The response of benthic communities and ecosystem functioning to the ecological restoration of freshwater streams

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

Ecological restoration of freshwater ecosystems is now being implemented around the world to prevent further damage and mitigate anthropogenic disruption. In many regions of the world including China, most emphasis is placed on assessing physico-chemical and hydromorphological properties in the monitoring of the restoration progress, but less is known about the structural integrity and ecosystem health of the restored ecosystems. In particular, little is known about how aquatic community and ecosystem function respond to river ecological restoration, if the restored rivers can persist/resist to future environment changes.

In this study, I used biofilm bacteria and macroinvertebrate as bioindicators, and applied ecosystem functioning leaf breakdown rates and ecosystem resilience as indicator of ecosystem health to assess the progress of ecological restoration in urban rivers in south China. By comparing the bacterial, macroinvertebrate community composition, and leaf breakdown rates in urban rivers undergoing ecological restoration with that in degraded urban rivers and rivers in forested areas (i.e., reference conditions), linking the community composition and leaf decomposition with abiotic and biotic factors through a field study, and comparing the biofilm bacterial community in intermittent streams with that in permanent streams under different habitat characteristic through an Ex-Stream experiment, I aimed to investigate: (i) how ecological restoration affected benthic biofilm bacterial community composition? (ii) the response of benthic macroinvertebrate communities to ecological restoration in urban rivers; (iii) the impact of stream ecological restoration on leaf litter decomposition; (iv) the contributing factors (i.e. abiotic or biotic) for the shifts in community composition and leaf decomposition; and (v) the resilience of restored stream to flow intermittent caused by anthropogenic disturbance and climate changes.

The field research results demonstrated a positive effect of ecological restoration on the structure and function of the previously degraded streams. A reduced bacterial diversity and an increased richness, diversity of macroinvertebrate and leaf breakdown rate were detected in rivers undergoing habitat restoration in contrast to previous degraded ones, while the bacterial and macroinvertebrate community composition in restored rivers differed from those in degraded rivers, which was developing towards that of the reference conditions (forested rivers). The turnover of these communities was mainly shaped by habitