pathogenic bacteria. For fecal markers, the target genes were PCR-amplified from respective fecal DNA extracts using the primers designed in previous studies (Table 5.1). For pathogenic bacteria, the target genes were PCR-amplified from respective genomic DNA of target organisms (*Salmonella* ATCC 14028, *Arcobacter butzleri* ATCC 49616, *Campylobacter jejuni* sub sp. *jejuni* ATCC 29428, *Escherichia coli* ATCC 35150, *Shigella sonnei* ATCC 9290 and *Enterococcus* ATCC 29212) using the primers previously reported (Table 5.1). The amplified PCR products were purified using a QIAquick PCR purification kit (Axygen Biosciences, CA, USA) following the manufacturer's instructions. The purified PCR products were cloned separately into pMD19 T-Vectors (Takara, Bio Inc., Shiga, Japan), followed by transformation of the recombinant plasmid into *E. coli* JM109 competent cells (Takara, Bio Inc., Shiga, Japan). The plasmid DNA was extracted from

positive clones using QIAprep Spin Miniprep Kit (Qiagen, Mississauga, ON) and target gene presence in the recombinant plasmid was confirmed by PCR and sequencing (Sangon Biotech, China). Plasmid DNA was quantified with a NanoDrop ND 2000C UV spectrophotometer to analyze the purity and concentration for calculating the gene copy number using the formula mentioned by Oster (Oster et al., 2014).

#### 5.2.2.3. Quantitative Polymerase Chain Reaction (qPCR)

A seven-point 10-fold serial diluted plasmid DNA with target sequence was used to generate standard curve (with a range of $10^1$ to $10^7$ copies/reaction) in each qPCR assay. All qPCR reactions were run in triplicates with a final reaction volume of 20 μL. The sequences of the primers and probes along with concentrations are presented in Table 5.1. The accuracy and efficiency of the standard curve were determined by including a positive control of $10^3$ copies of plasmid standard as unknown in each assay (Oster et al., 2014).

Three qPCR assays targeting total, human and avian associated fecal sources were quantified in water samples collected from Suzhou canals and Huangshan. Two TaqMan based assays (BacUni and HF183 Taqman) were selected for detection of total and human-associated *Bacteroidale*s, and one SYBR-Green based assay (GFD) was selected for detection of avian-associated fecal marker (unclassified *Helicobacter* spp.) (Green et al., 2012, Green et al., 2014, Kildare et al., 2007). These markers were validated previously for Taihu watershed region by our research group (Vadde et al., 2019).

Six qPCR assays targeting *Enterococcus* spp., *Arcobacter butzleri*, *Shigella* sp., *Campylobacter jejuni*, *Salmonella* spp. and Shiga toxin producing *E. coli* (STEC) were selected for this study and all these assays were based on TaqMan chemistry (Holland et al., 1991).

Taqman based qPCR assays (20 µL of master mix) contained 10 µL of Premix Ex Taq$^{TM}$ (Probe qPCR) (Takara Bio Inc.), 0.4 µL of ROX Reference Dye Ⅱ (Takara Bio Inc.), 2 µL of template DNA, 6 µL nuclease-free water and 2 µL of primers and probe set with the final concentrations as shown in Table 5.1. SYBR Green assays (20 µL of master mix) contained 10 µL of SYBR Green PCR Master Mix (Thermofisher Technologies, Foster City, CA), 7 µL nuclease-free water, 2 µL of template DNA and 1 µL of primer mixture with a final concentration as shown in Table 5.1.

### 5.2.3. Statistical analyses

All the assays with $R^2$ values of above 0.95 and efficiencies between 85 and 110% were considered as acceptable for detection and quantification of target markers in environmental samples. If these criteria were not meet by any assay, the samples were tested again. The details of the limit of detection (LOD), limit of quantification (LOQ) and final assessment of qPCR results for each fecal marker and pathogen assays are provided in Table 5.2. The qPCR results for each assay of fecal markers and pathogens were processed based on LOD in the table as described by Oster (Oster et al., 2014). For statistical analysis, the abundances of fecal markers and pathogens were log transformed. Detected but not quantifiable (DNQ) and non-detected (ND) were substituted with one or 1/2 limit of detection (LOD), respectively, as described previously (Cao et al., 2017). Then these data were analyzed statisticallly with "IBM SPSS Statistics 20" software. Tukey two-way ANOVA were was used to analyze variations with seasons (winter and summer) and urban intensifications (High, Medium and Low) (Fujikoshi, 1993, Taylor, 2012, Zaiontz, 2018).

**Table 5.1.** qPCR primers and probes used in this study for quantification of fecal markers and bacterial pathogens.

| Target source/organism | Assay/ Gene | Primer/Probe | Sequence (5'-3') | Concentration (nM) | Product size (bp) | Annealing temp (°C) | References |
|---|---|---|---|---|---|---|---|
| Total *Bacteroidales* | BacUni | BacUni-520F | CGTTATCCGGATTTATTGGGTTTA | 400 | 170 | 60 | (Kildare et al., 2007) |
| | | BacUni-690R | CAATCGGAGTTCTTCGTGATATCTA | 400 | | | |
| | | BacUni-656P | FAM-TGGTGTAGCGGTGAAA-MGB | 80 | | | |
| Human-associated *Bacteroidales* | HF183 | HF183F | ATCATGAGTTCACATGTCCG | 1000 | 105 | 60 | (Green et al., 2014) |
| | | BacR287R | CTTCCTCTCAGAACCCCTATCC | 1000 | | | |
| | | BacP234P | FAM-CTAATGGAACGCATCCC-MGB | 80 | | | |
| Avian-associated fecal marker | GFD | GFD F | TCGGCTGAGCACTCTAGGG | 100 | 123 | 57 | (Green et al., 2012) |
| | | GFD R | GCGTCTCTTTGTACATCCCA | 100 | | | |
| *Salmonella* spp. | NA[a] | Sal F | GCTATTTTCGTCCGGCATGA | 200 | 261 | 60 | (Wang et al., 2007a) |
| | | Sal R | GCGACTATCAGGTTACCGTGGA | 200 | | | |
| | | Sal Probe | FAM-TAGCCAGCGAGGTGAAAACGACAAAGG-TAMRA | 250 | | | |
| *Arcobacter butzleri* | *hsp60* | hsp60 F | CTCTTCATTAAAAGAGATGTTACCAATTTT | 300 | 89 | 60 | (de Boer et al., 2013) |
| | | hsp60 R | CACCATCTACATCTTCWGCAATAATTACT | 300 | | | |
| | | hsp60 Probe | FAM-CTTCCTGATTGATTTACTGATT-NFQ-MGB | 100 | | | |
| *Campylobacter jejuni* | *mapA* | mapA F | CTGGTGGTTTTGAAGCAAAGATT | 400 | 96 | 60 | (Best et al., 2003) |
| | | mapA R | CAATACCAGTGTCTAAAGTGCGTTTAT | 400 | | | |
| | | mapA Probe | FAM-TTGAATTCCAACATCGCTAATGTATAAAAGCCCTTT-TAMRA | 80 | | | |
| STEC | *stx2* | Stx2 F | CAGGCAGATACAGAGAGAATTTCG | 200 | 68 | 61 | (Beutin et al., 2008) |
| | | Stx2 R | CCGGCGTCATCGTATACACA | 200 | | | |
| | | Stx2 Probe | VIC-ACTGTCTGAAACTGCTC-MGB | 160 | | | |
| *Shigella* sp. | *ipaH* | ipaH F | CTTGACCGCCTTTCCGATA | 200 | 117 | 64 | (Ma et al., 2014) |
| | | ipaH R | AGCGAAAGACTGCTGTCGAAG | 200 | | | |
| | | ipaH Probe | CY3-AACAGGTCGCTGCATGGCTGGAA-BHQ1 | 160 | | | |
| *Enterococcus* spp. | ENT1A | Entero F1A | AGAAATTCCAAACGAACTTG | 200 | 92 | 60 | (Xue et al., 2018) |
| | | Entero R1 | CAGTGCTCTACCTCCATCATT | 200 | | | |
| | | Entero Probe | FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA | 80 | | | |

[a] NA, not available.

**Table 5.2.** The limit of detection (LOD), limit of quantification (LOQ) and final assessment of qPCR results for each fecal marker and pathogen assays.

| **Assay** | **Compiled Slope** | **Compiled Y-intercept** | **Compiled $R^2$ value** | **Compiled Efficiency (%)** | **LOQ (cp/µl)** | **LOD (cp/µl)** |
|---|---|---|---|---|---|---|
| BacUni | -3.076 | 39.777 | 0.999 | 111.386 | 100[a] | 100 [a] |
| HF183 | -3.218 | 38.449 | 0.996 | 104.509 | 10 [a] | 10 [a] |
| GFD | -3.658 | 36.997 | 0.995 | 87.651 | 10 [a] | 10 [a] |
| *Enterococcus* spp. (ENT1A) | -3.563 | 41.004 | 0.999 | 90.847 | 10 [b] | 3 [b] |
| *Arcobacter butzleri (hsp60)* | -3.3308 | 42.647 | 0.998 | 100.601 | 10 [b] | 3 [b] |
| *Campylobacter jejuni* (*mapA*) | -3.408 | 39.608 | 0.998 | 96.541 | 10 [b] | 3 [b] |
| *Shigella* sp. (*ipaH*) | -3.249 | 37.779 | 0.988 | 103.14 | 10 [b] | 3 [b] |
| STEC (*stx2*) | -3.155 | 38.84 | 0.999 | 107.447 | 10 [b] | 3 [b] |
| *Salmonella* spp. | -3.512 | 40.579 | 0.993 | 92.633 | 10 [b] | 3 [b] |

[a] Based on MST (Microbial source tracing) validation study in our group (Vadde et al., 2019).

[b] Based on Oster et al. 2014.

## 5.3. Results

In total, 36 water samples collected during four seasons (winter and summer 2015 and 2016) from nine sampling locations of Suzhou canals with three different urban gradients and 3 water samples collected from Huangshan (as control or pristine area) during summer 2016 were investigated to assess the impact of urbanization on the detection frequency and abundances of fecal markers and bacterial pathogens.

### 5.3.1. Performance of qPCR assays

The amplification efficiency and linear range of quantification ($R^2$) for all the qPCR assays were determined using standard curves generated by serial dilutions of known copy numbers. The qPCR amplification efficiencies for fecal markers and genes of bacterial pathogens ranged from 88.22 to 111.386%, with $R^2$ values between 0.987 and 1. The compiled amplification efficiencies and linear range of quantification for all qPCR assays carried out are given in Table 5.2.

### 5.3.2. Detection and quantification of fecal markers in canals of Suzhou and Huangshan

The presence and abundance of fecal markers in water samples collected from different sampling locations of Suzhou canals and Huangshan determined by qPCR is shown in Table 5.3. Total *Bacteroidales* markers were detected in all the water samples (100%), and the concentrations ranged from 5.52 to 9.63-$\log_{10}$ gene copies/100 mL (Figure 5.1a). The highest concentration (9.63-$\log_{10}$ gene copies/100 mL) of total *Bacteroidales* was detected at location 1-2 during winter 2015. In general, higher levels of total *Bacteroidales* (6.37 to 9.63-$\log_{10}$ gene copies/100 mL) were observed in water samples colleced from locations with high urbanization than medium (5.52 to 9.37-$\log_{10}$ gene copies/100 mL) and low (5.54 to 8.63-$\log_{10}$ gene copies/100 mL) urbanization. Statistically significant in total *Bacteroidales*

concentration was observed among the locations with varying urbanization (P = 0.008) rather than seasons (Table 5.4A). Among three urban intensifications (High, Medium and Low) in Suzhou, statistically significant was observed only between High vs. Medium and High vs. Low, but not for Medium vs. Low (Figure 5.2a and Table 5.4B). For water samples collected from Huangshan, low levels of total *Bacteroidales* were observed (6.14 to 7.00-$\log_{10}$ gene copies/100 mL), except at location H-1, in which the concentration was 8.49-$\log_{10}$ gene copies/100 mL.

Human-associated markers were frequently detected in most of the samples tested (36 out of 39 water samples, 92%) (Table 5.3), and the concentrations ranged from 2.15 to 7.65-$\log_{10}$ gene copies/100 mL (Figure 5.1b). The highest concentration of human-associated *Bacteroidales* (7.65-$\log_{10}$ gene copies/100 mL) was also detected at location 1-2 during winter 2015. Similar to total *Bacteroidales*, higher levels of human-associated *Bacteroidales* were observed in water samples collected from locations with high urbanization (3.95 to 7.65-$\log_{10}$ gene copies/100 mL) than medium (3.19 to 5.71-$\log_{10}$ gene copies/100 mL) and low (2.15 to 4.97-$\log_{10}$ gene copies/100 mL) urbanization. There was a statistically significant in human-associated *Bacteroidales* concentration among sampling locations with varying urbanization (P = 0.002) rather than seasons (Table 5.4A), and statistically significant was only observed between High vs. Medium and High vs. Low, but not for Medium vs. Low (Figure 5.2b and Table 5.4B). For water samples collected from Huangshan, human-associated *Bacteroidales* was only detected in location H-1 (4.57-$\log_{10}$ gene copies/100 mL) but not at H-2 and H-3 locations.

Avian fecal markers (GFD) were detected in 14 out of 39 water samples (36%) tested (Table 5.3). However, they were at the quantifiable range in only 2 water samples with extremely low cocentrations (2.64 to 2.89-$\log_{10}$ gene copies/100 mL) (Figure 5.1c) compared to total and human-associated *Bacteroidales* markers. The highest concentration (2.89-$\log_{10}$

gene copies/100 mL) of avian marker was detected at location 2-2 during summer 2015. However, there was no statistically significant in avian marker concentration either with varying urban intensifications nor seasons, and avian markers were not in detectable limit in water samples collected from locations H-2 and H-3 in Huangshan.

**Table 5.3.** Detection frequencies of fecal markers in water samples collected from Suzhou canals and Huangshan (2015-2016).

| Sample Type | No. of samples tested (n) | No. of positive samples (%) [a] | | |
|---|---|---|---|---|
| | | **Total *Bacteroidales*** | **Human-associated *Bacteroidales*** | **Avian-associated fecal marker** |
| **Suzhou** | | | | |
| Winter 2015 | 9 | 9 (100%) | 9 (100%) | 1 (11%) |
| Summer 2015 | 9 | 9 (100%) | 9 (100%) | 6 (67%) |
| Winter 2016 | 9 | 9 (100%) | 9 (100%) | 4 (44%) |
| Summer 2016 | 9 | 9 (100%) | 8 (89%) | 2 (22%) |
| **Huangshan** | | | | |
| Summer 2016 | 3 | 3 (100%) | 1 (33%) | 1 (11%) |
| **Total** | **39** | **39 (100%)** | **36 (92%)** | **10 (36%)** |

[a] limit of detection as cutoff.

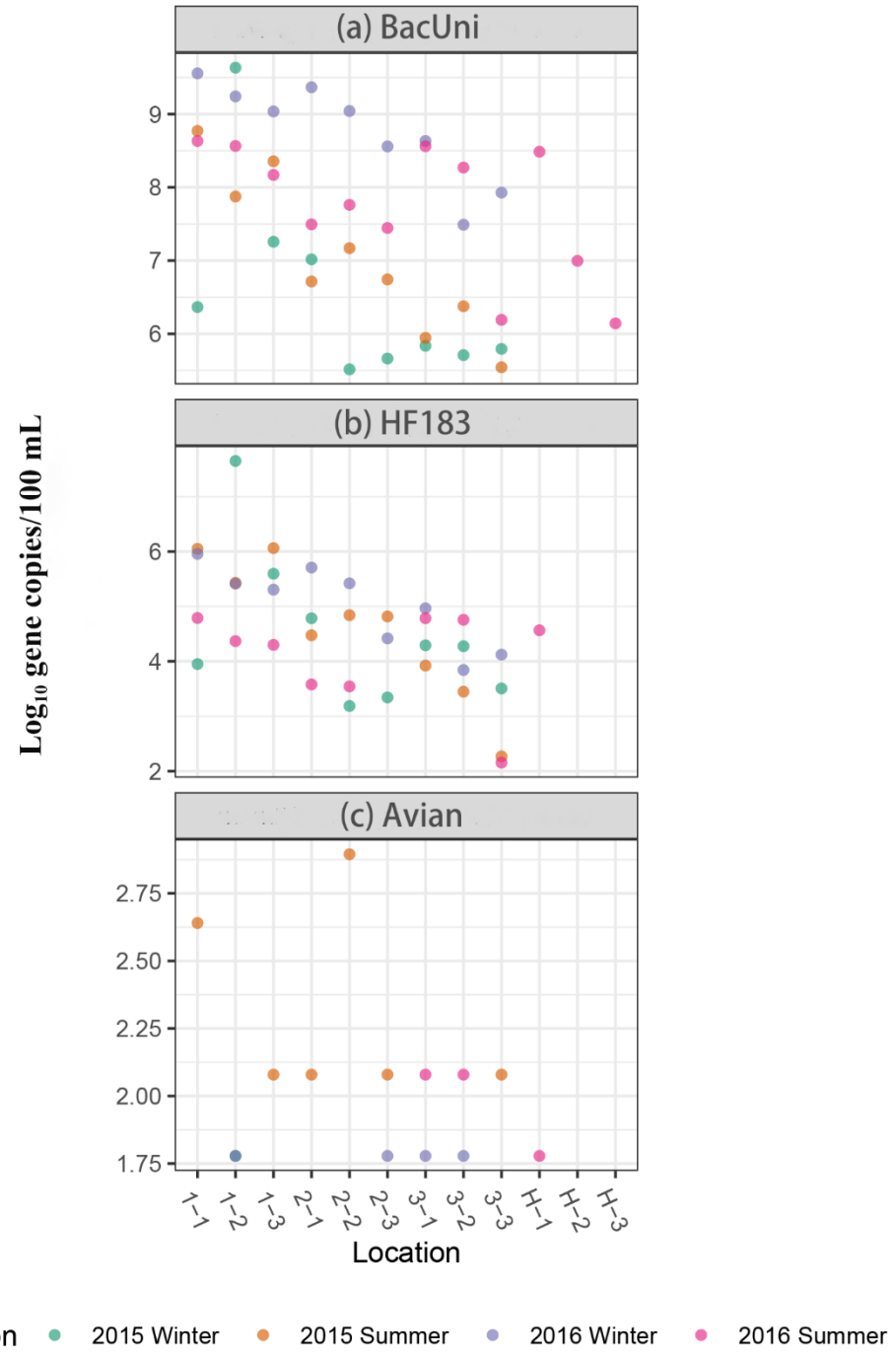



**Figure 5.1.** Concentrations of total *Bacteroidales* (BacUni) (a), human-associated *Bacteroidales* (HF183) (b), and avian-associated fecal markers (GFD) (c) in water samples collected from different locations in Suzhou and Huangshan.

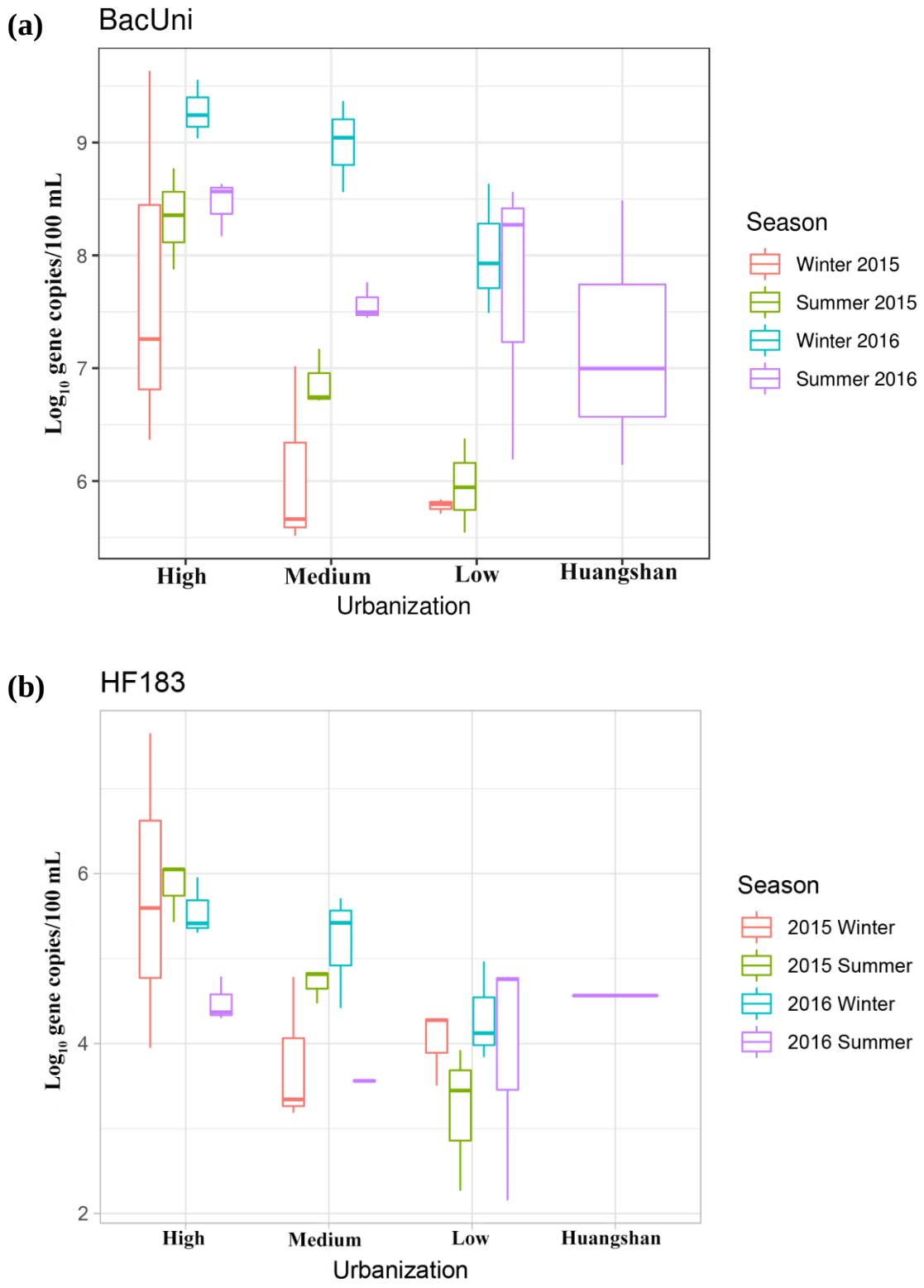



**Figure 5.2.** Comparison of fecal marker concentrations (boxplots with median value (line within each box), quartile interval (box), the minimum and maximum value (whiskers)) in Suzhou canals across different urban intensifications and Huangshan. a) Total *Bacteroidales* (BacUni) and b) human-associated *Bacteroidales* (HF183).

**Table 5.4A.** Two-way ANOVA for variations of fecal markers (BacUni and HF183).

| Source | Df | SS | MS | F | P |
|---|---|---|---|---|---|
| **Bac Uni** | | | | | |
| A. Urbanization† | 2 | 15.964 | 7.982 | 5.752 | **0.008** |
| B. Season‡ | 1 | 0.261 | 0.261 | 0.188 | 0.668 |
| A×B | 2 | 0.085 | 0.042 | 0.031 | 0.970 |
| **HF183** | | | | | |
| A. Urbanization | 2 | 16.134 | 8.067 | 7.930 | **0.002** |
| B. Season | 1 | 2.989 | 2.989 | 2.938 | 0.097 |
| A×B | 2 | 0.044 | 0.022 | 0.021 | 0.979 |

† Urbanization: High vs. Medium vs. Low; ‡ Season: Winter vs. Summer;
df: degree of freedom; SS: Sum Square; MS: Mean Square

**Table 5.4B.** Urbanization variation of fecal markers (BacUni and HF183).

| Urbanization | Mean Difference | Std. Error | Sig. (P) | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower Bound | Upper Bound |
| **Bac Uni** | | | | | |
| High vs. Medium | 1.081 | 0.481 | **0.032** | 0.099 | 2.063 |
| High vs. Low | 1.598 | 0.481 | **0.002** | 0.616 | 2.581 |
| Medium vs. Low | 0.518 | 0.481 | 0.290 | -0.464 | 1.500 |
| **HF183** | | | | | |
| High vs. Medium | 1.249 | 0.412 | **0.005** | 0.408 | 2.090 |
| High vs. Low | 1.545 | 0.412 | **0.001** | 0.704 | 2.386 |
| Medium vs. Low | 0.296 | 0.412 | 0.477 | -0.545 | 1.137 |

### 5.3.3. Detection frequencies and abundances of bacterial pathogens in canals of Suzhou and Huangshan

The detection frequencies and abundances of gene markers for six bacterial pathogens (*Enterococcus* spp., *Arcobacter butzleri*, *Shigella* sp., *Campylobacter jejuni*, *Salmonella* spp. and Shiga toxin producing *E. coli* (STEC)) are presented in Table 5.5. When detected but not quantificable (DNQ) samples were considered as positive, the most frequently detected pathogen was *Enterococcus* spp. (100%), followed by *Arcobacter butzleri* (74%), STEC (41%), *Shigella* sp. (36%), *Campylobacter jejuni* (10%) and *Salmonella* spp. (10%). The concentrations of genes of bacterial pathogens detected in water samples at each location is given in Table 5.6A-F. Considering limit of quantification (LOQ) as selection criteria, *Enterococcus* spp. was quantified in all the water samples (100%), and the concentrations ranged from 3.37 to 7.76-$\log_{10}$ gene copies/100 mL (Table 5.6A and Figure 5.3a). The highest concentration (7.76-$\log_{10}$ gene copies/100 mL) of *Enterococcus* spp. was observed at location 1-1 in winter 2016. Higher levels of *Enterococcus* spp. were observed in water samples colleced from locations with high urbanization. The concentrations of *Enterococcus* spp. showed statistically significant among locations with varying urbanization ($P = 0.034$) than seasonal variation (Table 5.7A). Among three urban intensifications (High, Medium and Low) in Suzhou, statistical significance was observed between High vs. Medium ($P = 0.031$) and High vs. Low ($P = 0.019$), but not for Medium vs. Low (Figure 5.4a and Table 5.7B). The detection frequency of *Arcobacter butzleri* (*A. butzleri*) was 74% (29 out of 39 water samples tested) (Table 5.6B and Figure 5.3b), and the concentrations ranged from 2.92 to 6.21-$\log_{10}$ gene copies/100 mL, with highest concentration (6.21-$\log_{10}$ gene copies/100 mL) at location 1-3 in summer 2015. *A. butzleri* showed statistically seasonal variation ($P = 0.000$). All the water samples collected in summer were quantified (3.36 to 6.21-$\log_{10}$ gene copies/100 mL), but only one sample was quantified (2.92-$\log_{10}$ gene copies/100 mL) in

winter 2015 (though seven more samples were detected but not quantifiable) and none of the samples collected from winter 2016 were at detectable levels. Statistically significant concentration of *A. butzleri* was observed in locations with varying urbanization (P = 0.019), and the variation was observed between High vs. Low (P = 0.005) (Figure 5.4b and Table 5.7b). Other pathogens, *Shigella* sp., *Campylobacter jejuni*, *Salmonella* spp. and Shiga toxin producing *E. coli* (STEC), were detected in few samples (10-41%) with very low concentrations (2.31 to 3.65-$\log_{10}$ gene copies/100 mL). These 4 pathogens were detected more frequently or quantified in higher levels at high urbanization locations (Table 5.6C-F, Figure 5.3c-f and Figure 5.5). The highest concentrations of *Shigella* sp. (2.81-$\log_{10}$ gene copies/100 mL) and STEC (3.44-$\log_{10}$ gene copies/100 mL) were observed at location 1-2 in summer 2016. The highest concentration of *Campylobacter jejuni* (3.65-$\log_{10}$ gene copies/100 mL) was observed at location 1-2 in winter 2015. The highest concentration of *Salmonella* spp. (3.17-$\log_{10}$ gene copies/100 mL) was observed at location 1-3 in summer 2015.

**Table 5.5.** Detection frequencies of pathogenic bacterial genes in water samples collected from Suzhou canals and Huangshan locations.

| Sample type | No. of samples tested (n) | No. of positive samples [a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | ***Enterococcus* spp.** | ***Arcobacter butzleri* (hsp60)** | ***Shigella* sp. (ipaH)** | ***Campylobacter jejuni* (mapA)** | ***Salmonella* spp.** | **STEC (stx2)** |
| **Suzhou** | | | | | | | |
| Winter 2015 | 9 | 9 (100%) | 8 (89%) | 2 (22%) | 1 (11%) | 0 | 1 (11%) |
| Summer 2015 | 9 | 9 (100%) | 9 (100%) | 3 (33%) | 0 | 1 (11%) | 0 |
| Winter 2016 | 9 | 9 (100%) | 0 | 2 (22%) | 3 (33%) | 2 (22%) | 7 (78%) |
| Summer 2016 | 9 | 9 (100%) | 9 (100%) | 6 (67%) | 0 | 1 (11%) | 6 (67%) |
| **Huangshan** | | | | | | | |
| Summer 2016 | 3 | 3 (100%) | 3 (100%) | 1 (33%) | 0 | 0 | 2 (67%) |
| **Total** | 39 | **39 (100%)** | **29 (74%)** | **14 (36%)** | **4 (10%)** | **4 (10%)** | **16 (41%)** |

[a] Considering DNQ's (Detected but Not Quantifiable) as positive samples.

**Table 5.6A.** Concentration of pathogenic bacterial genes (*Enterococcus* spp.) in water samples collected from Suzhou canals and Huangshan locations.

| ***Enterococcus* spp.** **Water ($log_{10}$ gene copies/100 mL)** | | | | |
|---|---|---|---|---|
| Location | Winter 2015 | Summer 2015 | Winter 2016 | Summer 2016 |
| 1-1 | **3.67** | **5.85** | **7.76** | **4.98** |
| 1-2 | **5.23** | **5.79** | **6.97** | **5.20** |
| 1-3 | **4.25** | **6.09** | **6.51** | **4.99** |
| 2-1 | **3.37** | **4.75** | **5.83** | **4.12** |
| 2-2 | **3.65** | **5.06** | **6.53** | **4.30** |
| 2-3 | **3.61** | **4.55** | **6.15** | **4.23** |
| 3-1 | **4.38** | **4.73** | **6.33** | **4.87** |
| 3-2 | **4.01** | **4.65** | **4.73** | **4.47** |
| 3-3 | **4.37** | **4.03** | **5.05** | **3.43** |
| H-1 | | | | **5.17** |
| H-2 | | | | **5.16** |
| H-3 | | | | **4.76** |

DNQ: Detected but Not Quantifiable; ND: Non-Detected.

**Table 5.6B.** Concentration of pathogenic bacterial genes (*Arcobacter butzleri*) in water samples collected from Suzhou canals and Huangshan locations.

| ***Arcobacter butzleri*** **Water ($log_{10}$ gene copies/100 mL)** | | | | |
|---|---|---|---|---|
| Location | Winter 2015 | Summer 2015 | Winter 2016 | Summer 2016 |
| 1-1 | **DNQ** | **5.97** | ND | **4.82** |
| 1-2 | ND | **5.03** | ND | **4.89** |
| 1-3 | **DNQ** | **6.21** | ND | **4.79** |
| 2-1 | **DNQ** | **4.34** | ND | **4.54** |
| 2-2 | **DNQ** | **4.94** | ND | **4.64** |
| 2-3 | **2.92** | **4.36** | ND | **4.33** |
| 3-1 | **DNQ** | **4.24** | ND | **5.04** |
| 3-2 | **DNQ** | **3.88** | ND | **4.42** |
| 3-3 | **DNQ** | **3.36** | ND | **4.05** |
| H-1 | | | | **4.25** |
| H-2 | | | | **3.80** |
| H-3 | | | | **3.69** |

DNQ: Detected but Not Quantifiable; ND: Non-Detected.

**Table 5.6C.** Concentration of pathogenic bacterial genes (*Shigella* sp.) in water samples collected from Suzhou canals and Huangshan locations.

| ***Shigella* sp.** **Water ($log_{10}$ gene copies/100 mL)** | | | | |
|---|---|---|---|---|
| Location | Winter 2015 | Summer 2015 | Winter 2016 | Summer 2016 |
| 1-1 | ND | ND | ND | ND |
| 1-2 | **DNQ** | **DNQ** | ND | **2.81** |
| 1-3 | ND | **DNQ** | ND | **2.79** |
| 2-1 | ND | ND | ND | **DNQ** |
| 2-2 | **DNQ** | ND | ND | **2.63** |
| 2-3 | ND | ND | **2.52** | **DNQ** |
| 3-1 | ND | ND | ND | **DNQ** |
| 3-2 | ND | **DNQ** | ND | ND |
| 3-3 | ND | ND | **2.31** | ND |
| H-1 | | | | ND |
| H-2 | | | | **DNQ** |
| H-3 | | | | ND |

DNQ: Detected but Not Quantifiable; ND: Non-Detected.

**Table 5.6D.** Concentration of pathogenic bacterial genes (*Campylobacter jejuni*) in water samples collected from Suzhou canals and Huangshan locations.

| ***Campylobacter jejuni*** **Water ($log_{10}$ gene copies/100 mL)** | | | | |
|---|---|---|---|---|
| Location | Winter 2015 | Summer 2015 | Winter 2016 | Summer 2016 |
| 1-1 | ND | ND | ND | ND |
| 1-2 | **3.65** | ND | ND | ND |
| 1-3 | ND | ND | ND | ND |
| 2-1 | ND | ND | **2.31** | ND |
| 2-2 | ND | ND | **2.35** | ND |
| 2-3 | ND | ND | ND | ND |
| 3-1 | ND | ND | ND | ND |
| 3-2 | ND | ND | **DNQ** | ND |
| 3-3 | ND | ND | ND | ND |
| H-1 | | | | ND |
| H-2 | | | | ND |
| H-3 | | | | ND |

DNQ: Detected but Not Quantifiable; ND: Non-Detected.

**Table 5.6E.** Concentration of pathogenic bacterial genes (*Salmonella* spp.) in water samples collected from Suzhou canals and Huangshan locations.

| ***Salmonella* spp.** | | | | |
|---|---|---|---|---|
| **Water ($log_{10}$ gene copies/100 mL)** | | | | |
| Location | Winter 2015 | Summer 2015 | Winter 2016 | Summer 2016 |
| 1-1 | ND | ND | ND | ND |
| 1-2 | ND | ND | ND | ND |
| 1-3 | ND | **3.17** | ND | ND |
| 2-1 | ND | ND | **DNQ** | **DNQ** |
| 2-2 | ND | ND | ND | ND |
| 2-3 | ND | ND | ND | ND |
| 3-1 | ND | ND | ND | ND |
| 3-2 | ND | ND | **3.16** | ND |
| 3-3 | ND | ND | ND | ND |
| H-1 | | | | ND |
| H-2 | | | | ND |
| H-3 | | | | ND |

DNQ: Detected but Not Quantifiable; ND: Non-Detected.

**Table 5.6F.** Concentration of pathogenic bacterial genes (STEC) in water samples collected from Suzhou canals and Huangshan locations.

| **STEC** | | | | |
|---|---|---|---|---|
| **Water ($log_{10}$ gene copies/100 mL)** | | | | |
| Location | Winter 2015 | Summer 2015 | Winter 2016 | Summer 2016 |
| 1-1 | ND | ND | ND | **3.23** |
| 1-2 | **2.92** | ND | **2.88** | **3.44** |
| 1-3 | ND | ND | **3.05** | **3.36** |
| 2-1 | ND | ND | ND | ND |
| 2-2 | ND | ND | **2.91** | **3.02** |
| 2-3 | ND | ND | **2.87** | ND |
| 3-1 | ND | ND | **3.19** | **3.28** |
| 3-2 | ND | ND | **2.96** | **3.14** |
| 3-3 | ND | ND | **2.89** | ND |
| H-1 | | | | **2.86** |
| H-2 | | | | **2.88** |
| H-3 | | | | ND |

DNQ: Detected but Not Quantifiable; ND: Non-Detected.

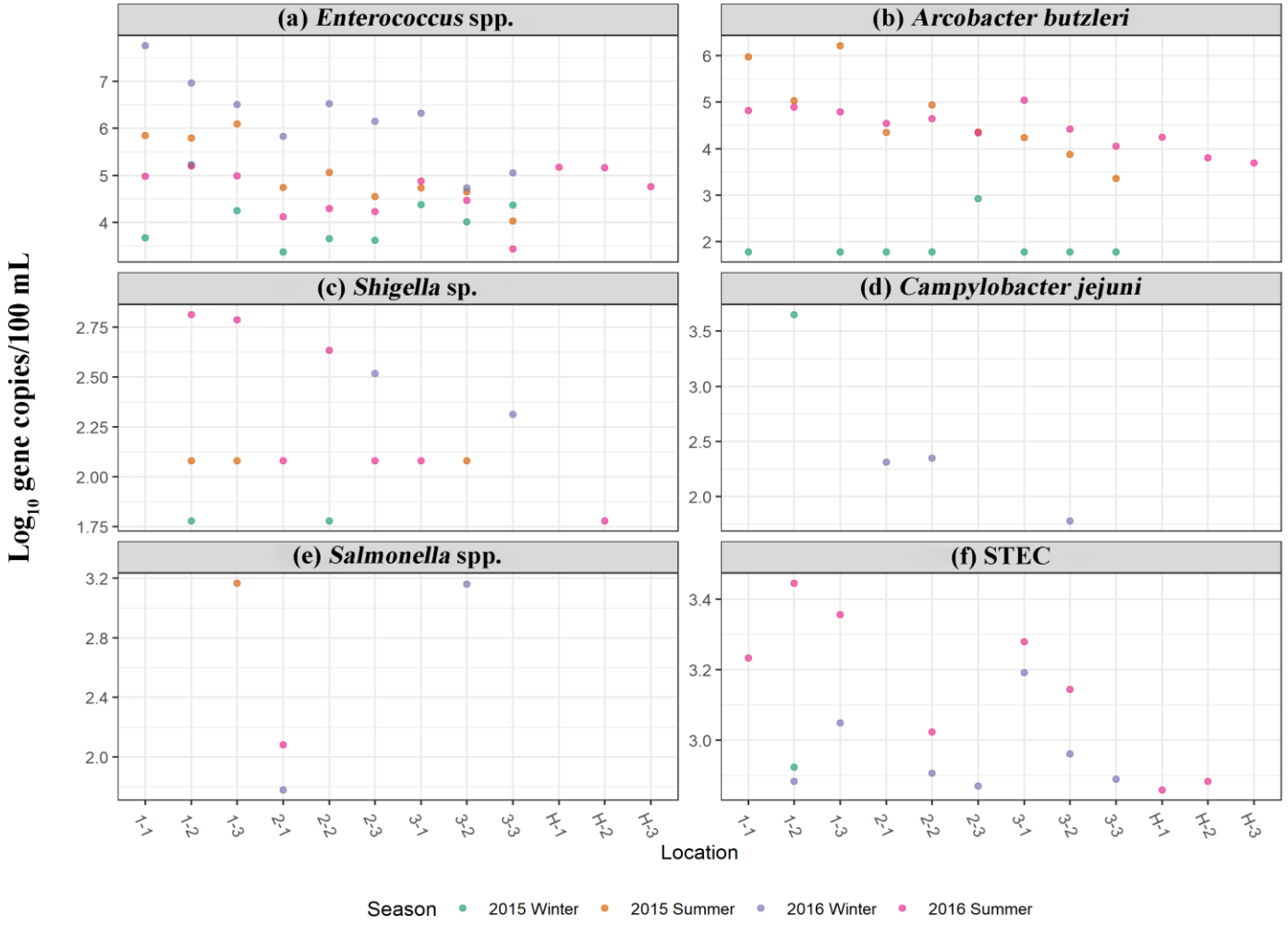



**Figure 5.3.** The abundances of pathogens (*Enterococcus* spp., *Arcobacter butzleri*, *Shigella* sp., *Campylobacter jejuni*, *Salmonella* spp. and STEC) in water samples collected from Suzhou canals and Huangshan.

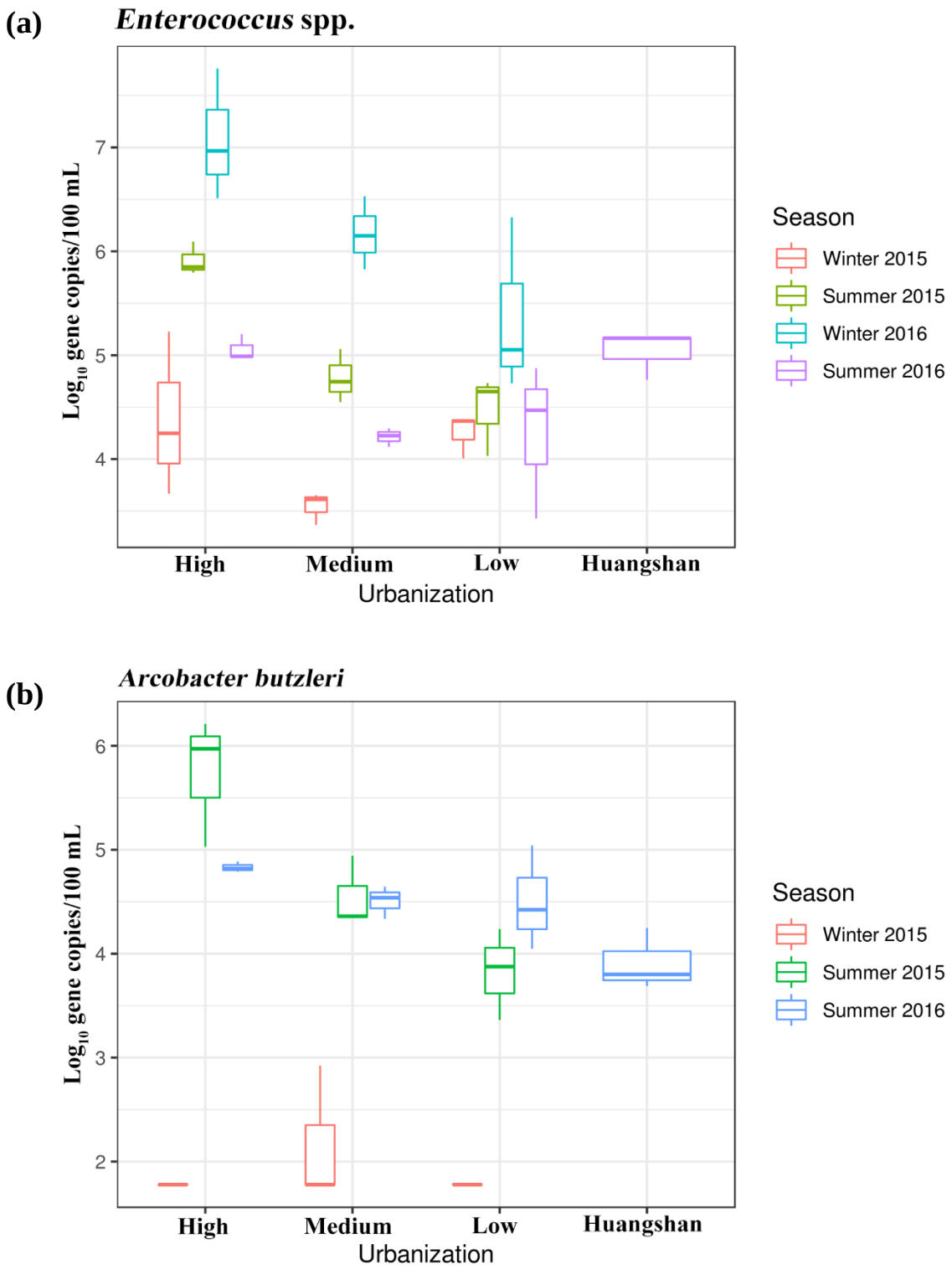



**Figure 5.4.** Comparison of bacterial pathogen concentrations (boxplots with median value (line within each box), quartile interval (box), the minimum and maximum value (whiskers)) in Suzhou canals across different urban intensifications and Huangshan. a) *Enterococcus* spp. and b) *Arcobacter butzleri*.

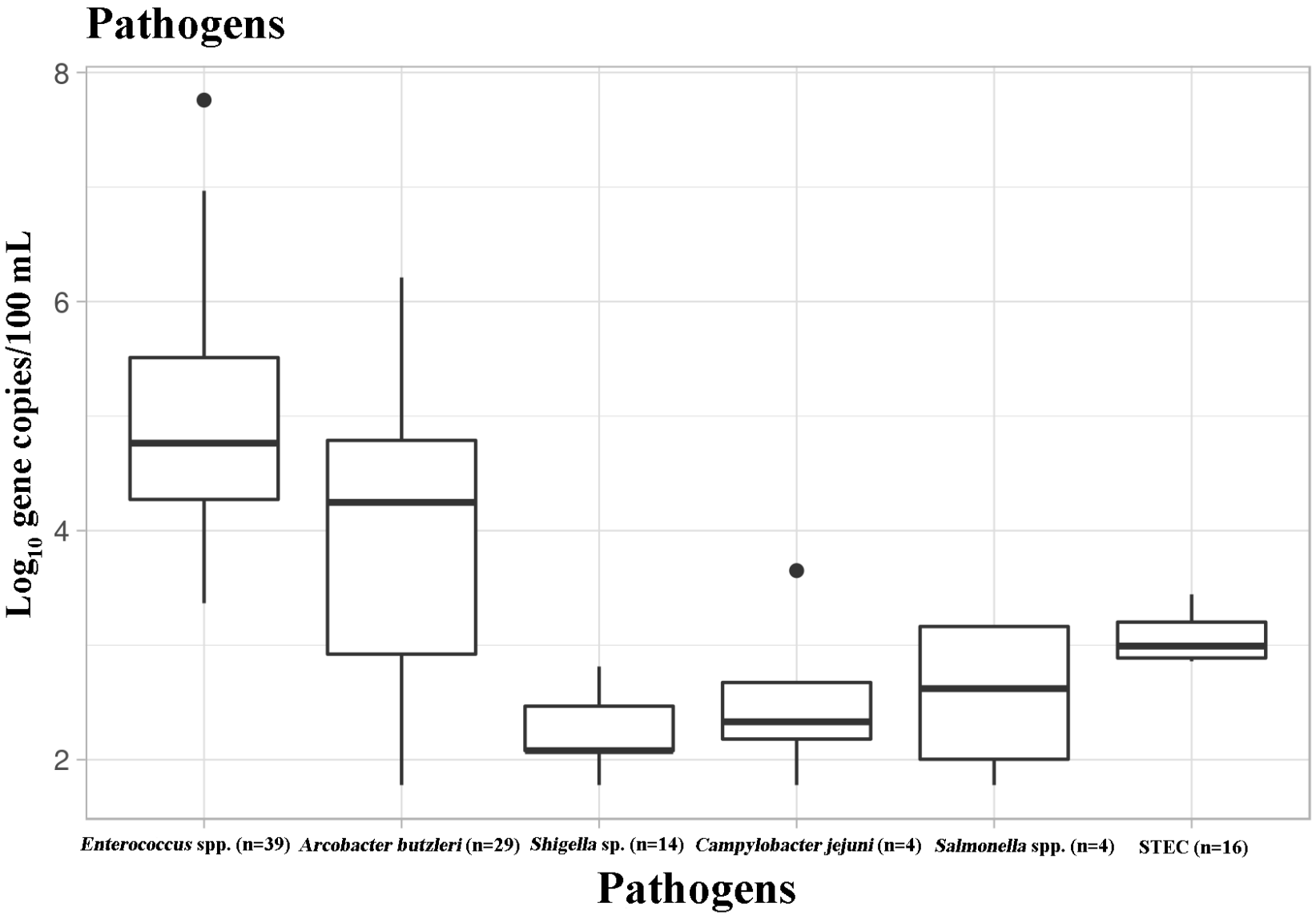



**Figure 5.5.** Comparison of six pathogenic gene markers in all the water samples collected from Suzhou canals and Huangshan.

**Table 5.7A.** Two-way ANOVA for abundances of *Enterococcus* spp. and *Arcobacter butzleri*.

| Source | df | SS | MS | F | P |
|---|---|---|---|---|---|
| ***Enterococcus* spp.** | | | | | |
| A. Urbanization† | 2 | 7.653 | 3.826 | 3.792 | **0.034** |
| B. Season‡ | 1 | 1.098 | 1.098 | 1.088 | 0.305 |
| A×B | 2 | 0.061 | 0.030 | 0.030 | 0.970 |
| ***Arcobacter butzleri*** | | | | | |
| A. Urbanization† | 2 | 1.718 | 0.859 | 4.518 | 0.019 |
| B. Season‡ | 1 | 80.149 | 80.149 | 421.466 | 0.000 |
| A×B | 2 | 2.396 | 1.198 | 6.299 | 0.005 |

† Urbanization: High vs. Medium vs. Low; ‡ Season: Winter vs. Summer;
df: degree of freedom; SS: Sum Square; MS: Mean Square.

**Table 5.7B.** Variations in abundances of *Enterococcus* spp. and *Arcobacter butzleri* in different sampling locations.

| Urbanization | Mean Difference | Std. Error | Sig. (P) | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower Bound | Upper Bound |
| ***Enterococcus* spp.** | | | | | |
| High vs. Medium | 0.930 | 0.410 | **0.031** | 0.092 | 1.767 |
| High vs. Low | 1.020 | 0.410 | **0.019** | 0.182 | 1.857 |
| Medium vs. Low | 0.090 | 0.410 | 0.828 | -0.748 | 0.928 |
| ***Arcobacter butzleri*** | | | | | |
| High vs. Medium | 0.258 | 0.178 | 0.157 | -0.105 | 0.622 |
| High vs. Low | 0.535 | 0.178 | **0.005** | 0.171 | 0.899 |
| Medium vs. Low | 0.277 | 0.178 | 0.130 | -0.087 | 0.640 |

## 5.4. Discussion

Fecal pollution of surface waters is a serious issue for the aquatic ecosystem and human health. Fecal sources enter into freashwater ecosystem through several sources such as effluents from wastewater treatment plants, septic leaks, urban and storm runoff water (Marsalek and Rochfort, 2004, Litton et al., 2010, Newton et al., 2013, Molina et al., 2014, Kapoor et al., 2015). In this study, the impact of urbanization on the abundance of fecal markers and assoiated pathogens in Suzhou canals was studied.

In case of fecal pollution, impact of urbanization was observed for total *Bacteroidales* and human-associated *Bacteroidales*, although avian-associated markers did not show any trend. Higher concentrations of total and human-associated markers were observed in water samples collected from locations with high urbanization than medium and low urbanization in Suzhou, indicating that canals in high urbanization areas are affected with fecal pollution and human sewage was a key contributor for the pollution. This could be due to discharge of sewer and septic waste in the water bodies directly or indirectly (Ohad et al., 2015). Runoff after rain fall could also lead to fecal source entry into watersheds (Kim et al., 2013). However, it should be noted that variation of season on precipitation in Suzhou was observed based on local government published reports about climate parameters (Supplementary Figures S2.1 and S2.2). There were more precipitations in summer than in winter, but no seasonal variation on both BacUni and HF183 was observed in this study, which indicated that their presence and abuanances were not affected by the rainfall. Similar trend of water impairment due to urbanization was reported elsewhere for urbanized tropical watershed (Kirs et al., 2017). High Density Residential Land (36-57%) represented the highest percentage of land use pattern in locations with high urbanization (See Chapter 2), and excess domestic sewage caused by local

resident and more human activities were observed in the area. High level of nutrients and fecal indicator bacteria (total coliform and fecal coliform) were observed in water samples collected from locations with high urbanization in Suzhou (See Chapter 2). All of these correlate well with the qPCR results obtained in this study. At the same time, by applying microbial source tracking (MST) methods, Molina assessed the presence and high levels of human contaminations in urban runoff (Molina et al., 2014). Among three urban intensifications (High, Medium and Low) in Suzhou, the variations between of High vs. Medium and High vs. Low could be observed. However, no significant variation in the abundance between Medium vs. Low was observed. This could be related to the population density of each urban classification used in this study: High, Medium and Low were >8000, 1700-2100 and 800-1100 persons/$km^2$, respectively. The gap of population density between Medium vs. Low is not large enough as High vs. Medium and High vs. Low, which might have caused no significant variation in the abundance between Medium vs. Low was observed in this. Since almost no poultry or other related animals activity were observed near the sampling locations, avian-associated fecal markers were only detected in some samples with very low concentrations. As a result, human sewage, rather than animals, was the main source of fecal contaminations in urbanized canals of Suzhou. Huangshan is a good control area, as the waterways are protected by local government in natural reserve. Low levels of fecal markers were observed in water samples colleted from there, except location H-1. This location is close to a village at the foot of the mountain, where some family farming activities (e.g. poultry) were observed during sampling, which caused some fecal contaminations in one of the control locations (H-1).

For pathogens, variation in the abundance of *Enterococcus* spp. was observed with respect to urbanization, and significantly higher levels of *Enterococcus* spp. were observed in water

samples colleced from locations with high urbanization than medium and low urbanization in Suzhou. Many species of *Enterococcus* are prevalent in the gastrointestinal (GI) tract of mammals, making them widely used as bacterial indicators for faecal pollution in water. Although *Enterococcus* spp. usually do not pose any health risks to humans, their presence in water could suggest possible presence of enteric pathogens (Martzy et al., 2017, Zimmer-Faust et al., 2017). Therefore, the quantification of *Enterococcus* spp. by qPCR should be related to the results of fecal markers, expecially human specific fecal markers. It was comfired in this study that both *Enterococcus* spp. and human associated *Bacteroidales* were represented at higher levels in water samples colleced from locations with high urbanization than medium and low urbanization in Suzhou. These results correlate with extremely high levels of multiple nutrients and also total and fecal coliforms in high urbanization sampling locations. The optimal growth temperature range for *Arcobacter butzleri* is from 26 to 30 $^{o}$C (Fernandez et al., 2015), which matches with the summer temperature observed in Suzhou canals (27-33 $^{o}$C, Chapter 2). This is in support of the findings observed in this study; *Arcobacter butzleri* was quantified in all the water samples collected in summer, but was detected at the quantifiable range in only one sample during winter, as temperature in winter (5-11 $^{o}$C, Chapter 2) is probably too low for this bacterium. Variation in the abundance of *Arcobacter butzleri* with respect to urbanization was also observed. Significantly higher levels of *Arcobacter butzleri* were observed in water samples colleced from locations with high urbanization than low urbanization in Suzhou. The genus *Arcobacter* was reported to be associated with human illness and fecal contamination, and human fecal source was likely to be a key contributor to *Arcobacter* contamination (Lee et al., 2012). Some species of the genus *Arcobacter* were considered as emerging food pathogens (Hausdorf et al., 2013). Among these species, *Arcobacter butzleri* was just an underestimated

enteropathogen (Prouzet-Mauléon et al., 2006). Therefore, it is an notable observation in this study. Variation of urbanization on *Arcobacter butzleri* is positively raleted to *Enterococcus* spp., total *Bacteroidales*, human host-associated *Bacteroidales* and water quality results. *Shigella* sp. is one of the major food-borne pathogens that caused human shigellosis worldwide (Ma et al., 2014). Shiga toxin producing *E. coli* (STEC) is associated with production of Shiga toxin or Vero toxin, and STEC plays an important role as pathogens in humans (Beutin et al., 2008). *Campylobacter jejuni* is a clinically important bacteria (Best et al., 2003). *Salmonella* spp., a leading cause of morbidity and mortality due to food and water borne diseases in many countries, causes gastroenteritis, typhoid and diarrheal illnesses for human beings (Jyoti et al., 2010, Ma et al., 2014). However, all these four pathogens were only detected in several samples with very low levels as compared to *Enterococcus* spp. and *Arcobacter butzleri*, and these pathogens were detected in higher levels in locations with high urbanization in Suzhou. Therefore, this study confirms that high urbanization impacted and increased the abundances of pathogens, as well as fecal markers.

### 5.5. Conclusions

In general, for fecal markers, variation in the abundances of total *Bacteroidales* and human-associated *Bacteroidales* were observed with respect to urbanization. Higher levels of these two fecal markers were observed in water samples collected from locations with high urbanization as compared to medium and low urbanization in Suzhou and Huangshan. Avian-associated markers were only found at very low levels. It can be concluded that high urbanization caused fecal pollution in Suzhou canals. Sewage and wastewater input in to the canals would appear to be one of the main sources, rather than livestock and poultry.

In this study, *Enterococcus* spp. was the most frequently detected pathogenic marker in water samples (100%), followed by *Arcobacter butzleri* (74%), Shiga toxin-producing *E. coli* (STEC) (41%), *Shigella* sp. (36%), whereas *Campylobacter jejuni* and *Salmonella* spp. were least frequently detected (10%). Variation in the abundances of *Enterococcus* spp. and *Arcobacter butzleri* with respect to urbanization were observed, and higher levels of both pathogens were detected in water samples collected from locations with high urbanization than medium and low urbanization in Suzhou and locations in Huangshan.

In conclusion, high urbanization impacted on the presence and abundances of both fecal and pathogen markers in Suzhou canals. Significantly higher levels of total *Bacteriodales*, human-associated *Bacteroidales* and bacterial pathogens (mainly *Enterococcus* spp. and *Arcobacter butzleri*) were observed in locations with high urbanization, which has public health significance.

# Chapter 6 - Summary and Future Directions

## 6.1. Summary

The overall aim of this PhD thesis was to investigate the influence of urbanization on water quality, microbial abundance, microbial diversity and ecosystem function using Suzhou canals as a model system. Nine sampling locations in Suzhou covering three urbanization gradients (high, medium and low) were selected for the study and three sampling locations in the natural reserve mountains in Huangshan were used as control locations for this study. This thesis comprised of:

1) Assessment of physico-chemical and microbiological parameters in Suzhou canals across a gradient of urban intensification, and comparison of the water quality results with locations in Huangshan;
2) Assessment of microbial (bacterial and fungal) diversity in water and leaf samples by next generation sequencing of specific target genes (16S rRNA and ITS1), and assessment of organic matter (leaf-litter) breakdown to study the influence of urbanization on the microbial diversity and ecosystem function;
3) Laboratory microcosm experiments to study the influence of key environmental parameters on the microbial diversity and ecosystem function;
4) Quantitative assessment of the presence and abundance of selected fecal markers and bacterial pathogens in Suzhou canals and Huangshan sampling locations.

In Chapter 2, detailed physico-chemical and microbiological analyses were carried out to assess the impact of urbanization on the water quality of canals in Suzhou and results compared with control locations in Huangshan. Fourteen water quality parameters including temperature, pH, conductivity, multiple nutrients and microbiological parameters were analyzed on four occasions over a two-year period. The results showed that many of the parameters such as pH, EC, TN, TP, $PO_4$-P, $NH_4$-N, TVC, TC and FC varied significantly with urban intensification, and

parameters such as water temperature, EC, TN, $PO_4$-P, $NO_3$-N, $NO_2$-N, Chl *a* and FC showed significant seasonal variations. Higher levels of nutrients, particularly TN, TP, $NH_4$-N and $PO_4$-P, were usually observed in locations with high urban intensification as compared to medium and low urbanization. The nutrient values in samples from Huangshan were extremely low in the absence of any influence from urbanization and human activities as compared to Suzhou canals. TVC, TC and FC counts were higher in locations with high urbanization as compared to locations with low urbanization in Suzhou and Huangshan, which correlates well with physico-chemical parameters. Land use types in locations with high urbanization areas were mainly high density residential lands. The overall study indicates that urbanization had a high impact on the water quality of canals, and sources like domestic wastewater or sewage probably were the main contributors leading to pollution and serious eutrophication in Suzhou canals.

In Chapter 3, detailed study on the influence of urbanization on the bacterial and fungal diversity and organic matter breakdown was carried out. The bacterial and fungal diversity were studied by NGS of 16S rRNA gene and ITS1 region amplicons using Illumina PE250 platform followed by the detailed alpha and beta diversity analyses. The bacterial and fungal communities in water were distinct from the leaf associated community and a strong seasonal variation in the diversity was also observed. The phylum Proteobacteria was dominant (20-80%) in almost all the water and leaf samples, followed by either Bacteriodetes in winter or Actinobacteria during summer in water samples, and Bacteroidetes during winter and Firmicutes and/or Chloroflexi during summer in leaf samples. At order level, the members of Burkholderiales were dominant (5-50%) in most samples, followed by Flavobacteriales, Pseudomonadales in water and leaf samples in winter, and Frankiales, Rhodocyclales, Sphingobacteriales in water samples in summer whereas, Pseudomonadales co-dominated the leaf samples in some locations in summer.

At genus level, *Flavobacterium, Pseudomonas* and members of Comamonadaceae were represented at higher percentages in both water and leaf samples in winter than in summer. *Flavobacterium* was dominant (3-47%) in most water and leaf samples in winter 2015. *Milikia, Arcobacter* and *Polynucleobacter* were represented at higher percentages in water samples than in leaf samples whereas, *Acinetobacter* was present at higher percentage in leaf samples than in water samples. Some bacterial genera (*Arcobacter, Massilia* and *Acinetobacter*) typically found in wastewater or associated with human / animal microbiomes, were represented at high percentages in high and medium urban intensification areas. In the fungal community at phylum level, Ascomycota was dominant (2-99%) in most water and leaf samples, and Chytridiomycota was the dominant phylum (67-81%) in water samples collected in two of the medium urbanization locations (2-1 and 2-2) in winter 2015. Pleosporales was dominant (1-99%) in most leaf samples at order level, and Hypocreales was dominant (56-98%) in most leaf samples collected from locations with high urbanization in winter 2016. Some of the fungal genera (e.g. *Trichothecium*) associated with pathogens / microbiomes and human health were represented at high percentages in high urban intensification areas whereas, natural fungal flora (*Alternaria*) associated with decomposition / ecosystem function represented at high percentages in low urban intensification areas and natural reserve areas which indicated ecosystem functions and services were well maintained in fresh watersheds with less human eutrophic activities. Beta diversity (NMDS and cluster) analysis showed more variation in bacterial / fungal community with regards to urbanization in Suzhou canals as compared to OTU and other community analysis. The relationship between bacterial / fungal community and temperature / nutrient parameters was confirmed by RDA / CCA that nutrient parameters could affect bacterial / fungal composition. The effect of nutrients on bacterial/fungal community was more clearly observed between

Huangshan and Suzhou, as compared to urban intensifications in Suzhou, except locations with heavy pollution (e.g. locations 1-2 and 2-2 where high levels of nutrients or algal blooms observed). Ecosystem function (AFDW loss rate) was significantly affected by season rather than urbanization; correlation between AFDW loss rate and temperature was also confirmed, and high temperature accelerate AFDW loss rate. In summary, the results showed that microbial diversity and ecosystem function in Suzhou canals were affected by both season and urbanization, but season had a greater influence than urbanization.

In Chapter 4, the influence of some key environmental parameters such as temperature, nutrients and heavy metals on the relationship of microbial diversity and ecosystem function (OM breakdown rate) were studied by laboratory microcosm experiments. Based on OM breakdown rates and bacterial community results, temperature was found to be a key factor that affects the composition of the bacterial community. Bacterial diversity increased at high temperature (35 °C) with more OTUs compared to medium (20 °C) or low (5 °C) temperature, and OM decomposition rate was also accelerated at higher temperature. Whereas, nutrients (ammonia and phosphate) had fewer effects on the composition of bacterial community and nutrients accelerated only the OM breakdown rate in short term. The heavy metals had effects on the shifts in bacterial community composition, especially high concentration of Cu (0.1mM) reduced OUT diversity and also OM biodegradation rate significantly.

In Chapter 5, the abundances of fecal markers and pathogens in water samples collected from Suzhou canals with varying urbanization were assessed by qPCR and the results compared with the samples collected from Huangshan locations. The data showed that the presence and abundance of total *Bacteroidales* and human-associated *Bacteroidales* varied with respect to urbanization, and higher levels of both fecal markers were observed in water samples collected

from locations with high urbanization than medium and low urbanization in Suzhou and in locations in Huangshan. The avian-associated markers were only found at very low levels as compared to total *Bacteroidales* and human-associated *Bacteroidales*. High urbanization caused fecal pollution in Suzhou canals, and human sewage would appear to be one of the main sources, rather than livestock and poultry. The bacterial pathogens, *Enterococcus* spp. were the most frequently detected pathogens in water samples (100%), followed by *Arcobacter butzleri* (54%), *Shigella* sp. and Shiga toxin-producing *E. coli* (STEC) (41%), whereas *Campylobacter jejuni* and *Salmonella* spp. were least frequently detected (10% and 5%, respectively). The abundances of *Enterococcus* spp. and *Arcobacter butzleri* significantly varied with respect to urbanization, and higher levels of both pathogens were observed in water samples collected from locations with high urbanization than medium and low urbanization in Suzhou and control locations in Huangshan. In general, high urbanization had an impact on the presence and abundances of both fecal and pathogen contaminations in Suzhou canals and significantly high levels of total *Bacteroidales*, human-associated *Bacteroidales* and bacterial pathogens (mainly *Enterococcus* spp. and *Arcobacter butzleri*) were observed in locations with high urbanization.

In summary, the results obtained in this study demonstrated that urbanization impacted canal water quality and led to the presence of fecal markers and enteric pathogenic gene markers. Although overall microbial diversity was affected more by season than urbanization, high levels of nutrients and increased temperature were found to be key factors which affect both bacterial / fungal diversity and the organic matter (OM) breakdown rate.

## 6.2. Future Research Directions

The research findings in this thesis have provided ideas for further improvement and new research directions. In this study, nine sampling locations covering three urban gradients were used, and the population densities in each location were >8000, 1700-2100 and 800-1100 persons/km$^2$ for High, Medium and Low urbanization categories, respectively. The population density difference between Medium vs. Low is limited, which is probably why variations in the nutrients, fecal markers and pathogens between these two categories were not found to be significant. Maps also indicated less variation in land use pattern between the sampling locations in the medium and low urbanization locations. Therefore, future studies should include more sampling locations with clear differences in the population density among the urban gradients to provide a better picture of the impact of urbanization on water quality and other parameters.

Secondly, no significant variation in the organic matter breakdown rate with regards to urbanization was observed in field studies. Leaf bags were used for the experiments, but no expected result was obtained (no variation in OM breakdown rate with respect to urbanization was found), which indicated leaf bag experiments may not be an effective and precise method to assess ecosystem function in this study. Alternative methods could be tested for comparison and better measurement of organic matter breakdown, for example the polymer PCP2000 (used for microcosm studies in this project), could be used for field studies as complement of leaf bags to assess the organic matter breakdown rate. In addition, other functional genes can be quantified by qPCR as the index for ecosystem function to assess the effect of bacterial/fungal species on decomposition of organic matter (e.g. cellulose degrading genes and ammonia oxidizing bacteria). Cellulose is one of the major components in leaf samples and the abundance of cellulose degrading genes could be an index of leaf litter degradation. Ammonium oxidizing

bacteria (AOB) are associated with the bio-geochemically important process of nitrification (Bouskill et al., 2011), and the abundance and composition of AOB has been previously used as an indicator for environmental impact from wastes, heavy metals and hydrocarbon (Kowalchuk and Stephen, 2001, Bouskill et al., 2011, Zhang et al., 2015). As the major sources for ammonia and organic nitrogen in water bodies are sewage effluent and / or run off from agriculture, the measurement of AOB and associated genes can be used as pollution indicators. Furthermore, new molecular techniques, such as Geo-Chip analysis through functional gene arrays (Stormer et al., 2013, Wang et al., 2014), metagenomics (Steffen et al., 2012, Low-Decarie et al., 2015, Gourmelon et al., 2016), metatranscriptomics (Helbling et al., 2012, Vikram et al., 2016) and proteomics (Russo et al., 2019, Schneider and Riedel, 2010), are potential approaches to investigate the functional structure of microbial communities and the relationship between functional microbial groups and total microbial community.

In the present study, only limited parameters were tested in the microcosm studies on the impact on bacterial diversity and ecosystem function due to limited time availability for lab studies, and other resources. Additional parameters could be tested in microcosm studies to find out the effect of multiple factors on microbial diversity and ecosystem function. Besides nutrients and heavy metals, various chemicals (e.g. endocrine disrupting chemicals), antibiotics, steroid hormones, pharmaceuticals, personal care products, pesticides, fecal matters and pathogens affect the ecosystem function. Many of these parameters could be tested in microcosm studies subject to getting approval for health and lab safety.

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# Supplementary Files

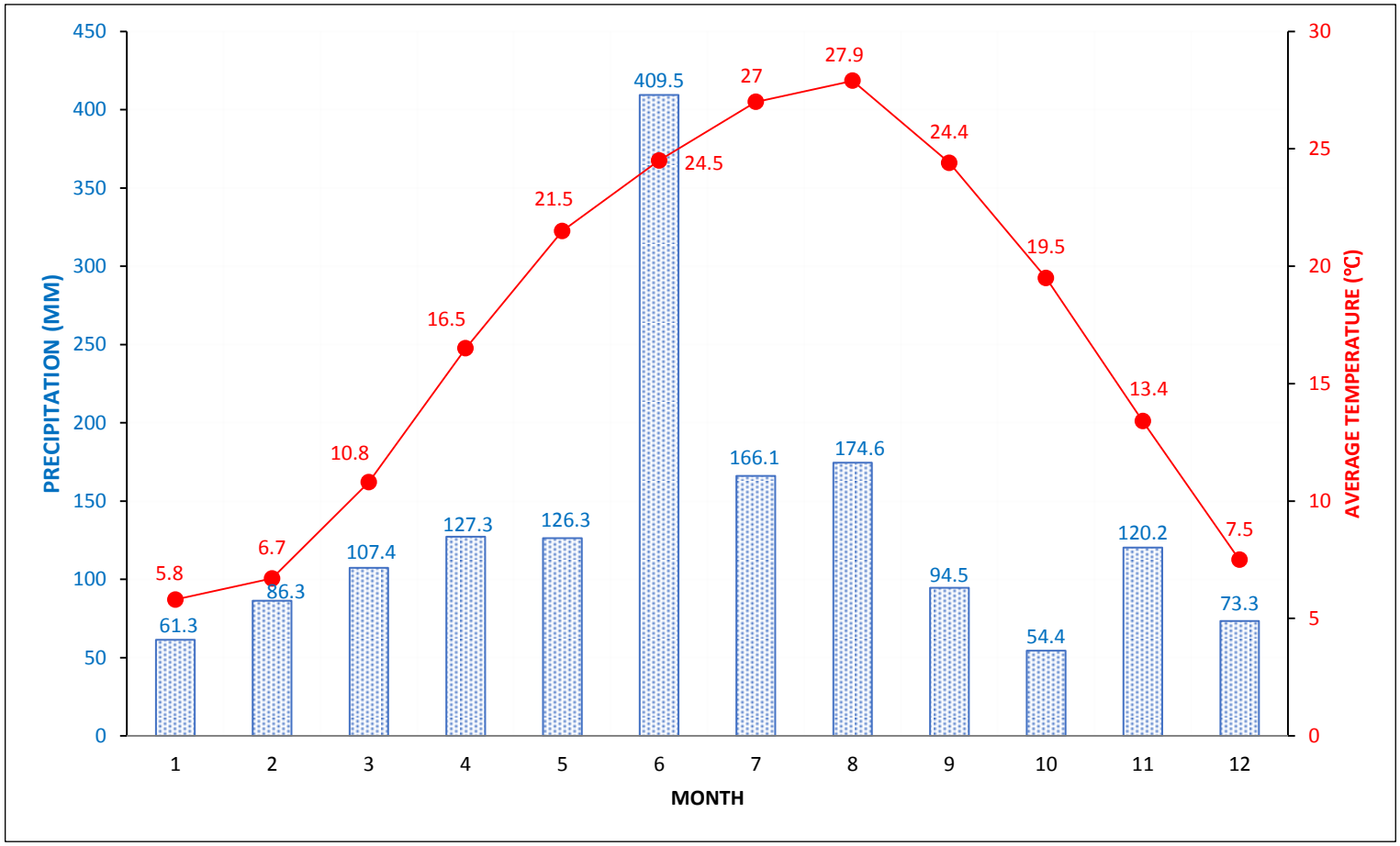



**Figure S2.1.** The average air temperature and precipitation in Suzhou during 2015**.**

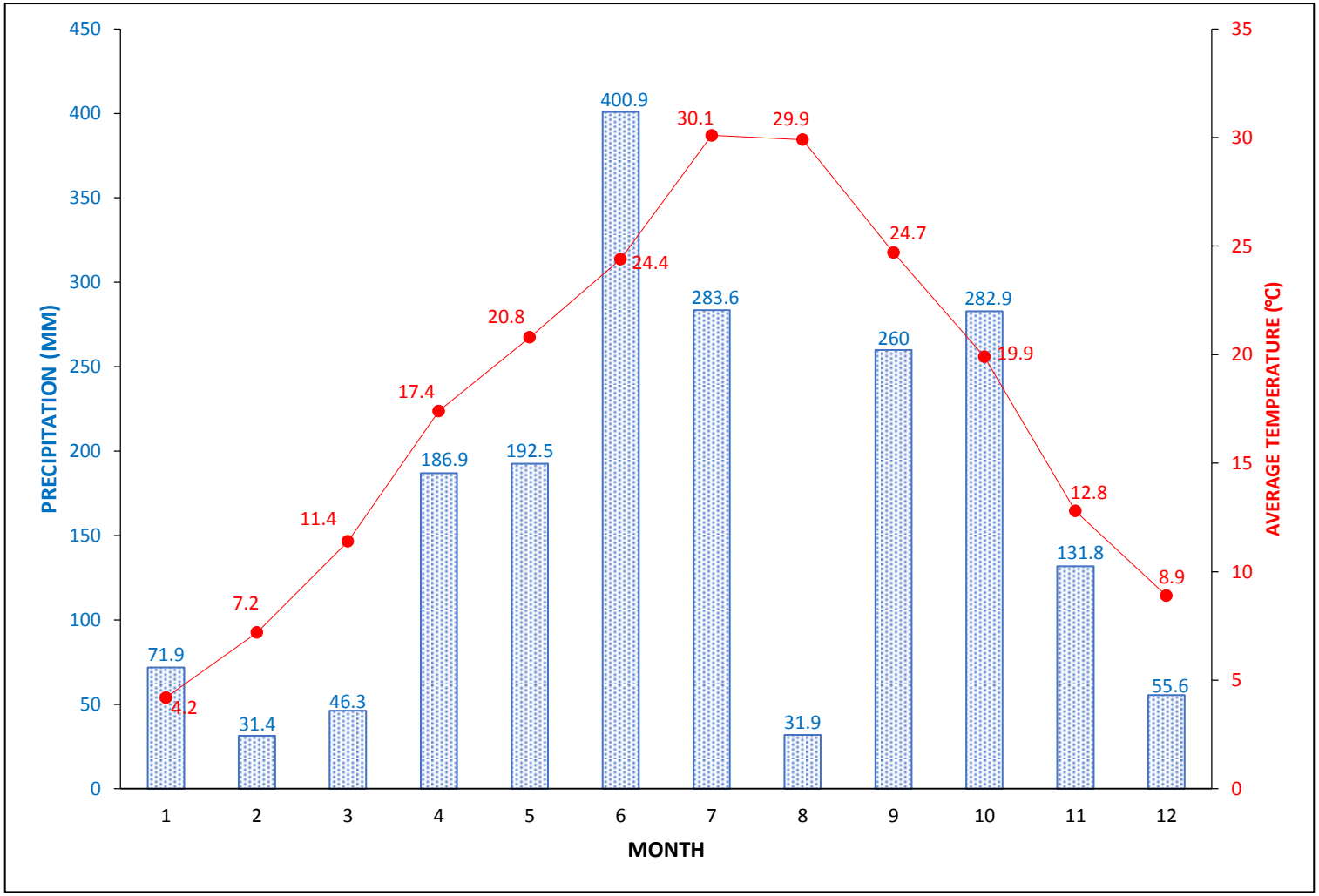



**Figure S2.2.** The average air temperature and precipitation in Suzhou during 2016.

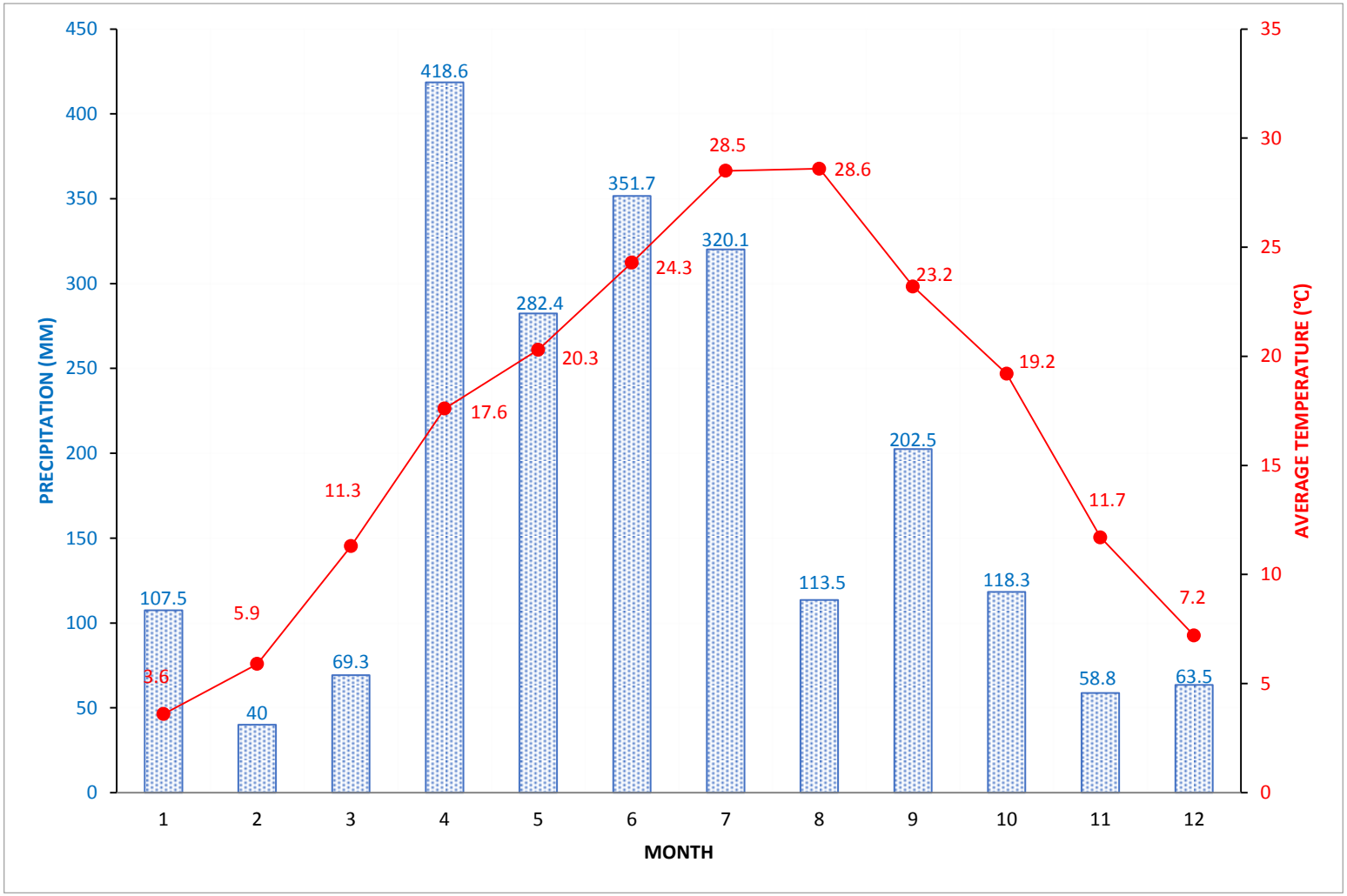
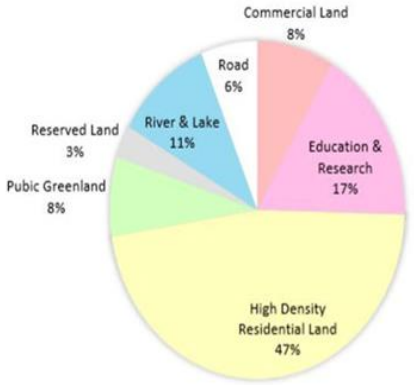
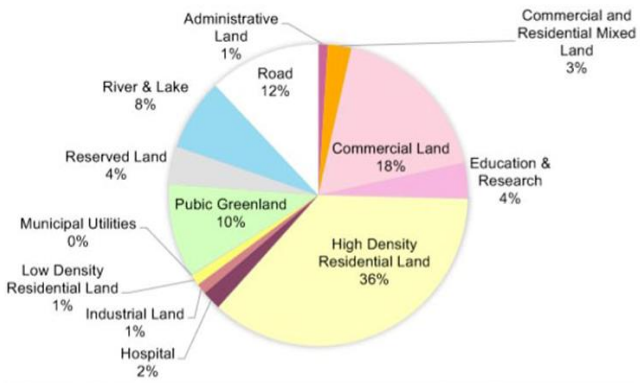



**Figure S2.3.** The average air temperature and precipitation in Huangshan during 2016.

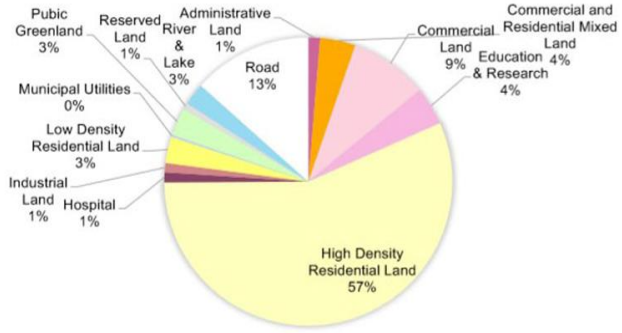

| Land Types | Area (㎡) |
|---|---|
| Commercial Land | 264183 |
| Education & Research | 536691 |
| High Density Residential Land | 1481056 |
| Pubic Greenland | 239262 |
| Reserved Land | 97218 |
| River & Lake | 334659 |
| Road | 194978 |
| **Grand Total** | **3148046** |

**Figure S2.4.** Map and data statistics of land types within 1 km buffer for location 1-1.

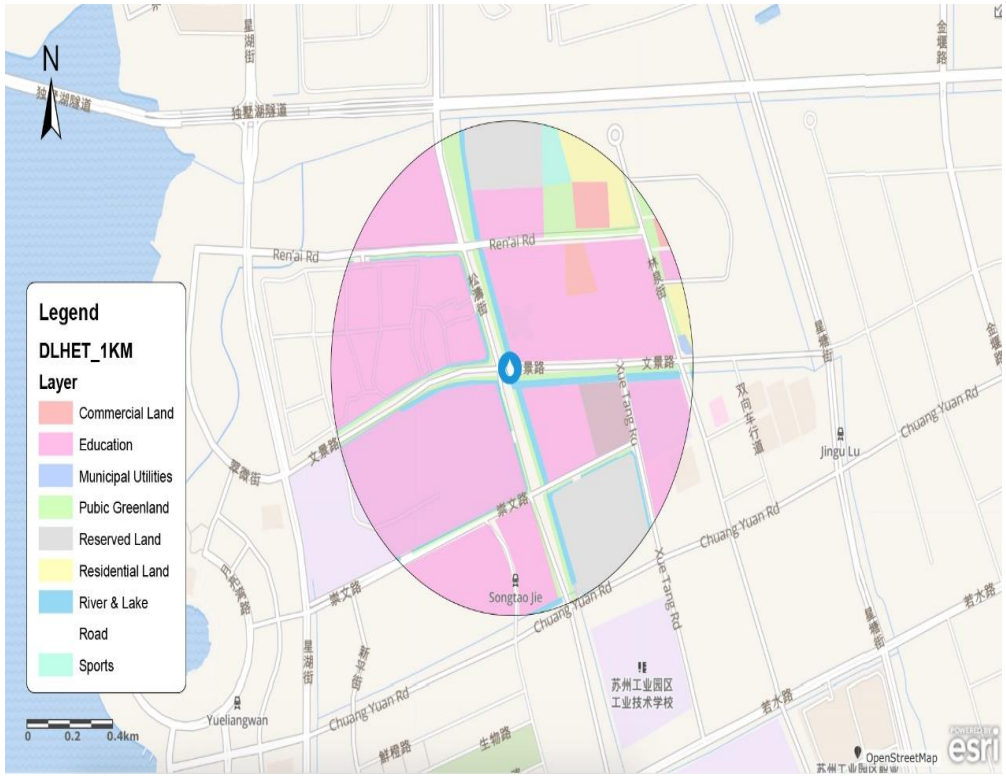

| Land Types | Area (㎡) |
|---|---|
| Administrative Land | 31953 |
| Commercial and Residential Mixed Land | 79836 |
| Commercial Land | 564756 |
| Education & Research | 120914 |
| High Density Residential Land | 1139974 |
| Hospital | 65320 |
| Industrial Land | 35899 |
| Low Density Residential Land | 36539 |
| Municipal Utilities | 2415 |
| Pubic Greenland | 314404 |
| Reserved Land | 127339 |
| River & Lake | 242774 |
| Road | 378798 |
| **Grand Total** | **3140922** |

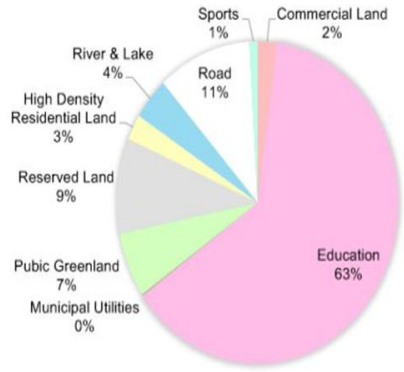
**Figure S2.5.** Map and data statistics of land types within 1 km buffer for location 1-2.


| Land Types | Area (㎡) |
|---|---|
| Administrative Land | 41417 |
| Commercial and Residential Mixed Land | 124876 |
| Commercial Land | 275993 |
| Education & Research | 134428 |
| High Density Residential Land | 1777736 |
| Hospital | 35508 |
| Industrial Land | 32687 |
| Low Density Residential Land | 93054 |
| Municipal Utilities | 7619 |
| Pubic Greenland | 98007 |
| Reserved Land | 22665 |
| River & Lake | 78333 |
| Road | 420922 |
| **Grand Total** | **3143244** |

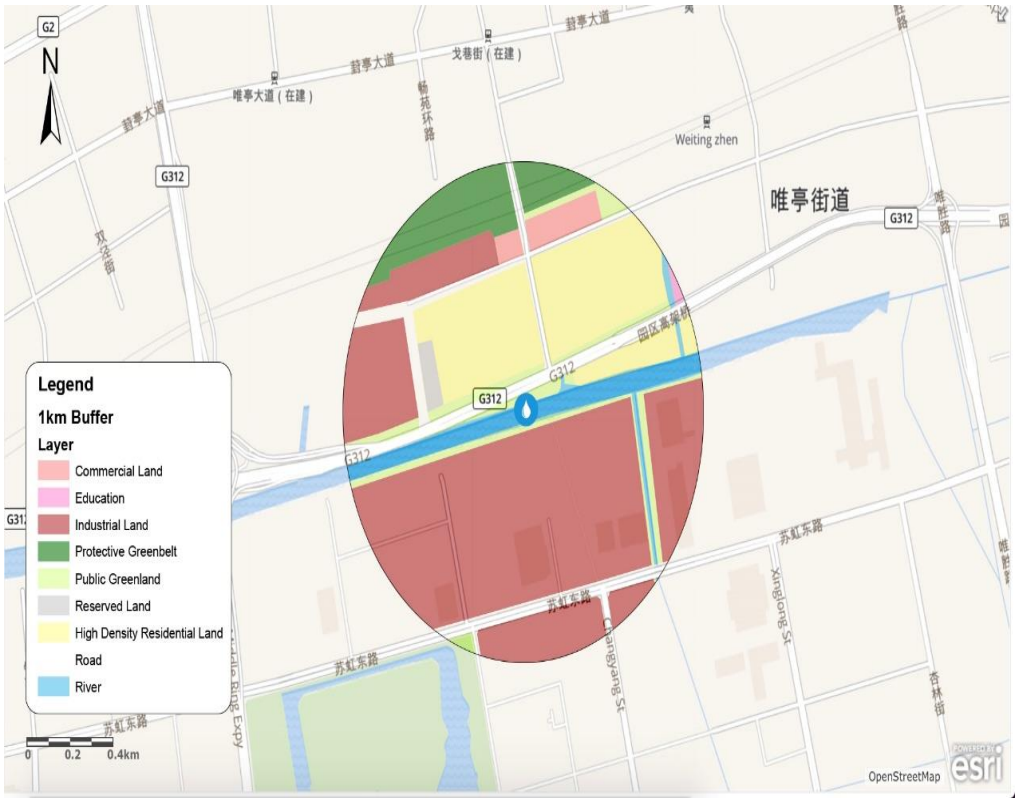
**Figure S2.6.** Map and data statistics of land types within 1 km buffer for location 1-3.

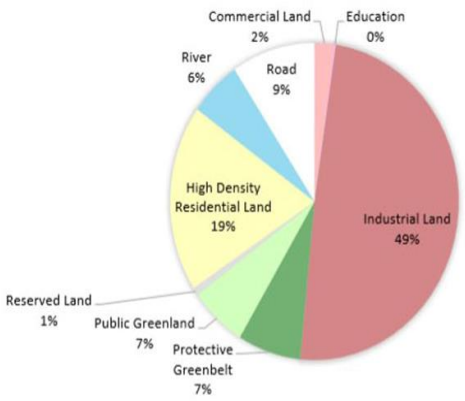



| Land Types | Area (㎡) |
|---|---|
| Commercial Land | 69028 |
| Education & Research | 1977672 |
| Municipal Utilities | 4245 |
| High Density Residential Land | 81199 |
| Reserved Land | 299472 |
| Pubic Greenland | 192055 |
| River & Lake | 133521 |
| Road | 338084 |
| Sports | 29265 |
| **Grand Total** | **3138787** |

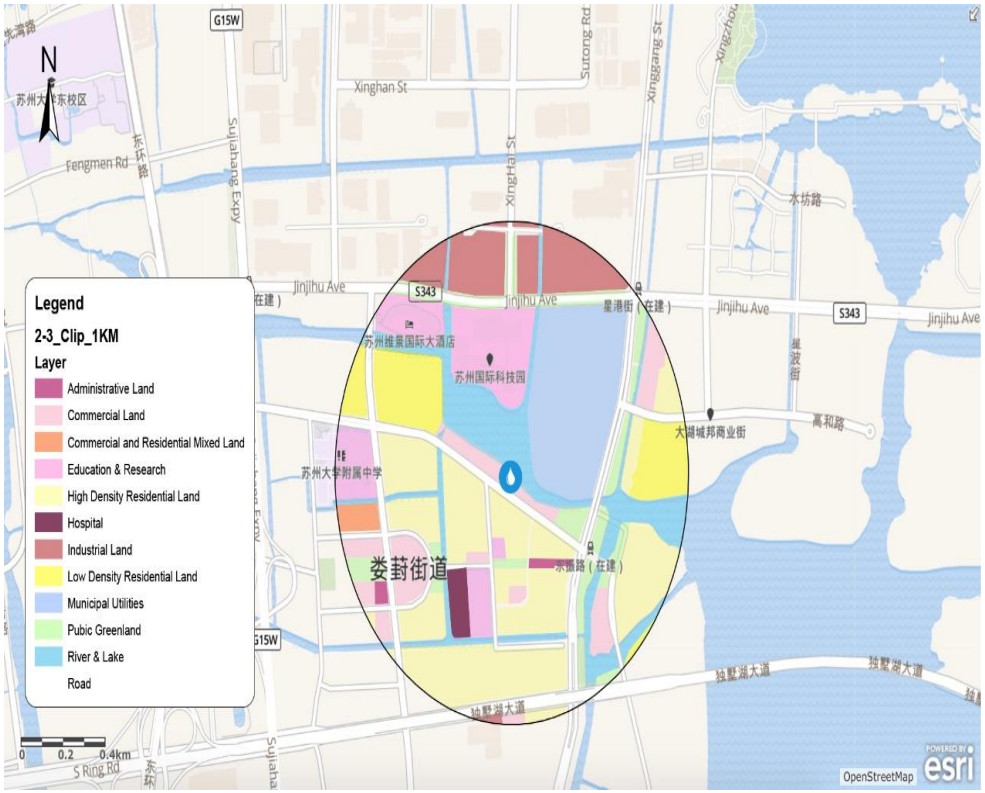
**Figure S2.7.** Map and data statistics of land types within 1 km buffer for location 2-1.

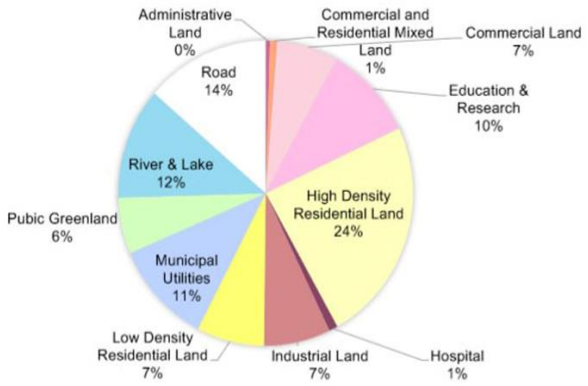



| Land Types | Area (m²) |
|---|---|
| Commercial Land | 68524 |
| Education & Research | 5709 |
| Industrial Land | 1544818 |
| Protective Greenbelt | 222239 |
| Pubic Greenland | 199862 |
| Reserved Land | 23823 |
| High Density Residential Land | 599433 |
| River & Lake | 181045 |
| Road | 293461 |
| **Grand Total** | **3138914** |

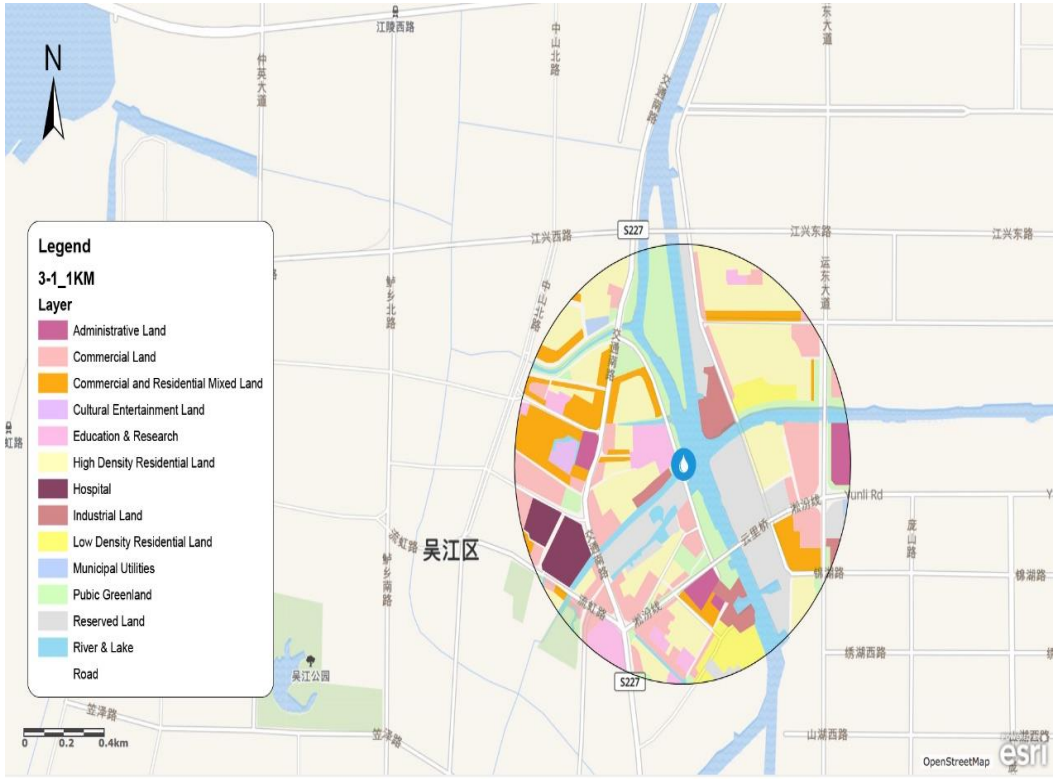
**Figure S2.8.** Map and data statistics of land types within 1 km buffer for location 2-2.

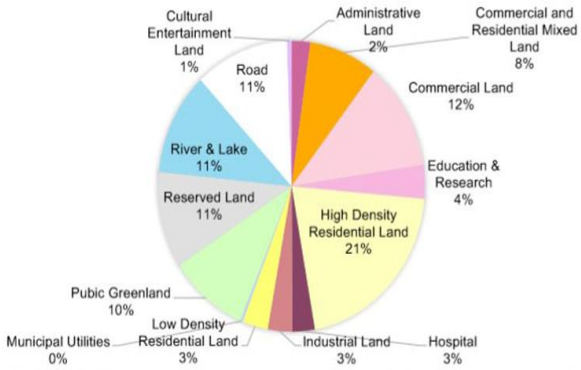



| Land Types | Area (㎡) |
|---|---|
| Administrative Land | 14813 |
| Commercial and Residential Mixed Land | 24992 |
| Commercial Land | 211992 |
| Education & Research | 314008 |
| High Density Residential Land | 750905 |
| Hospital | 29996 |
| Industrial Land | 229664 |
| Low Density Residential Land | 232848 |
| Municipal Utilities | 344742 |
| Pubic Greenland | 187759 |
| River & Lake | 371722 |
| Road | 427847 |
| **Grand Total** | **3141286** |

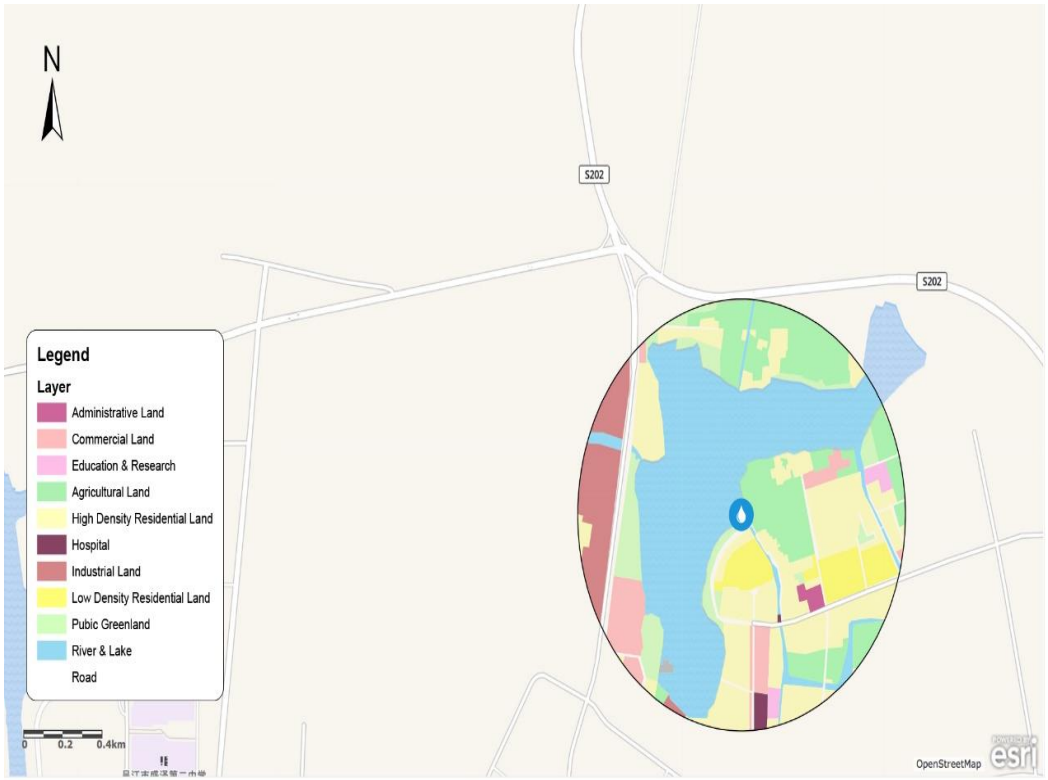
**Figure S2.9.** Map and data statistics of land types within 1 km buffer for location 2-3.

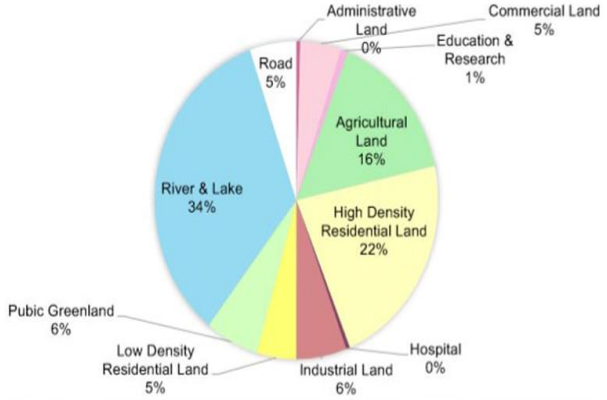



| Land Types | Area (m²) |
|---|---|
| Administrative Land | 41417 |
| Commercial and Residential Mixed Land | 124876 |
| Commercial Land | 275993 |
| Education & Research | 134428 |
| Cultural Entertainment Land | 16930 |
| High Density Residential Land | 1777736 |
| Hospital | 35508 |
| Industrial Land | 32687 |
| Low Density Residential Land | 93054 |
| Municipal Utilities | 7619 |
| Pubic Greenland | 98007 |
| Reserved Land | 22665 |
| River & Lake | 78333 |
| Road | 420922 |
| **Grand Total** | **3143244** |

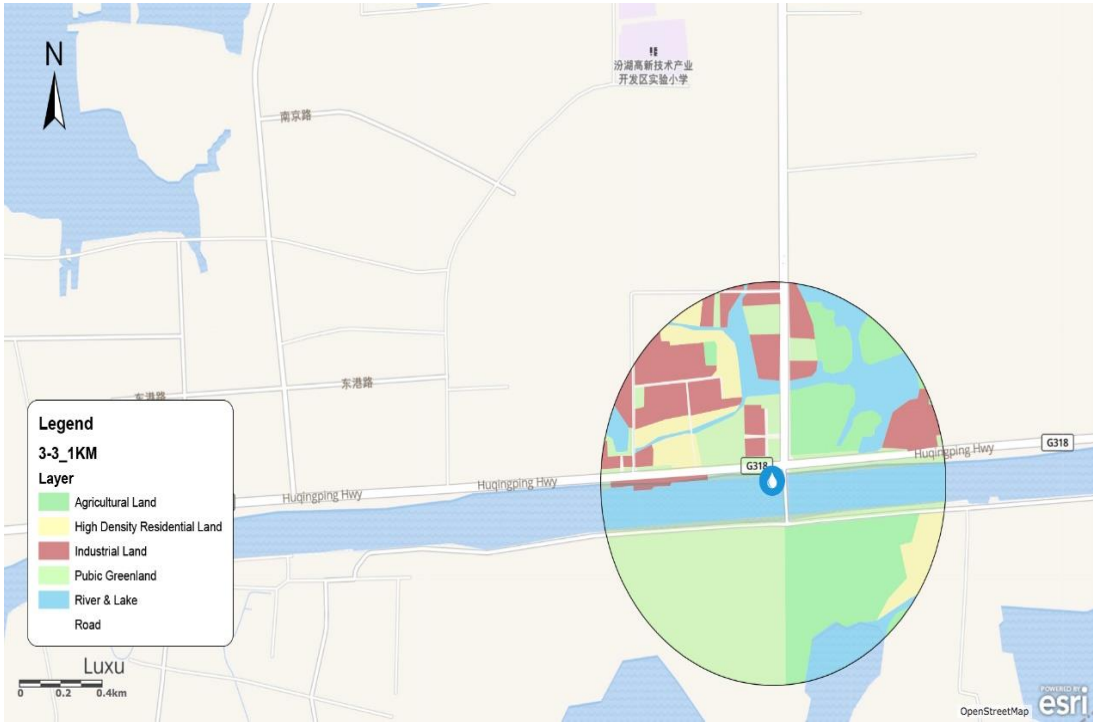
**Figure S2.10.** Map and data statistics of land types within 1 km buffer for location 3-1.

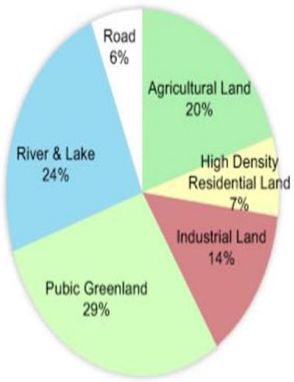



| Land Types | Area (㎡) |
|---|---|
| Administrative Land | 14063 |
| Commercial Land | 146756 |
| Education & Research | 25473 |
| Agricultural Land | 489943 |
| High Density Residential Land | 700748 |
| Hospital | 13872 |
| Industrial Land | 179888 |
| Low Density Residential Land | 141248 |
| Pubic Greenland | 192055 |
| River & Lake | 1070399 |
| Road | 166592 |
| **Grand Total** | **3141039** |

**Figure S2.11.** Map and data statistics of land types within 1 km buffer for location 3-2.




| Land Types | Area (㎡) |
|---|---|
| Agricultural Land | 630616 |
| High Density Residential Land | 220489 |
| Industrial Land | 431477 |
| Pubic Greenland | 910607 |
| River & Lake | 755693 |
| Road | 192550 |
| **Grand Total** | **3141433** |

**Figure S2.12.** Map and data statistics of land types within 1 km buffer for location 3-3.

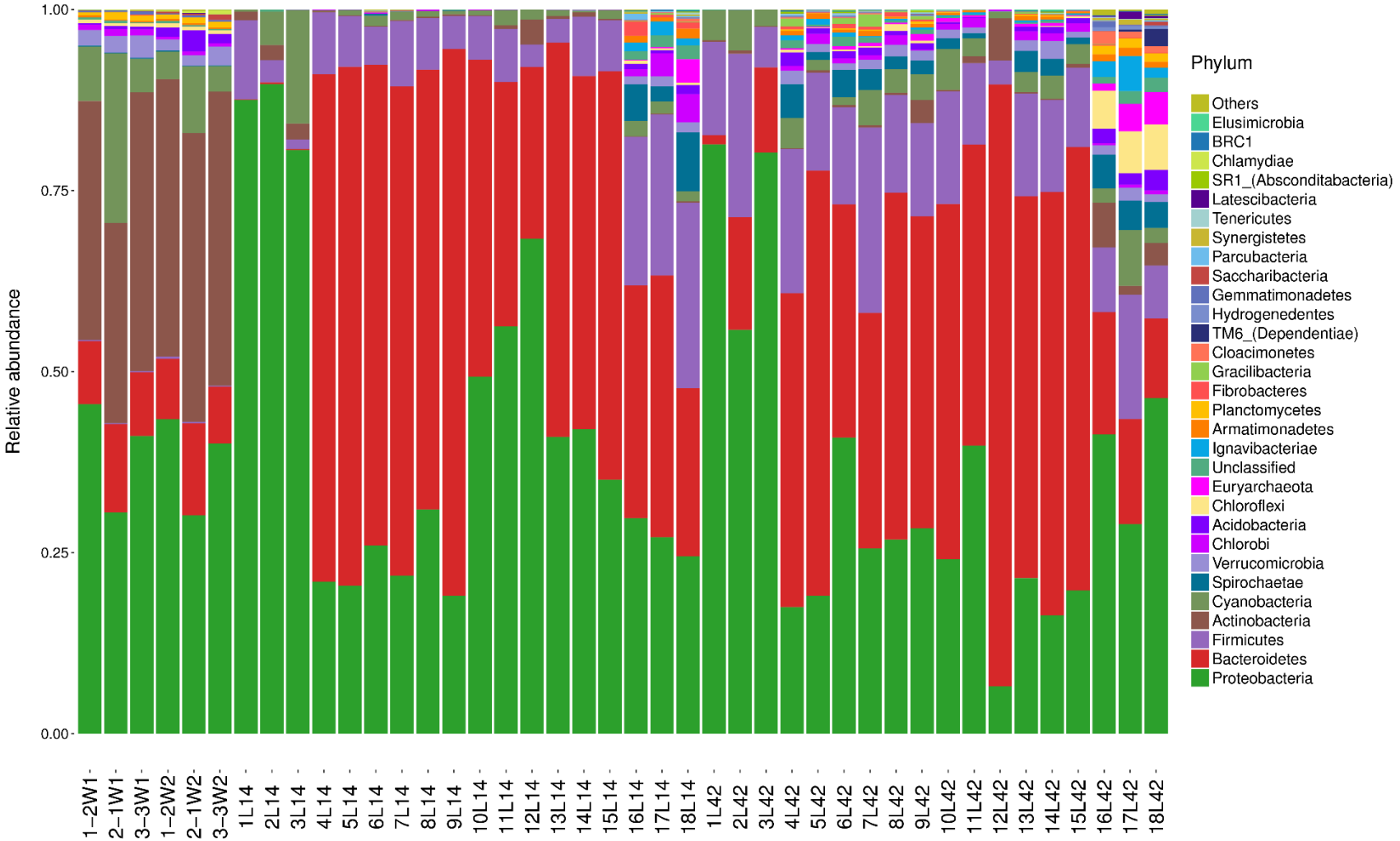



**Figure S4.1.** Bacterial community composition (phylum level) in water and leaf samples collected during microcosm studies.

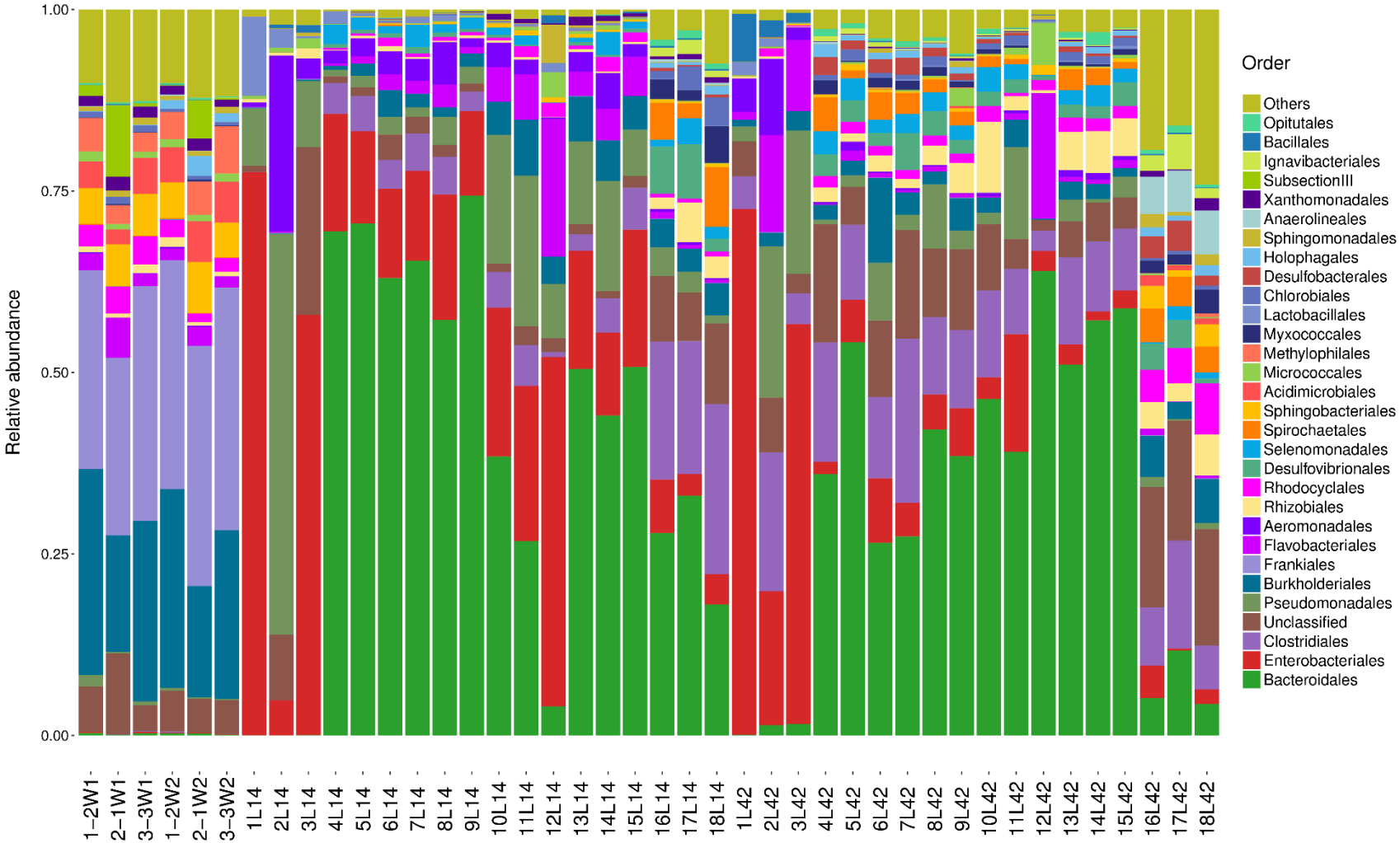



**Figure S4.2.** Bacterial community composition (order level) in water and leaf samples collected during microcosm studies.

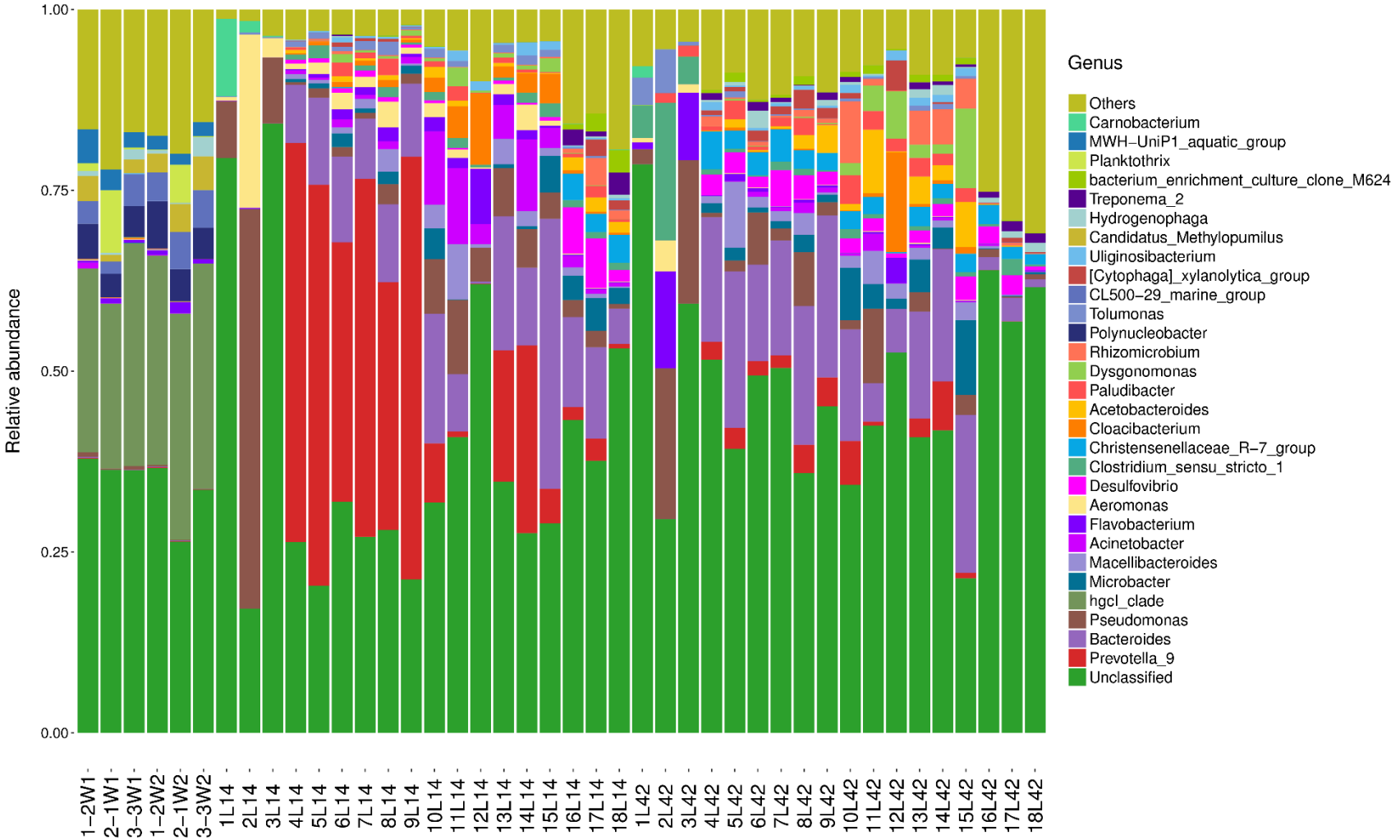



**Figure S4.3.** Bacterial community composition (genus level) in water and leaf samples collected during microcosm studies.

**Table S2.1.** P values of Spearman's correlation coefficient (r) between different water quality parameters.

| | WT | pH | EC | TN | TP | $NO_3$-N | $NO_2$-N | $PO_4$-P | $NH_4$-N | TOC | Chl *a* | TVC | TC | FC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **WT** | | | | | | | | | | | | | | |
| **pH** | 0.1732098 | | | | | | | | | | | | | |
| **EC** | 2.068E-06 | 0.642183 | | | | | | | | | | | | |
| **TN** | 0.0113452 | 0.0462495 | 7.403E-07 | | | | | | | | | | | |
| **TP** | 0.1310214 | 0.0361173 | 0.0006644 | 4.574E-14 | | | | | | | | | | |
| **$NO_3$-N** | 6.128E-12 | 0.7135741 | 3.273E-07 | 9.588E-08 | 0.8188419 | | | | | | | | | |
| **$NO_2$-N** | 1.738E-06 | 0.0049127 | 0.9611735 | 1.881E-05 | 6.618E-07 | 0.7042439 | | | | | | | | |
| **$PO_4$-P** | 0.2405977 | 0.0131624 | 0.0022933 | 3.791E-11 | 8.442E-28 | 0.6068479 | 9.038E-07 | | | | | | | |
| **$NH_4$-N** | 0.1316037 | 0.0061895 | 6.438E-06 | 3.173E-20 | 1.394E-16 | 0.0123609 | 1.528E-05 | 1.567E-18 | | | | | | |
| **TOC** | 0.3902751 | 0.0754037 | 0.0027599 | 0.0657068 | 0.0012183 | 0.9950904 | 0.1049634 | 0.0964926 | 0.0524696 | | | | | |
| **Chl *a*** | 4.393E-05 | 0.7953584 | 0.1505071 | 0.0624939 | 0.0002279 | 0.1192654 | 3.66E-08 | 0.0012383 | 0.10495 | 0.2550725 | | | | |
| **TVC** | 0.1511464 | 0.001209 | 0.4057009 | 0.7816433 | 0.0704201 | 0.0797223 | 0.0549225 | 0.0198908 | 0.7129466 | 0.222877 | 0.0064433 | | | |
| **TC** | 0.5177751 | 0.0006437 | 0.0047 | 0.1070436 | 0.0012717 | 0.3659224 | 0.1561144 | 4.628E-06 | 0.0102943 | 0.5291477 | 0.2628125 | 0.0025582 | | |
| **FC** | 0.5264753 | 0.0319433 | 0.0715311 | 0.1038779 | 4.196E-05 | 0.8075224 | 0.0130454 | 1.722E-05 | 0.0980369 | 0.0396602 | 0.0178584 | 3.152E-06 | 6.522E-07 | |

**Table S3.1.** Similarity-based OTUs and species richness and diversity estimates (97% cut-off) for bacterial community in water and leaf samples collected from different urban gradients (High, Medium and Low) in winter and summer 2015.

| Group | Sample ID | Reads | OTUs | ace | Chao1 | Shannon | Simpson | Coverage |
|---|---|---|---|---|---|---|---|---|
| Winter, Water, High urbanization | WI1_1W0 | 16925 | 943 | 2230 | 1760 | 4.55 | 0.0388 | 0.973 |
| | WI1_1W42 | 16925 | 546 | 1280 | 960 | 3.89 | 0.0507 | 0.985 |
| | WI1_2W0 | 16925 | 286 | 533 | 450 | 3.23 | 0.0971 | 0.994 |
| | WI1_2W42 | 16925 | 386 | 902 | 689 | 3.21 | 0.0997 | 0.990 |
| | WI1_3W0 | 16925 | 888 | 2020 | 1528 | 4.68 | 0.0234 | 0.978 |
| | WI1_3W42 | 16925 | 637 | 1605 | 1332 | 4.14 | 0.0466 | 0.982 |
| **Mean ± SD** | | | **614± 264** | **1428± 652** | **1120± 506** | **3.95± 0.63** | **0.059± 0.032** | **0.983± 0.008** |
| Winter, Water, Medium urbanization | WI2_1W0 | 16925 | 696 | 1503 | 1178 | 4.71 | 0.0197 | 0.983 |
| | WI2_1W42 | 16925 | 478 | 1044 | 771 | 4.39 | 0.03 | 0.989 |
| | WI2_2W0 | 16925 | 705 | 1509 | 1157 | 4.19 | 0.0557 | 0.982 |
| | WI2_2W42 | 16925 | 440 | 916 | 707 | 4.09 | 0.0386 | 0.990 |
| | WI2_3W0 | 16925 | 440 | 903 | 705 | 4.37 | 0.0238 | 0.990 |
| | WI2_3W42 | 16925 | 567 | 1171 | 889 | 4.32 | 0.0305 | 0.987 |
| **Mean ± SD** | | | **554± 122** | **1174± 275** | **901± 217** | **4.35± 0.21** | **0.033± 0.013** | **0.987± 0.004** |
| Winter, Water, Low urbanization | WI3_1W0 | 16925 | 862 | 2364 | 1680 | 4.77 | 0.0233 | 0.975 |
| | WI3_1W42 | 16925 | 872 | 2134 | 1621 | 4.53 | 0.0286 | 0.975 |
| | WI3_2W0 | 16925 | 517 | 1101 | 836 | 4.38 | 0.0314 | 0.988 |
| | WI3_2W42 | 16925 | 557 | 1207 | 923 | 4.46 | 0.0227 | 0.987 |
| | WI3_3W0 | 16925 | 821 | 1790 | 1410 | 4.54 | 0.0385 | 0.978 |
| | WI3_3W42 | 16925 | 884 | 2404 | 1715 | 4.77 | 0.0202 | 0.974 |
| **Mean ± SD** | | | **752± 168** | **1833± 571** | **1364± 391** | **4.58± 0.16** | **0.027± 0.007** | **0.980± 0.006** |
| Summer, Water, High urbanization | SU1_1W0 | 16925 | 949 | 1973 | 1636 | 4.96 | 0.0173 | 0.976 |
| | SU1_1W42 | 16925 | 780 | 1413 | 1189 | 4.6 | 0.0261 | 0.982 |
| | SU1_2W0 | 16925 | 1004 | 1928 | 1621 | 5.02 | 0.0192 | 0.975 |
| | SU1_2W42 | 16925 | 840 | 1622 | 1343 | 4.48 | 0.0349 | 0.979 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | SU1_3W0 | 16925 | 719 | 1563 | 1303 | 4.7 | 0.0205 | 0.982 |
| | SU1_3W42 | 16925 | 718 | 1387 | 1094 | 4.37 | 0.0417 | 0.983 |
| **Mean ± SD** | | | **835± 120** | **1648± 251** | **1364± 223** | **4.69± 0.26** | **0.027± 0.010** | **0.980± 0.003** |
| Summer, Water, Medium urbanization | SU2_1W0 | 16925 | 692 | 1370 | 1209 | 4.69 | 0.0206 | 0.984 |
| | SU2_1W42 | 16925 | 888 | 1622 | 1321 | 4.92 | 0.0188 | 0.980 |
| | SU2_2W0 | 16925 | 729 | 1553 | 1163 | 4.27 | 0.0556 | 0.982 |
| | SU2_2W42 | 16925 | 803 | 1639 | 1319 | 4.6 | 0.0326 | 0.980 |
| | SU2_3W0 | 16925 | 830 | 1520 | 1233 | 4.74 | 0.0239 | 0.981 |
| | SU2_3W42 | 16925 | 932 | 1868 | 1452 | 4.62 | 0.0422 | 0.976 |
| **Mean ± SD** | | | **812± 92** | **1595± 164** | **1283± 104** | **4.64± 0.21** | **0.032± 0.014** | **0.980± 0.002** |
| Summer, Water, Low urbanization | SU3_1W0 | 16925 | 890 | 1898 | 1573 | 4.85 | 0.0193 | 0.977 |
| | SU3_1W42 | 16925 | 844 | 1836 | 1401 | 4.42 | 0.0313 | 0.978 |
| | SU3_2W0 | 16925 | 795 | 1400 | 1163 | 4.63 | 0.0267 | 0.982 |
| | SU3_2W42 | 16925 | 882 | 1765 | 1577 | 4.21 | 0.0683 | 0.977 |
| | SU3_3W0 | 16925 | 793 | 1553 | 1305 | 4.68 | 0.0269 | 0.981 |
| | SU3_3W42 | 16925 | 841 | 2030 | 1478 | 4.14 | 0.0682 | 0.976 |
| **Mean ± SD** | | | **841± 41** | **1747± 232** | **1416± 162** | **4.49± 0.28** | **0.040± 0.022** | **0.978± 0.002** |
| Winter, Leaf, High urbanization | WI1_1L14 | 16925 | 515 | 1232 | 947 | 3.73 | 0.0552 | 0.986 |
| | WI1_1L42 | 16925 | 414 | 609 | 638 | 3.61 | 0.0738 | 0.991 |
| | WI1_2L14 | 16925 | 209 | 390 | 320 | 3.11 | 0.095 | 0.996 |
| | WI1_2L42 | 16925 | 344 | 624 | 551 | 3.87 | 0.0494 | 0.993 |
| | WI1_3L14 | 16925 | 252 | 535 | 396 | 3.34 | 0.0707 | 0.994 |
| | WI1_3L42 | 16925 | 447 | 936 | 722 | 4.16 | 0.0301 | 0.989 |
| **Mean ± SD** | | | **364± 118** | **721± 308** | **596± 228** | **3.64± 0.38** | **0.062± 0.022** | **0.991± 0.004** |
| Winter, Leaf, Medium urbanization | WI2_1L14 | 16925 | 238 | 441 | 408 | 3.11 | 0.1019 | 0.995 |
| | WI2_1L42 | 16925 | 421 | 908 | 715 | 3.98 | 0.0396 | 0.990 |
| | WI2_2L14 | 16925 | 224 | 414 | 317 | 3.21 | 0.0735 | 0.995 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | WI2_2L42 | 16925 | 423 | 759 | 621 | 3.93 | 0.0491 | 0.991 |
| | WI2_3L14 | 16925 | 228 | 473 | 431 | 3.1 | 0.0803 | 0.994 |
| | WI2_3L42 | 16925 | 297 | 536 | 465 | 3.4 | 0.0831 | 0.994 |
| **Mean ± SD** | | | **305± 94** | **589± 200** | **493± 147** | **3.46± 0.40** | **0.071± 0.023** | **0.993± 0.002** |
| Winter, Leaf, Low urbanization | WI3_1L14 | 16925 | 121 | 236 | 174 | 2.38 | 0.1753 | 0.997 |
| | WI3_1L42 | 16925 | 270 | 568 | 438 | 2.87 | 0.1424 | 0.994 |
| | WI3_2L14 | 16925 | 276 | 510 | 461 | 3.34 | 0.075 | 0.994 |
| | WI3_2L42 | 16925 | 405 | 591 | 580 | 3.81 | 0.0505 | 0.991 |
| | WI3_3L14 | 16925 | 237 | 497 | 403 | 2.85 | 0.1385 | 0.994 |
| | WI3_3L42 | 16925 | 552 | 1031 | 908 | 4.31 | 0.0294 | 0.987 |
| **Mean ± SD** | | | **310± 149** | **572± 258** | **494± 242** | **3.26± 0.71** | **0.102± 0.058** | **0.993± 0.003** |
| Summer, Leaf, High urbanization | SU1_1L14 | 16925 | 1021 | 1871 | 1563 | 4.94 | 0.0349 | 0.976 |
| | SU1_1L42 | 16925 | 1922 | 2488 | 2443 | 6.14 | 0.0103 | 0.964 |
| | SU1_2L14 | 16925 | 1110 | 1947 | 1618 | 5.02 | 0.0225 | 0.974 |
| | SU1_2L42 | 16925 | 2068 | 2293 | 2328 | 6.7 | 0.0037 | 0.976 |
| | SU1_3L14 | 16925 | 896 | 1694 | 1317 | 4.9 | 0.0198 | 0.980 |
| | SU1_3L42 | 16925 | 1884 | 2153 | 2121 | 6.46 | 0.0053 | 0.976 |
| **Mean ± SD** | | | **1484± 528** | **2074± 292** | **1898± 460** | **5.69± 0.83** | **0.016± 0.012** | **0.974± 0.005** |
| Summer, Leaf, Medium urbanization | SU2_1L14 | 16925 | 601 | 1218 | 1028 | 3.21 | 0.184 | 0.984 |
| | SU2_1L42 | 16925 | 1720 | 2390 | 2331 | 5.93 | 0.011 | 0.964 |
| | SU2_2L14 | 16925 | 1008 | 2145 | 1719 | 4.7 | 0.0291 | 0.973 |
| | SU2_2L42 | 16925 | 2161 | 2796 | 2779 | 6.51 | 0.0046 | 0.959 |
| | SU2_3L14 | 16925 | 1053 | 2181 | 1698 | 4.92 | 0.0197 | 0.973 |
| | SU2_3L42 | 16925 | 1845 | 2234 | 2204 | 6.08 | 0.0181 | 0.971 |
| **Mean ± SD** | | | **1398± 599** | **2161± 520** | **1960± 610** | **5.23± 1.21** | **0.044± 0.069** | **0.971± 0.009** |
| Summer, Leaf, Low | SU3_1L14 | 16925 | 1304 | 2507 | 2021 | 5.08 | 0.0269 | 0.967 |
| | SU3_2L14 | 16925 | 1356 | 2050 | 1915 | 5.3 | 0.0166 | 0.968 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| urbanization | SU3_2L42 | 16925 | 1982 | 2369 | 2352 | 6.41 | 0.0059 | 0.970 |
| | SU3_3L42 | 16925 | 1810 | 2273 | 2263 | 6.27 | 0.0061 | 0.969 |
| **Mean ± SD** | | | **1613± 335** | **2300± 192** | **2138± 204** | **5.77± 0.67** | **0.014± 0.010** | **0.968± 0.001** |

**Table S3.2.** Similarity-based OTUs and species richness and diversity estimates (97% cut-off) for bacterial community in water and leaf samples collected from different urban gradients (High, Medium and Low) in winter and summer 2016. The data for samples collected from the control locations in Huangshan during summer 2016 are also included.

| Group | Sample ID | Reads | OTU | ace | Chao1 | Shannon | Simpson | Coverage |
|---|---|---|---|---|---|---|---|---|
| Winter, Water, High urbanization | WI1_1W | 27966 | 553 | 944 | 897 | 4.32 | 0.0308 | 0.993 |
| | WI1_2W | 27966 | 654 | 1001 | 901 | 4.65 | 0.0203 | 0.993 |
| **Mean ± SD** | | | **604± 71** | **973± 40** | **899± 3** | **4.49± 0.23** | **0.026± 0.007** | **0.993± 0.000** |
| Winter, Water, Medium urbanization | WI2_1W | 27966 | 415 | 681 | 604 | 3.89 | 0.0587 | 0.995 |
| | WI2_2W | 27966 | 491 | 915 | 754 | 3.63 | 0.0712 | 0.994 |
| **Mean ± SD** | | | **453± 54** | **798± 165** | **679± 106** | **3.76± 0.18** | **0.065± 0.009** | **0.995± 0.001** |
| Winter, Water, Low urbanization | WI3_2W | 27966 | 579 | 1180 | 952 | 4.35 | 0.028 | 0.992 |
| | WI3_3W | 27966 | 625 | 1214 | 1060 | 4.05 | 0.0582 | 0.991 |
| **Mean ± SD** | | | **602± 33** | **1197± 24** | **1006± 76** | **4.20± 0.21** | **0.043± 0.021** | **0.992± 0.001** |
| Summer, Water, High urbanization | SU1_1W | 27966 | 851 | 1413 | 1236 | 4.72 | 0.0245 | 0.989 |
| | SU1_2W | 27966 | 778 | 1039 | 1045 | 4.53 | 0.0453 | 0.992 |
| **Mean ± SD** | | | **815± 52** | **1226± 264** | **1141± 135** | **4.63± 0.13** | **0.035± 0.015** | **0.990± 0.002** |
| Summer, Water, Medium urbanization | SU2_1W | 27966 | 794 | 1074 | 1074 | 4.58 | 0.0307 | 0.991 |
| | SU2_2W | 27966 | 606 | 774 | 802 | 4.18 | 0.047 | 0.994 |
| **Mean ± SD** | | | **700± 133** | **924± 212** | **938± 192** | **4.38± 0.28** | **0.039± 0.012** | **0.993± 0.002** |
| Summer, Water, Low urbanization | SU3_2W | 27966 | 809 | 1117 | 1139 | 4.58 | 0.0311 | 0.991 |
| | SU3_3W | 27966 | 728 | 926 | 964 | 4.67 | 0.0246 | 0.993 |
| **Mean ± SD** | | | **769± 57** | **1022± 135** | **1052± 124** | **4.63± 0.06** | **0.028± 0.005** | **0.992± 0.002** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Summer, Water, Huangshan | SUH_1W | 27966 | 412 | 964 | 727 | 2.95 | 0.1322 | 0.993 |
| | SUH_2W | 27966 | 471 | 1518 | 999 | 3.11 | 0.1132 | 0.991 |
| | SUH_3W | 27966 | 1109 | 2211 | 1820 | 3.44 | 0.1211 | 0.982 |
| **Mean ± SD** | | | **664± 387** | **1564± 625** | **1182± 569** | **3.17± 0.25** | **0.122± 0.010** | **0.989± 0.006** |
| Winter, Leaf, High urbanization | WI1_1L14 | 27966 | 448 | 623 | 653 | 3.94 | 0.0427 | 0.995 |
| | WI1_1L42 | 27966 | 884 | 1421 | 1185 | 4.79 | 0.021 | 0.989 |
| | WI1_2L14 | 27966 | 397 | 664 | 597 | 3.84 | 0.0457 | 0.995 |
| | WI1_2L42 | 27966 | 896 | 1256 | 1269 | 4.89 | 0.0172 | 0.989 |
| **Mean ± SD** | | | **656± 271** | **991± 407** | **926± 350** | **4.37± 0.55** | **0.032± 0.015** | **0.992± 0.003** |
| Winter, Leaf, Medium urbanization | WI2_1L14 | 27966 | 438 | 804 | 657 | 3.84 | 0.0428 | 0.994 |
| | WI2_1L42 | 27966 | 767 | 1461 | 1142 | 4.28 | 0.0431 | 0.989 |
| | WI2_2L14 | 27966 | 447 | 965 | 806 | 3.62 | 0.0602 | 0.993 |
| | WI2_2L42 | 27966 | 445 | 780 | 669 | 3.64 | 0.0586 | 0.994 |
| **Mean ± SD** | | | **524± 162** | **1003± 317** | **819± 226** | **3.85± 0.31** | **0.051± 0.010** | **0.993± 0.002** |
| Winter, Leaf, Low urbanization | WI3_2L14 | 27966 | 387 | 721 | 623 | 3.36 | 0.0711 | 0.995 |
| | WI3_2L42 | 27966 | 707 | 991 | 974 | 4.28 | 0.0373 | 0.992 |
| | WI3_3L14 | 27966 | 234 | 316 | 316 | 2.87 | 0.1046 | 0.997 |
| | WI3_3L42 | 27966 | 640 | 895 | 897 | 4.39 | 0.0268 | 0.992 |
| **Mean ± SD** | | | **492± 220** | **731± 298** | **703± 298** | **3.73± 0.73** | **0.060± 0.035** | **0.994± 0.003** |
| Summer, Leaf, High urbanization | SU1_1L14 | 27966 | 1517 | 1977 | 2036 | 5.63 | 0.0136 | 0.984 |
| | SU1_1L42 | 27966 | 1723 | 2217 | 2253 | 5.91 | 0.0086 | 0.982 |
| | SU1_2L14 | 27966 | 1626 | 2164 | 2166 | 5.61 | 0.0141 | 0.982 |
| | SU1_2L42 | 27966 | 1835 | 2249 | 2257 | 5.97 | 0.0089 | 0.983 |
| **Mean ± SD** | | | **1675 ±136** | **2152± 122** | **2178± 104** | **5.78± 0.19** | **0.011± 0.003** | **0.982± 0.001** |
| Summer, Leaf, Medium | SU2_1L14 | 27966 | 1714 | 2193 | 2221 | 5.95 | 0.0072 | 0.982 |
| | SU2_1L42 | 27966 | 1790 | 2241 | 2295 | 6.11 | 0.0061 | 0.982 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| urbanization | SU2_2L14 | 27966 | 1725 | 2260 | 2273 | 5.8 | 0.0092 | 0.981 |
| | SU2_2L42 | 27966 | 1564 | 2005 | 2087 | 5.59 | 0.0258 | 0.984 |
| **Mean ± SD** | | | **1698 ±96** | **2175± 117** | **2219± 93** | **5.86± 0.22** | **0.012± 0.009** | **0.982± 0.001** |
| Summer, Leaf, Low urbanization | SU3_2L14 | 27966 | 1748 | 2327 | 2257 | 5.71 | 0.0125 | 0.980 |
| | SU3_2L42 | 27966 | 2031 | 2629 | 2752 | 5.88 | 0.0181 | 0.978 |
| | SU3_3L14 | 27966 | 1811 | 2320 | 2362 | 5.79 | 0.0105 | 0.980 |
| **Mean ± SD** | | | **1863 ±149** | **2425± 176** | **2457± 261** | **5.79± 0.09** | **0.014± 0.004** | **0.979± 0.001** |
| Summer, Leaf, Huangshan | SUH_1L14 | 27966 | 1369 | 1863 | 1927 | 5.11 | 0.0407 | 0.984 |
| | SUH_2L14 | 27966 | 1430 | 1853 | 1830 | 5.51 | 0.0128 | 0.985 |
| | SUH_2L42 | 27966 | 1774 | 2115 | 2157 | 6.16 | 0.0066 | 0.985 |
| | SUH_3L14 | 27966 | 1643 | 1896 | 1919 | 5.93 | 0.0106 | 0.987 |
| | SUH_3L42 | 27966 | 1838 | 2121 | 2119 | 6.4 | 0.0043 | 0.986 |
| **Mean ± SD** | | | **1611 ±206** | **1970± 136** | **1990± 141** | **5.82± 0.52** | **0.015± 0.015** | **0.986± 0.001** |

**Table S3.3.** Similarity-based OTUs and species richness and diversity estimates (97% cut-off) for fungal community in water and leaf samples collected from different urban gradients (High, Medium and Low) in winter and summer 2015.

| Group | Sample ID | Reads | OTU | ace | Chao1 | Shannon | Simpson | Coverage |
|---|---|---|---|---|---|---|---|---|
| Winter, Water, High | WI1_1W | 29600 | 381 | 548 | 522 | 3.37 | 0.0779 | 0.995372 |
| | WI1_2W | 29600 | 496 | 595 | 598 | 3.95 | 0.0421 | 0.996014 |
| **Mean ± SD** | | | **439± 81** | **572± 33** | **560± 54** | **3.66± 0.41** | **0.060± 0.025** | **0.996± 0.000** |
| Winter, Water, Medium | WI2_1W | 29600 | 242 | 345 | 318 | 1.26 | 0.5596 | 0.996926 |
| | WI2_2W | 29600 | 169 | 227 | 219 | 1.25 | 0.4785 | 0.998142 |
| **Mean ± SD** | | | **206± 52** | **286± 83** | **269± 70** | **1.26± 0.01** | **0.519± 0.057** | **0.998± 0.001** |
| Winter, Water, Low | WI3_2W | 29600 | 336 | 454 | 466 | 2.15 | 0.349 | 0.99625 |
| | WI3_3W | 29600 | 741 | 872 | 862 | 3.72 | 0.1108 | 0.994257 |
| **Mean ± SD** | | | **539± 286** | **663± 296** | **664± 280** | **2.94± 1.11** | **0.230± 0.168** | **0.995± 0.001** |
| Summer, Water, High | SU1_1W | 29600 | 1014 | 1089 | 1083 | 5.11 | 0.0179 | 0.995135 |
| | SU1_2W | 29600 | 1111 | 1166 | 1173 | 5.47 | 0.011 | 0.995845 |
| **Mean ± SD** | | | **1063 ±69** | **1128± 54** | **1128± 64** | **5.29± 0.25** | **0.014± 0.005** | **0.995± 0.001** |
| Summer, Water, Medium | SU2_1W | 29600 | 1019 | 1085 | 1081 | 4.82 | 0.0351 | 0.995439 |
| | SU2_2W | 29600 | 818 | 912 | 901 | 4.37 | 0.0639 | 0.995135 |
| **Mean ± SD** | | | **919± 142** | **999± 122** | **991± 127** | **4.60± 0.32** | **0.050± 0.020** | **0.995± 0.000** |
| Summer, Water, Low | SU3_2W | 29600 | 646 | 815 | 805 | 3.35 | 0.1398 | 0.99375 |
| | SU3_3W | 29600 | 950 | 1019 | 1004 | 4.65 | 0.0452 | 0.995608 |
| **Mean ± SD** | | | **798± 215** | **917± 144** | **905± 141** | **4.00± 0.92** | **0.093± 0.067** | **0.995± 0.001** |
| Winter, Leaf, High | WI1_1L14 | 29600 | 571 | 749 | 711 | 3.65 | 0.0696 | 0.994324 |
| | WI1_1L42 | 29600 | 414 | 541 | 520 | 3.43 | 0.0674 | 0.995811 |
| | WI1_2L14 | 29600 | 600 | 778 | 773 | 3.75 | 0.0914 | 0.994122 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | WI1_2L42 | 29600 | 344 | 484 | 461 | 2.8 | 0.1609 | 0.996115 |
| **Mean ± SD** | | | **482± 123** | **638± 147** | **616± 149** | **3.41± 0.43** | **0.097± 0.044** | **0.995± 0.001** |
| Winter, Leaf, Medium | WI2_1L14 | 29600 | 76 | 126 | 130 | 0.84 | 0.483 | 0.998818 |
| | WI2_1L42 | 29600 | 71 | 96 | 87 | 1.26 | 0.379 | 0.999291 |
| | WI2_2L14 | 29600 | 47 | 77 | 70 | 0.75 | 0.4944 | 0.999223 |
| | WI2_2L42 | 29600 | 81 | 202 | 149 | 1.23 | 0.3975 | 0.998615 |
| **Mean ± SD** | | | **69± 15** | **125± 55** | **109± 37** | **1.02± 0.26** | **0.438± 0.059** | **0.999± 0.000** |
| Winter, Leaf, Low | WI3_2L14 | 29600 | 89 | 229 | 143 | 1.38 | 0.3069 | 0.998412 |
| | WI3_2L42 | 29600 | 138 | 261 | 221 | 1.75 | 0.2678 | 0.998041 |
| | WI3_3L14 | 29600 | 75 | 104 | 117 | 1.23 | 0.5091 | 0.999223 |
| | WI3_3L42 | 29600 | 93 | 296 | 150 | 1.42 | 0.4117 | 0.998581 |
| **Mean ± SD** | | | **99± 27** | **223± 84** | **158± 44** | **1.45± 0.22** | **0.374± 0.109** | **0.999± 0.000** |
| Summer, Leaf, High | SU1_1L14 | 29600 | 574 | 1057 | 872 | 2.19 | 0.3303 | 0.991824 |
| | SU1_1L42 | 29600 | 997 | 1172 | 1158 | 3.91 | 0.122 | 0.992027 |
| | SU1_2L14 | 29600 | 186 | 462 | 374 | 1.05 | 0.4411 | 0.99652 |
| | SU1_2L42 | 29600 | 727 | 789 | 782 | 3.44 | 0.1671 | 0.996284 |
| **Mean ± SD** | | | **621± 339** | **870± 316** | **797± 324** | **2.65± 1.29** | **0.265± 0.148** | **0.994± 0.003** |
| Summer, Leaf, Medium | SU2_1L14 | 29600 | 364 | 819 | 610 | 2.17 | 0.226 | 0.994223 |
| | SU2_1L42 | 29600 | 1015 | 1230 | 1176 | 3.88 | 0.1027 | 0.991047 |
| | SU2_2L14 | 29600 | 360 | 782 | 598 | 1.71 | 0.3744 | 0.994324 |
| | SU2_2L42 | 29600 | 773 | 899 | 872 | 3.65 | 0.1337 | 0.994189 |
| **Mean ± SD** | | | **628± 323** | **933± 204** | **814± 272** | **2.85± 1.07** | **0.209± 0.122** | **0.993± 0.002** |
| Summer, Leaf, Low | SU3_1L14 | 29600 | 708 | 950 | 908 | 3.57 | 0.0935 | 0.992162 |
| | SU3_2L14 | 29600 | 902 | 1157 | 1143 | 4.48 | 0.0302 | 0.991182 |
| | SU3_2L42 | 29600 | 389 | 541 | 532 | 2.63 | 0.1545 | 0.995304 |
| | SU3_3L42 | 29600 | 177 | 411 | 310 | 2.4 | 0.1366 | 0.997399 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Mean ± SD** | | | **544± 323** | **765± 348** | **723± 373** | **3.27± 0.95** | **0.104± 0.055** | **0.994± 0.003** |

**Table S3.4.** Similarity-based OTUs and species richness and diversity estimates (97% cut-off) for fungal community in water and leaf samples collected from different urban gradients (High, Medium and Low) in winter and summer 2016. The data for samples collected from the control locations in Huangshan during summer 2016 are also included.

| Group | Sample ID | Reads | OTU | ace | Chao1 | Shannon | Simpson | Coverage |
|---|---|---|---|---|---|---|---|---|
| Winter, Water, High | 16WI1_1W | 37620 | 439 | 549 | 547 | 3.38 | 0.0644 | 0.997 |
| | 16WI1_2W | 37620 | 396 | 515 | 535 | 3.33 | 0.0665 | 0.997 |
| **Mean ± SD** | | | **418± 30** | **532± 24** | **541± 8** | **3.36± 0.04** | **0.065± 0.001** | **0.997± 0.000** |
| Winter, Water, Medium | 16WI2_1W | 37620 | 395 | 491 | 500 | 2.48 | 0.2641 | 0.997 |
| | 16WI2_2W | 37620 | 423 | 499 | 500 | 3.22 | 0.133 | 0.997 |
| **Mean ± SD** | | | **409± 20** | **495± 6** | **500± 0** | **2.85± 0.52** | **0.199± 0.093** | **0.997± 0.000** |
| Winter, Water, Low | 16WI3_2W | 37620 | 395 | 563 | 560 | 2.67 | 0.1716 | 0.996 |
| | 16WI3_3W | 37620 | 846 | 928 | 923 | 4.74 | 0.0267 | 0.996 |
| **Mean ± SD** | | | **621± 319** | **746± 258** | **742± 257** | **3.71± 1.46** | **0.099± 0.102** | **0.996± 0.000** |
| Summer, Water, High | 16SU1_1W | 37620 | 989 | 1118 | 1104 | 5.08 | 0.0178 | 0.995 |
| | 16SU1_2W | 37620 | 827 | 904 | 917 | 4.68 | 0.0378 | 0.997 |
| **Mean ± SD** | | | **908± 115** | **1011± 151** | **1011± 132** | **4.88± 0.28** | **0.028± 0.014** | **0.996± 0.001** |
| Summer, Water, Medium | 16SU2_1W | 37620 | 974 | 1163 | 1152 | 4.78 | 0.0228 | 0.994 |
| | 16SU2_2W | 37620 | 536 | 618 | 621 | 3.93 | 0.053 | 0.997 |
| **Mean ± SD** | | | **755± 310** | **891± 385** | **887± 375** | **4.36± 0.60** | **0.038± 0.021** | **0.996± 0.002** |
| Summer, Water, Low | 16SU3_2W | 37620 | 686 | 800 | 792 | 4.16 | 0.0402 | 0.996 |
| | 16SU3_3W | 37620 | 1020 | 1158 | 1146 | 5.09 | 0.0164 | 0.995 |
| **Mean ± SD** | | | **853± 236** | **979± 253** | **969± 250** | **4.63± 0.66** | **0.028± 0.017** | **0.995± 0.001** |
| Summer, | 16SUH_1W | 37620 | 998 | 1161 | 1148 | 4.22 | 0.0631 | 0.994 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Water, Huangshan | 16SUH_2W | 37620 | 885 | 979 | 962 | 4.77 | 0.0209 | 0.996 |
| | 16SUH_3W | 37620 | 963 | 1056 | 1048 | 4.12 | 0.0585 | 0.996 |
| **Mean ± SD** | | | **949± 58** | **1065 ±91** | **1053± 93** | **4.37± 0.35** | **0.048± 0.023** | **0.995± 0.001** |
| Winter, Leaf, High | 16WI1_1L14 | 37620 | 48 | 141 | 99 | 0.28 | 0.9073 | 0.999 |
| | 16WI1_1L42 | 37620 | 49 | 85 | 71 | 0.12 | 0.9714 | 1.000 |
| | 16WI1_2L14 | 37620 | 64 | 111 | 92 | 1.25 | 0.3879 | 0.999 |
| | 16WI1_2L42 | 37620 | 67 | 94 | 88 | 0.27 | 0.9227 | 0.999 |
| **Mean ± SD** | | | **57± 10** | **108± 25** | **88± 12** | **0.48± 0.52** | **0.797± 0.274** | **0.999± 0.000** |
| Winter, Leaf, Medium | 16WI2_1L14 | 37620 | 89 | 165 | 124 | 1.23 | 0.387 | 0.999 |
| | 16WI2_1L42 | 37620 | 126 | 248 | 175 | 1.79 | 0.2617 | 0.999 |
| | 16WI2_2L14 | 37620 | 85 | 234 | 145 | 1.11 | 0.3831 | 0.999 |
| | 16WI2_2L42 | 37620 | 131 | 184 | 190 | 2 | 0.2591 | 0.999 |
| **Mean ± SD** | | | **108± 24** | **208± 40** | **159± 30** | **1.53± 0.43** | **0.323± 0.072** | **0.999± 0.000** |
| Winter, Leaf, Low | 16WI3_2L14 | 37620 | 73 | 149 | 100 | 1.22 | 0.3643 | 0.999 |
| | 16WI3_2L42 | 37620 | 106 | 216 | 174 | 0.85 | 0.6905 | 0.999 |
| | 16WI3_3L14 | 37620 | 53 | 125 | 82 | 1.21 | 0.3854 | 0.999 |
| | 16WI3_3L42 | 37620 | 124 | 263 | 196 | 1.65 | 0.294 | 0.999 |
| **Mean ± SD** | | | **89± 32** | **188± 63** | **138± 55** | **1.23± 0.33** | **0.434± 0.176** | **0.999± 0.000** |
| Summer, Leaf, High | 16SU1_1L14 | 37620 | 1198 | 1433 | 1406 | 4.6 | 0.0365 | 0.992 |
| | 16SU1_1L42 | 37620 | 936 | 1067 | 1056 | 4.26 | 0.0756 | 0.995 |
| | 16SU1_2L14 | 37620 | 1125 | 1342 | 1337 | 4.47 | 0.0482 | 0.993 |
| | 16SU1_2L42 | 37620 | 572 | 818 | 804 | 1.63 | 0.4835 | 0.994 |
| **Mean ± SD** | | | **958± 280** | **1165± 279** | **1151± 276** | **3.74± 1.41** | **0.161± 0.216** | **0.994± 0.001** |
| Summer, Leaf, Medium | 16SU2_1L14 | 37620 | 875 | 1200 | 1178 | 3.23 | 0.1233 | 0.992 |
| | 16SU2_1L42 | 37620 | 665 | 898 | 859 | 1.84 | 0.4842 | 0.994 |
| | 16SU2_2L14 | 37620 | 352 | 801 | 591 | 2.41 | 0.1466 | 0.996 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 16SU2_2L42 | 37620 | 542 | 659 | 679 | 3.96 | 0.0486 | 0.996 |
| **Mean ± SD** | | | **609± 219** | **890± 229** | **827± 259** | **2.86± 0.93** | **0.201± 0.194** | **0.995± 0.002** |
| Summer, Leaf, Low | 16SU3_2L14 | 37620 | 507 | 744 | 723 | 2.43 | 0.2084 | 0.995 |
| | 16SU3_2L42 | 37620 | 903 | 1096 | 1096 | 4.41 | 0.0316 | 0.994 |
| | 16SU3_3L14 | 37620 | 160 | 294 | 290 | 1.62 | 0.3105 | 0.998 |
| **Mean ± SD** | | | **523± 372** | **711± 402** | **703± 403** | **2.82± 1.44** | **0.184± 0.141** | **0.996± 0.002** |
| Summer, Leaf, Huangshan | 16SUH_1L14 | 37620 | 615 | 1113 | 966 | 3.09 | 0.1817 | 0.994 |
| | 16SUH_2L14 | 37620 | 200 | 359 | 279 | 1.78 | 0.3793 | 0.998 |
| | 16SUH_2L42 | 37620 | 579 | 789 | 774 | 3.46 | 0.0939 | 0.995 |
| | 16SUH_3L14 | 37620 | 257 | 497 | 388 | 1.42 | 0.4669 | 0.997 |
| | 16SUH_3L42 | 37620 | 255 | 491 | 381 | 2.43 | 0.1725 | 0.997 |
| **Mean ± SD** | | | **381± 199** | **650± 303** | **558± 296** | **2.44± 0.86** | **0.259± 0.157** | **0.996± 0.002** |

**Table S3.5.** Relative abundance of important bacterial genera observed in water at different sampling locations in winter 2015.

| Taxon | WI1.1 W0 | WI1.1 W42 | WI1.2 W0 | WI1.2 W42 | WI1.3 W0 | WI1.3 W42 | WI2.1 W0 | WI2.1 W42 | WI2.2 W0 | WI2.2 W42 | WI2.3 W0 | WI2.3 W42 | WI3.1 W0 | WI3.1 W42 | WI3.2 W0 | WI3.2 W42 | WI3.3 W0 | WI3.3 W42 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Acinetobacter* | 0.22 | 0.42 | 2.52 | 0.34 | 0.41 | 0.35 | 0.92 | 0.10 | 1.13 | 0.02 | 0.08 | 0.21 | 0.18 | 0.17 | 0.06 | 0.05 | 0.25 | 0.02 |
| *Acidibacter* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.01 | 0.11 |
| *Arcobacter* | 0.35 | 0.45 | 50.0 | 42.2 | 0.77 | 1.88 | 1.06 | 2.59 | 0.21 | 3.18 | 0.01 | 0.35 | 0.14 | 1.75 | 0.02 | 0.35 | 0.24 | 0.28 |
| *Bacteroides* | 0.21 | 0.15 | 1.58 | 0.90 | 0.19 | 0.66 | 0.15 | 0.22 | 0.02 | 0.25 | 0.00 | 0.09 | 0.02 | 0.17 | 0.00 | 0.24 | 0.04 | 0.03 |
| *Comamonadaceae* | 10.9 | 17.9 | 3.16 | 8.94 | 12.2 | 16.2 | 4.34 | 9.85 | 3.48 | 14.8 | 13.5 | 16.9 | 10.7 | 10.4 | 10.6 | 12.9 | 7.27 | 17.1 |
| *Cyanobacteria_norank* | 25.57 | 13.74 | 0.02 | 0.19 | 14.64 | 6.69 | 16.96 | 3.62 | 24.95 | 14.10 | 2.03 | 11.29 | 0.97 | 9.73 | 2.45 | 4.56 | 4.21 | 2.26 |
| *Dechloromonas* | 0.00 | 0.00 | 0.31 | 0.04 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.00 | 0.00 | 0.00 | 0.02 | 0.01 | 0.00 | 0.01 | 0.01 | 0.00 |
| *Duganella* | 0.02 | 0.01 | 0.01 | 0.02 | 0.03 | 0.05 | 0.01 | 0.01 | 0.01 | 0.01 | 0.09 | 0.02 | 0.11 | 0.02 | 0.02 | 0.04 | 0.02 | 0.02 |
| *Flavobacterium* | 16.2 | 30.7 | 6.38 | 14.9 | 17.4 | 38.8 | 3.92 | 23.9 | 3.33 | 25.5 | 17.1 | 21.4 | 8.4 | 24.4 | 11.8 | 28.7 | 20.1 | 9.9 |
| *hgcI_clade* | 3.91 | 2.51 | 0.01 | 0.14 | 3.60 | 1.3 | 13.3 | 8.4 | 13.4 | 2.52 | 6.21 | 4.38 | 6.1 | 4.4 | 10.5 | 2.07 | 6.88 | 4.38 |
| *Malikia* | 3.63 | 1.61 | 4.99 | 7.11 | 5.54 | 2.87 | 0.24 | 0.12 | 0.28 | 1.36 | 2.37 | 1.51 | 9.54 | 6.14 | 3.23 | 5.44 | 16.3 | 3.06 |
| *Mangroviflexus* | 0.00 | 0.01 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.02 | 0.00 | 0.00 | 0.01 | 0.00 |
| *Massilia* | 0.02 | 0.01 | 0.00 | 0.01 | 0.02 | 0.03 | 0.12 | 0.02 | 0.05 | 0.22 | 0.20 | 0.01 | 0.07 | 0.01 | 0.04 | 0.00 | 0.09 | 0.09 |
| *Novosphingobium* | 0.26 | 0.15 | 0.00 | 0.05 | 0.28 | 0.32 | 0.10 | 0.03 | 0.25 | 0.14 | 1.44 | 0.27 | 0.30 | 0.18 | 0.22 | 0.09 | 0.40 | 0.32 |
| *Paludibacter* | 0.05 | 0.01 | 0.58 | 0.11 | 0.02 | 0.01 | 0.01 | 0.02 | 0.01 | 0.04 | 0.00 | 0.01 | 0.01 | 0.06 | 0.01 | 0.31 | 0.02 | 0.01 |
| *Planktothricoides* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Polynucleobacter* | 1.56 | 3.79 | 0.08 | 0.64 | 2.16 | 1.70 | 1.58 | 1.00 | 0.42 | 1.58 | 2.77 | 3.81 | 1.96 | 2.82 | 3.66 | 2.77 | 1.36 | 4.31 |
| *Prevotella_9* | 0.37 | 0.12 | 0.19 | 0.43 | 0.32 | 1.23 | 0.18 | 0.19 | 0.02 | 0.11 | 0.01 | 0.03 | 0.04 | 0.77 | 0.00 | 0.02 | 0.05 | 0.15 |
| *Propionivibrio* | 0.02 | 0.01 | 0.27 | 0.08 | 0.01 | 0.02 | 0.00 | 0.00 | 0.01 | 0.01 | 0.00 | 0.01 | 0.01 | 0.02 | 0.01 | 0.03 | 0.01 | 0.00 |
| *Pseudomonas* | 2.22 | 2.02 | 11.7 | 12.80 | 2.69 | 4.24 | 6.58 | 0.74 | 5.14 | 0.86 | 2.15 | 0.63 | 7.35 | 1.58 | 0.25 | 0.59 | 0.48 | 0.37 |
| *Rhizobium* | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.01 | 0.01 | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.01 | 0.01 |
| *Sediminibacterium* | 1.25 | 0.46 | 0.02 | 0.09 | 1.12 | 0.63 | 0.26 | 0.35 | 0.20 | 0.35 | 1.49 | 1.18 | 0.56 | 0.55 | 0.59 | 0.25 | 0.43 | 0.27 |

**Table S3.6.** Relative abundance of important bacterial genera observed in leaf at different sampling locations in winter 2015.

| Taxon | WI1.1 L14 | WI1.1 L42 | WI1.2 L14 | WI1.2 L42 | WI1.3 L14 | WI1.3 L42 | WI2.1 L14 | WI2.1 L42 | WI2.2 L14 | WI2.2 L42 | WI2.3 L14 | WI2.3 L42 | WI3.1 L14 | WI3.1 L42 | WI3.2 L14 | WI3.2 L42 | WI3.3 L14 | WI3.3 L42 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Acinetobacter* | 1.00 | 0.77 | 6.17 | 3.14 | 9.79 | 4.7 | 4.21 | 5.42 | 1.00 | 0.44 | 0.67 | 0.26 | 3.17 | 0.50 | 1.55 | 6.42 | 0.34 | 0.4 |
| *Acidibacter* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.0 |
| *Arcobacter* | 0.64 | 0.23 | 1.18 | 0.63 | 0.81 | 0.7 | 0.02 | 0.08 | 0.07 | 0.02 | 0.11 | 0.06 | 0.06 | 0.01 | 0.09 | 0.02 | 0.02 | 0.0 |
| *Bacteroides* | 0.00 | 0.02 | 0.01 | 0.38 | 0.00 | 0.1 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.0 |
| *Comamonadaceae* | 29.0 | 26.7 | 10.9 | 12.6 | 17.9 | 21 | 12.2 | 14.8 | 22.5 | 14.0 | 18.9 | 20.1 | 16.4 | 33.1 | 9.18 | 11.6 | 21.9 | 23 |
| *Cyanobacteria_norank* | 1.57 | 0.02 | 0.06 | 2.00 | 4.28 | 7.8 | 1.33 | 6.69 | 0.67 | 4.79 | 0.06 | 0.34 | 0.37 | 0.59 | 4.05 | 2.83 | 4.82 | 2.6 |
| *Dechloromonas* | 0.02 | 0.01 | 0.00 | 0.02 | 0.00 | 0.0 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.0 |
| *Duganella* | 1.26 | 0.47 | 0.91 | 0.13 | 1.57 | 0.5 | 3.17 | 0.73 | 3.00 | 0.37 | 6.73 | 2.92 | 1.57 | 0.40 | 3.11 | 0.83 | 1.84 | 1.0 |
| *Flavobacterium* | 26.4 | 33.2 | 47.0 | 29.7 | 33.6 | 0.0 | 27.9 | 24.3 | 24.1 | 28.2 | 29.1 | 35.5 | 46.8 | 39.8 | 31.9 | 28.9 | 44.0 | 0.0 |
| *hgcI_clade* | 0.00 | 0.02 | 0.00 | 0.00 | 0.02 | 0.0 | 0.00 | 0.04 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 | 0.06 | 0.03 | 0.01 | 0.01 | 0.0 |
| *Malikia* | 0.34 | 0.18 | 0.07 | 0.14 | 0.14 | 0.4 | 0.11 | 0.26 | 0.18 | 0.81 | 0.15 | 0.08 | 0.05 | 0.77 | 0.19 | 0.26 | 0.09 | 0.5 |
| *Mangroviflexus* | 0.06 | 1.14 | 0.00 | 2.63 | 0.00 | 5.2 | 0.00 | 0.43 | 0.00 | 0.42 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.29 | 0.01 | 0.15 |
| *Massilia* | 1.17 | 4.40 | 0.74 | 0.56 | 2.57 | 2.0 | 30.9 | 10.0 | 8.56 | 3.01 | 2.76 | 4.44 | 3.62 | 3.55 | 5.73 | 8.06 | 11.5 | 13.2 |
| *Novosphingobium* | 0.26 | 0.22 | 0.06 | 0.11 | 0.11 | 0.37 | 0.63 | 0.59 | 0.24 | 1.02 | 0.52 | 0.27 | 0.02 | 1.80 | 0.08 | 0.09 | 0.17 | 1.21 |
| *Paludibacter* | 0.57 | 2.38 | 0.38 | 6.87 | 0.12 | 8.25 | 0.00 | 1.05 | 0.01 | 0.60 | 0.01 | 0.91 | 0.00 | 0.04 | 0.02 | 0.18 | 0.01 | 0.38 |
| *Planktothricoides* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Polynucleobacter* | 0.02 | 0.02 | 0.02 | 0.01 | 0.05 | 0.02 | 0.01 | 0.01 | 0.00 | 0.01 | 0.04 | 0.03 | 0.01 | 0.04 | 0.01 | 0.02 | 0.00 | 0.04 |
| *Prevotella_9* | 0.00 | 0.00 | 0.00 | 0.08 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Propionivibrio* | 1.84 | 2.75 | 1.47 | 4.43 | 0.71 | 1.96 | 0.02 | 1.21 | 0.09 | 0.34 | 0.08 | 0.76 | 0.05 | 0.02 | 0.15 | 0.69 | 0.09 | 0.57 |
| *Pseudomonas* | 4.61 | 3.25 | 11.39 | 4.01 | 7.41 | 9.87 | 9.26 | 8.14 | 22.91 | 11.63 | 14.96 | 10.88 | 21.72 | 3.62 | 27.21 | 13.80 | 6.52 | 3.62 |
| *Rhizobium* | 0.05 | 0.10 | 0.01 | 0.02 | 0.02 | 0.16 | 0.64 | 1.65 | 0.27 | 3.90 | 0.02 | 0.12 | 0.01 | 0.53 | 0.25 | 2.54 | 0.31 | 8.18 |
| *Sediminibacterium* | 1.89 | 1.47 | 0.60 | 0.58 | 1.35 | 2.14 | 0.84 | 1.35 | 1.16 | 1.58 | 6.98 | 1.57 | 0.25 | 3.70 | 0.72 | 0.77 | 0.65 | 1.51 |

**Table S3.7.** Relative abundance of important bacterial genera observed in water at different sampling locations in summer 2015.

| Taxon | SU1.1 W0 | SU1.1 W42 | SU1.2 W0 | SU1.2 W42 | SU1.3 W0 | SU1.3 W42 | SU2.1 W0 | SU2.1 W42 | SU2.2 W0 | SU2.2 W42 | SU2.3 W0 | SU2.3 W42 | SU3.1 W0 | SU3.1 W42 | SU3.2 W0 | SU3.2 W42 | SU3.3 W0 | SU3.3 W42 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Acinetobacter* | 0.02 | 0.04 | 0.12 | 0.18 | 0.61 | 0.18 | 0.01 | 0.05 | 0.02 | 0.18 | 0.35 | 0.58 | 0.09 | 0.07 | 0.15 | 0.03 | 0.05 | 0.00 |
| *Acidibacter* | 0.11 | 0.15 | 0.12 | 0.22 | 0.10 | 0.14 | 0.45 | 1.19 | 0.32 | 0.39 | 0.37 | 0.68 | 0.10 | 0.28 | 0.36 | 0.88 | 0.36 | 1.03 |
| *Arcobacter* | 10.2 | 2.45 | 0.66 | 2.42 | 3.20 | 2.22 | 0.15 | 1.15 | 0.83 | 1.25 | 0.15 | 4.97 | 0.18 | 0.28 | 0.09 | 0.08 | 0.01 | 0.02 |
| *Bacteroides* | 1.26 | 0.63 | 0.29 | 0.56 | 0.38 | 0.83 | 0.05 | 0.25 | 0.13 | 0.07 | 0.08 | 0.69 | 0.03 | 0.14 | 0.02 | 0.09 | 0.01 | 0.01 |
| *Comamonadaceae* | 10.0 | 12.8 | 11.8 | 11.4 | 14.9 | 11.0 | 10.7 | 10.1 | 10.9 | 7.35 | 12.21 | 9.12 | 10.91 | 11.90 | 9.97 | 11.30 | 8.83 | 5.44 |
| *Cyanobacteria_norank* | 2.45 | 6.27 | 3.29 | 1.80 | 3.68 | 0.45 | 0.66 | 0.74 | 0.38 | 2.38 | 2.93 | 0.37 | 4.98 | 1.20 | 6.97 | 6.39 | 3.58 | 1.77 |
| *Dechloromonas* | 3.11 | 1.57 | 0.57 | 0.82 | 0.50 | 1.23 | 0.08 | 0.39 | 0.19 | 0.53 | 0.29 | 2.53 | 0.17 | 0.55 | 0.19 | 0.02 | 0.04 | 0.02 |
| *Duganella* | 0.01 | 0.01 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 |
| *Flavobacterium* | 4.95 | 2.84 | 6.29 | 10.0 | 11.8 | 6.94 | 0.90 | 0.28 | 1.91 | 1.19 | 0.00 | 0.44 | 1.25 | 0.79 | 0.55 | 0.42 | 0.52 | 0.11 |
| *hgcI_clade* | 11.5 | 14.1 | 15.1 | 13.1 | 6.43 | 9.79 | 15.0 | 17.5 | 35.2 | 26.33 | 16.5 | 26.65 | 18.4 | 18.8 | 21.81 | 29.0 | 24.35 | 36.82 |
| *Malikia* | 3.51 | 2.68 | 4.11 | 10.9 | 7.10 | 15.7 | 0.22 | 0.85 | 2.42 | 2.75 | 7.73 | 1.83 | 1.44 | 4.78 | 5.44 | 3.88 | 0.47 | 0.47 |
| *Mangroviflexus* | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Massilia* | 0.01 | 0.02 | 0.00 | 0.05 | 0.01 | 0.01 | 0.00 | 0.03 | 0.00 | 0.01 | 0.01 | 0.02 | 0.01 | 0.00 | 0.00 | 0.02 | 0.00 | 0.01 |
| *Novosphingobium* | 1.14 | 2.14 | 1.88 | 1.77 | 2.95 | 3.19 | 1.42 | 1.45 | 0.56 | 1.12 | 0.55 | 1.97 | 1.79 | 2.22 | 0.49 | 0.66 | 0.99 | 0.58 |
| *Paludibacter* | 0.79 | 0.06 | 0.33 | 0.25 | 0.17 | 0.33 | 0.01 | 0.02 | 0.06 | 0.05 | 0.05 | 0.15 | 0.04 | 0.04 | 0.04 | 0.05 | 0.01 | 0.02 |
| *Planktothricoides* | 0.01 | 0.14 | 0.01 | 0.28 | 0.00 | 0.08 | 0.00 | 0.46 | 0.02 | 0.06 | 0.02 | 0.10 | 0.08 | 5.60 | 0.02 | 0.38 | 0.01 | 0.32 |
| *Polynucleobacter* | 2.55 | 3.21 | 2.32 | 2.92 | 2.36 | 2.30 | 5.31 | 5.94 | 3.73 | 2.59 | 2.37 | 2.18 | 2.72 | 2.98 | 1.91 | 2.00 | 2.93 | 2.46 |
| *Prevotella_9* | 3.12 | 0.86 | 0.19 | 0.74 | 0.26 | 1.49 | 0.01 | 0.04 | 0.26 | 0.05 | 0.01 | 0.33 | 0.04 | 0.11 | 0.00 | 0.04 | 0.01 | 0.01 |
| *Propionivibrio* | 0.18 | 0.02 | 0.04 | 0.04 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.11 | 0.01 | 0.02 | 0.00 | 0.01 | 0.00 | 0.01 |
| *Pseudomonas* | 0.48 | 0.43 | 0.25 | 0.73 | 0.38 | 0.96 | 0.04 | 0.24 | 0.05 | 0.14 | 0.37 | 0.97 | 0.14 | 0.12 | 0.21 | 0.04 | 0.08 | 0.02 |
| *Rhizobium* | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.01 | 0.01 | 0.00 | 0.00 | 0.01 | 0.06 | 0.04 |
| *Sediminibacterium* | 0.33 | 0.44 | 0.87 | 0.89 | 0.76 | 0.66 | 0.31 | 0.33 | 0.45 | 0.63 | 0.24 | 0.32 | 0.30 | 0.38 | 0.11 | 0.09 | 0.30 | 0.09 |

**Table S3.8.** Relative abundance of important bacterial genera observed in leaf at different sampling locations in summer 2015.

| Taxon | SU1.1 L14 | SU1.1 L42 | SU1.2 L14 | SU1.2 L42 | SU1.3 L14 | SU1.3 L42 | SU2.1 L14 | SU2.1 L42 | SU2.2 L14 | SU2.2 L42 | SU2.3 L14 | SU2.3 L42 | SU3.1 L14 | SU3.2 L14 | SU3.2 L42 | SU3.3 L42 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Acinetobacter* | 0.06 | 0.51 | 0.53 | 0.27 | 0.52 | 0.29 | 49.27 | 0.17 | 11.55 | 0.34 | 7.75 | 0.23 | 17.36 | 7.56 | 2.00 | 0.12 |
| *Acidibacter* | 0.01 | 0.03 | 0.03 | 0.15 | 0.00 | 0.24 | 0.00 | 0.09 | 0.01 | 0.02 | 0.01 | 0.11 | 0.00 | 0.00 | 0.17 | 0.33 |
| *Arcobacter* | 0.01 | 0.03 | 0.04 | 0.01 | 0.01 | 0.00 | 0.00 | 0.02 | 0.04 | 0.00 | 0.01 | 0.01 | 0.01 | 0.02 | 0.00 | 0.01 |
| *Bacteroides* | 0.34 | 0.25 | 1.22 | 0.67 | 4.27 | 0.22 | 0.02 | 0.80 | 0.01 | 0.24 | 0.84 | 0.27 | 0.19 | 0.08 | 0.64 | 0.26 |
| *Comamonadaceae* | 3.47 | 2.36 | 16.50 | 1.95 | 3.65 | 1.94 | 7.03 | 1.06 | 17.41 | 2.15 | 10.48 | 1.58 | 6.45 | 10.08 | 3.33 | 2.56 |
| *Cyanobacteria_norank* | 1.00 | 0.79 | 4.72 | 1.58 | 2.82 | 0.80 | 6.64 | 12.42 | 4.81 | 1.41 | 4.12 | 0.42 | 0.30 | 1.51 | 1.38 | 0.06 |
| *Dechloromonas* | 0.33 | 0.01 | 0.34 | 0.11 | 0.25 | 0.01 | 0.20 | 0.00 | 0.41 | 0.19 | 0.31 | 0.01 | 0.21 | 0.15 | 0.03 | 0.00 |
| *Duganella* | 0.00 | 0.05 | 0.00 | 0.02 | 0.01 | 0.01 | 0.02 | 0.00 | 0.01 | 0.02 | 0.00 | 0.00 | 0.01 | 0.02 | 0.00 | 0.00 |
| *Flavobacterium* | 0.12 | 0.50 | 0.87 | 0.41 | 0.08 | 0.38 | 1.39 | 0.02 | 2.92 | 0.56 | 3.08 | 0.32 | 3.88 | 2.63 | 0.44 | 0.34 |
| *hgcI_clade* | 0.01 | 0.03 | 0.79 | 0.01 | 0.02 | 0.04 | 0.02 | 0.03 | 0.38 | 0.03 | 0.93 | 0.00 | 0.01 | 0.03 | 0.03 | 0.00 |
| *Malikia* | 0.19 | 0.07 | 1.09 | 0.04 | 0.31 | 0.09 | 0.78 | 0.03 | 0.59 | 0.08 | 0.77 | 0.04 | 0.73 | 0.55 | 0.09 | 0.04 |
| *Mangroviflexus* | 0.38 | 0.05 | 0.25 | 0.06 | 0.40 | 0.26 | 2.88 | 0.06 | 1.17 | 1.18 | 0.32 | 0.04 | 2.61 | 3.78 | 0.47 | 0.02 |
| *Massilia* | 0.01 | 0.08 | 0.00 | 0.23 | 0.00 | 0.05 | 0.09 | 0.44 | 0.04 | 0.02 | 0.04 | 0.09 | 0.08 | 0.14 | 0.02 | 0.17 |
| *Novosphingobium* | 0.25 | 2.50 | 0.40 | 0.71 | 0.15 | 1.17 | 0.50 | 1.32 | 1.80 | 0.95 | 2.30 | 0.80 | 2.30 | 1.06 | 1.25 | 3.18 |
| *Paludibacter* | 0.42 | 0.04 | 0.55 | 0.02 | 0.81 | 0.01 | 0.09 | 0.00 | 0.13 | 0.06 | 0.18 | 0.00 | 0.22 | 0.97 | 0.02 | 0.08 |
| *Planktothricoides* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Polynucleobacter* | 0.01 | 0.01 | 0.10 | 0.01 | 0.01 | 0.00 | 0.01 | 0.01 | 0.02 | 0.01 | 0.05 | 0.02 | 0.00 | 0.01 | 0.03 | 0.00 |
| *Prevotella_9* | 0.83 | 0.06 | 0.36 | 0.14 | 1.48 | 0.09 | 0.00 | 0.00 | 0.00 | 0.12 | 0.27 | 0.10 | 0.05 | 0.04 | 0.04 | 0.06 |
| *Propionivibrio* | 1.99 | 0.08 | 1.21 | 0.22 | 2.73 | 0.24 | 0.43 | 0.01 | 0.87 | 0.56 | 0.54 | 0.10 | 0.49 | 0.53 | 0.17 | 0.08 |
| *Pseudomonas* | 0.06 | 1.13 | 0.06 | 3.11 | 0.04 | 2.58 | 0.34 | 1.00 | 0.23 | 0.77 | 0.16 | 1.05 | 1.47 | 2.29 | 1.06 | 2.00 |
| *Rhizobium* | 0.11 | 0.65 | 0.40 | 0.11 | 0.08 | 0.37 | 0.84 | 0.51 | 3.94 | 0.57 | 4.03 | 1.66 | 2.42 | 2.03 | 2.48 | 1.37 |
| *Sediminibacterium* | 0.01 | 0.01 | 0.01 | 0.02 | 0.00 | 0.06 | 0.00 | 0.00 | 0.00 | 0.01 | 0.01 | 0.05 | 0.01 | 0.01 | 0.02 | 0.02 |

**Table S3.9.** Relative abundance of important bacterial genera observed in water at different sampling locations in winter 2016.

| Taxon | WI1.1W | WI1.2W | WI2.1W | WI2.2W | WI3.2W | WI3.3W |
|---|---|---|---|---|---|---|
| *Acetobacteroides* | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 |
| *Actinoplanes* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Anaerolineaceae_unclassified* | 0.00 | 0.00 | 0.00 | 0.05 | 0.00 | 0.00 |
| *Anaerolineaceae_uncultured* | 0.01 | 0.03 | 0.00 | 0.03 | 0.04 | 0.03 |
| *Arcobacter* | 5.76 | 1.96 | 23.36 | 1.83 | 0.36 | 0.36 |
| *Bacteria_unclassified* | 0.33 | 1.11 | 0.55 | 0.27 | 0.11 | 0.11 |
| *Bacteroidetes_unclassified* | 0.00 | 0.02 | 0.00 | 0.00 | 0.01 | 0.00 |
| *CL500-29_marine_group* | 0.13 | 0.15 | 0.44 | 0.38 | 0.89 | 0.83 |
| *Comamonadaceae_unclassified* | 14.22 | 12.96 | 9.65 | 13.88 | 8.52 | 14.54 |
| *Cyanobacteria_norank* | 5.85 | 15.43 | 10.18 | 5.47 | 18.34 | 4.20 |
| *Dechloromonas* | 0.17 | 0.39 | 0.05 | 0.08 | 0.15 | 0.07 |
| *Polynucleobacter* | 3.19 | 4.52 | 0.92 | 1.14 | 3.36 | 4.02 |
| *Pseudomonas* | 3.45 | 2.01 | 1.85 | 0.47 | 0.41 | 0.38 |
| *Rhizobium* | 0.02 | 0.02 | 0.03 | 0.00 | 0.01 | 0.00 |
| *Rhodobacter* | 0.40 | 1.24 | 0.24 | 0.55 | 0.28 | 0.48 |
| *Rhodocyclaceae_unclassified* | 0.01 | 0.03 | 0.01 | 0.02 | 0.02 | 0.01 |
| *Sandaracinaceae_uncultured* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Sediminibacterium* | 1.62 | 0.89 | 1.11 | 0.78 | 1.76 | 1.00 |
| *Sphaerotilus* | 1.92 | 1.92 | 1.08 | 1.49 | 1.54 | 2.32 |
| *Sporichthyaceae_norank* | 3.87 | 5.11 | 2.27 | 3.94 | 6.18 | 4.70 |
| *Sporichthyaceae_unclassified* | 1.90 | 3.27 | 1.61 | 1.80 | 2.59 | 3.90 |
| *Tolumonas* | 0.31 | 0.41 | 0.17 | 0.34 | 0.00 | 0.01 |
| *Uliginosibacterium* | 0.05 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *hgcI_clade* | 2.87 | 3.73 | 3.54 | 2.26 | 10.27 | 3.50 |

**Table S3.10.** Relative abundance of important bacterial genera observed in leaf at different sampling locations in winter 2016.

| **Taxon** | **WI1.1 L14** | **WI1.1 L42** | **WI1.2 L14** | **WI1.2 L42** | **WI2.1 L14** | **WI2.1 L42** | **WI2.2 L14** | **WI2.2 L42** | **WI3.2 L14** | **WI3.2 L42** | **WI3.3 L14** | **WI3.3 L42** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Acetobacteroides* | 0.00 | 0.04 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Actinoplanes* | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.01 | 0.00 | 0.02 | 0.00 | 0.00 | 0.00 | 0.04 |
| *Anaerolineaceae_unclassified* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Anaerolineaceae_uncultured* | 0.01 | 0.18 | 0.01 | 0.29 | 0.03 | 0.19 | 0.02 | 0.01 | 0.04 | 0.05 | 0.00 | 0.21 |
| *Arcobacter* | 2.28 | 0.56 | 1.56 | 0.79 | 0.13 | 0.02 | 0.06 | 0.03 | 0.16 | 0.14 | 0.00 | 0.00 |
| *Bacteria_unclassified* | 0.00 | 0.08 | 0.04 | 0.07 | 0.04 | 0.16 | 0.02 | 0.00 | 0.02 | 0.04 | 0.00 | 0.36 |
| *Bacteroidetes_unclassified* | 0.01 | 0.14 | 0.00 | 0.29 | 0.01 | 0.03 | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 |
| *CL500-29_marine_group* | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.06 | 0.03 | 0.01 | 0.01 | 0.06 | 0.00 | 0.02 |
| *Comamonadaceae_unclassified* | 7.45 | 4.72 | 7.00 | 6.92 | 9.11 | 7.62 | 8.20 | 8.93 | 6.94 | 6.61 | 7.46 | 7.96 |
| *Cyanobacteria_norank* | 0.83 | 5.25 | 0.51 | 4.09 | 4.53 | 2.66 | 2.41 | 1.31 | 3.78 | 1.48 | 0.53 | 2.01 |
| *Dechloromonas* | 1.15 | 2.65 | 1.07 | 2.91 | 0.31 | 1.00 | 0.09 | 0.25 | 0.03 | 0.65 | 0.01 | 0.03 |
| *Polynucleobacter* | 0.03 | 0.04 | 0.01 | 0.02 | 0.05 | 0.03 | 0.01 | 0.02 | 0.06 | 0.04 | 0.01 | 0.06 |
| *Pseudomonas* | 5.46 | 2.39 | 6.73 | 5.01 | 10.62 | 6.66 | 8.40 | 9.15 | 8.53 | 5.48 | 5.16 | 1.87 |
| *Rhizobium* | 0.34 | 0.61 | 0.16 | 1.10 | 2.15 | 2.65 | 1.14 | 5.82 | 0.44 | 2.79 | 0.19 | 5.30 |
| *Rhodobacter* | 1.73 | 1.96 | 1.49 | 2.36 | 2.96 | 2.43 | 0.78 | 0.56 | 0.45 | 1.00 | 0.25 | 2.80 |
| *Rhodocyclaceae_unclassified* | 0.05 | 0.56 | 0.11 | 0.38 | 0.03 | 0.14 | 0.01 | 0.01 | 0.02 | 0.09 | 0.00 | 0.01 |
| *Sandaracinaceae_uncultured* | 0.00 | 0.05 | 0.00 | 0.02 | 0.00 | 0.10 | 0.00 | 0.10 | 0.00 | 0.03 | 0.00 | 0.08 |
| *Sediminibacterium* | 0.01 | 0.03 | 0.00 | 0.01 | 0.01 | 0.01 | 0.00 | 0.00 | 0.00 | 0.05 | 0.00 | 0.01 |
| *Sphaerotilus* | 6.74 | 5.51 | 6.89 | 6.64 | 4.29 | 4.56 | 7.97 | 4.67 | 5.57 | 6.38 | 7.81 | 2.41 |
| *Sporichthyaceae_norank* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Sporichthyaceae_unclassified* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Tolumonas* | 10.42 | 6.68 | 9.36 | 2.48 | 2.80 | 1.63 | 0.98 | 0.88 | 0.29 | 0.33 | 0.99 | 0.02 |
| *Uliginosibacterium* | 3.41 | 3.24 | 3.87 | 1.54 | 1.41 | 5.48 | 1.61 | 8.60 | 0.69 | 1.82 | 0.38 | 0.10 |
| *hgcI_clade* | 0.00 | 0.02 | 0.01 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

**Table S3.11.** Relative abundance of important bacterial genera observed in water at different sampling locations in summer 2016.

| Taxon | SU1.1W | SU1.2W | SU2.1W | SU2.2W | SU3.2W | SU3.3W | SUH.1W | SUH.2W | SUH.3W |
|---|---|---|---|---|---|---|---|---|---|
| *Acetobacteroides* | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.03 | 0.00 |
| *Actinoplanes* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.02 |
| *Anaerolineaceae_unclassified* | 0.03 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Anaerolineaceae_uncultured* | 0.16 | 0.06 | 0.06 | 0.05 | 0.08 | 0.01 | 0.01 | 0.00 | 0.06 |
| *Arcobacter* | 0.04 | 0.03 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 |
| *Bacteria_unclassified* | 0.23 | 0.29 | 0.15 | 0.38 | 0.12 | 0.13 | 0.04 | 0.02 | 1.55 |
| *Bacteroidetes_unclassified* | 0.04 | 0.02 | 0.06 | 0.16 | 0.07 | 0.07 | 0.00 | 0.00 | 0.04 |
| *CL500-29_marine_group* | 6.88 | 6.11 | 5.76 | 0.39 | 5.43 | 5.26 | 0.00 | 0.00 | 0.04 |
| *Comamonadaceae_unclassified* | 6.03 | 7.48 | 3.08 | 6.76 | 4.98 | 4.92 | 23.31 | 19.21 | 32.72 |
| *Cyanobacteria_norank* | 9.95 | 6.67 | 15.01 | 0.91 | 8.54 | 3.31 | 0.31 | 0.35 | 6.76 |
| *Dechloromonas* | 0.41 | 0.24 | 0.18 | 0.43 | 0.51 | 0.01 | 0.05 | 0.06 | 0.02 |
| *Polynucleobacter* | 3.13 | 2.92 | 1.73 | 1.53 | 2.45 | 3.02 | 0.74 | 0.19 | 0.18 |
| *Pseudomonas* | 0.09 | 0.87 | 0.08 | 0.86 | 0.02 | 0.02 | 0.05 | 0.01 | 0.04 |
| *Rhizobium* | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.02 | 0.02 | 0.01 | 0.11 |
| *Rhodobacter* | 0.09 | 0.11 | 0.07 | 0.45 | 0.09 | 0.11 | 0.24 | 1.31 | 0.31 |
| *Rhodocyclaceae_unclassified* | 0.08 | 0.04 | 0.08 | 0.01 | 0.06 | 0.01 | 0.07 | 0.04 | 0.06 |
| *Sandaracinaceae_uncultured* | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.06 |
| *Sediminibacterium* | 0.47 | 0.39 | 0.40 | 1.05 | 0.17 | 0.75 | 1.92 | 9.83 | 0.16 |
| *Sphaerotilus* | 1.24 | 0.80 | 0.44 | 2.10 | 0.04 | 1.45 | 2.45 | 3.21 | 3.29 |
| *Sporichthyaceae_norank* | 0.00 | 0.00 | 0.08 | 0.00 | 0.00 | 0.01 | 0.06 | 0.20 | 0.00 |
| *Sporichthyaceae_unclassified* | 0.56 | 0.71 | 0.71 | 0.23 | 0.05 | 0.53 | 0.29 | 0.36 | 0.00 |
| *Tolumonas* | 0.05 | 0.06 | 0.01 | 0.00 | 0.00 | 0.01 | 0.09 | 0.00 | 0.02 |
| *Uliginosibacterium* | 0.01 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.03 | 0.01 | 0.05 |
| *hgcI_clade* | 17.84 | 25.07 | 15.75 | 8.23 | 15.79 | 19.27 | 0.79 | 2.27 | 0.00 |

**Table S3.12.** Relative abundance of important bacterial genera observed in leaf at different sampling locations in summer 2016.

| Taxon | SU1.1 L14 | SU1.1 L42 | SU1.2 L14 | SU1.2 L42 | SU2.1 L14 | SU2.1 L42 | SU2.2 L14 | SU2.2 L42 | SU3.2 L14 | SU3.2 L42 | SU3.3 L42 | SUH.1 L14 | SUH.2 L14 | SUH.2 L42 | SUH.3 L14 | SUH.3 L42 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Acetobacteroides* | 4.49 | 0.35 | 8.79 | 3.78 | 4.75 | 2.45 | 4.97 | 0.02 | 2.52 | 0.47 | 4.00 | 0.00 | 0.03 | 0.01 | 0.00 | 0.00 |
| *Actinoplanes* | 0.00 | 0.00 | 0.03 | 0.01 | 0.00 | 0.00 | 0.01 | 0.03 | 0.08 | 0.06 | 0.65 | 2.91 | 4.54 | 1.67 | 4.03 | 3.35 |
| *Anaerolineaceae_unclassified* | 7.57 | 1.09 | 2.27 | 6.44 | 0.64 | 6.77 | 0.88 | 0.17 | 0.55 | 0.42 | 0.24 | 0.01 | 0.00 | 0.05 | 0.00 | 0.01 |
| *Anaerolineaceae_uncultured* | 5.07 | 5.22 | 1.82 | 6.77 | 1.13 | 6.81 | 2.35 | 7.25 | 1.64 | 3.35 | 3.44 | 1.57 | 5.34 | 4.64 | 0.75 | 3.65 |
| *Arcobacter* | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Bacteria_unclassified* | 4.05 | 0.53 | 1.15 | 2.40 | 0.65 | 5.25 | 0.74 | 0.34 | 1.51 | 0.75 | 0.48 | 0.12 | 0.53 | 0.24 | 0.93 | 0.90 |
| *Bacteroidetes_unclassified* | 2.73 | 0.74 | 3.60 | 2.45 | 2.73 | 2.39 | 3.21 | 0.49 | 2.22 | 1.82 | 1.78 | 0.21 | 0.12 | 0.10 | 0.13 | 0.31 |
| *CL500-29_marine_group* | 0.01 | 0.77 | 0.04 | 0.06 | 0.04 | 0.05 | 0.17 | 0.88 | 0.08 | 0.53 | 0.14 | 1.49 | 0.48 | 0.82 | 1.10 | 0.67 |
| *Comamonadaceae_unclassified* | 1.83 | 2.02 | 4.93 | 2.12 | 8.86 | 3.01 | 7.32 | 2.02 | 6.18 | 2.82 | 9.17 | 4.37 | 4.14 | 2.53 | 6.64 | 3.51 |
| *Cyanobacteria_norank* | 2.87 | 0.57 | 2.44 | 0.64 | 0.70 | 0.36 | 1.26 | 0.11 | 5.61 | 0.61 | 0.88 | 1.18 | 1.77 | 1.25 | 0.95 | 0.46 |
| *Dechloromonas* | 8.55 | 4.65 | 7.17 | 4.60 | 4.97 | 3.10 | 4.62 | 0.58 | 7.78 | 6.54 | 5.90 | 0.05 | 0.01 | 0.02 | 0.03 | 0.07 |
| *Polynucleobacter* | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 |
| *Pseudomonas* | 0.04 | 0.03 | 0.32 | 0.06 | 0.40 | 0.10 | 0.75 | 0.17 | 0.62 | 0.03 | 0.17 | 0.08 | 0.61 | 0.11 | 0.85 | 0.17 |
| *Rhizobium* | 0.01 | 0.24 | 0.12 | 0.04 | 0.04 | 0.10 | 0.97 | 1.42 | 0.98 | 0.80 | 0.99 | 1.43 | 3.60 | 1.42 | 0.81 | 0.47 |
| *Rhodobacter* | 0.17 | 3.21 | 0.89 | 0.36 | 0.29 | 0.16 | 0.84 | 1.24 | 0.74 | 1.00 | 0.80 | 4.27 | 3.44 | 2.90 | 2.44 | 1.73 |
| *Rhodocyclaceae_unclassified* | 2.51 | 2.55 | 3.42 | 5.13 | 2.50 | 4.61 | 1.98 | 0.81 | 0.92 | 2.66 | 1.35 | 0.04 | 0.07 | 0.08 | 0.02 | 0.03 |
| *Sandaracinaceae_uncultured* | 0.01 | 1.13 | 0.14 | 2.89 | 0.93 | 3.52 | 5.34 | 15.55 | 7.33 | 14.75 | 7.54 | 23.13 | 8.71 | 11.19 | 1.54 | 3.66 |
| *Sediminibacterium* | 0.00 | 0.04 | 0.01 | 0.06 | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.06 | 0.02 | 0.08 | 0.12 | 0.09 | 0.21 | 0.12 |
| *Sphaerotilus* | 0.08 | 0.04 | 0.04 | 0.03 | 0.06 | 0.01 | 0.11 | 0.01 | 0.04 | 0.02 | 0.03 | 0.03 | 0.09 | 0.02 | 0.50 | 0.08 |
| *Sporichthyaceae_norank* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Sporichthyaceae_unclassified* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Tolumonas* | 0.28 | 0.00 | 0.18 | 0.02 | 0.22 | 0.04 | 0.13 | 0.00 | 0.22 | 0.01 | 0.02 | 0.02 | 0.00 | 0.00 | 0.16 | 0.02 |
| *Uliginosibacterium* | 0.78 | 0.02 | 1.12 | 0.16 | 1.09 | 0.19 | 1.04 | 0.03 | 1.98 | 0.15 | 1.13 | 1.74 | 1.26 | 0.04 | 1.34 | 0.05 |
| *hgcI_clade* | 0.00 | 0.01 | 0.00 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 |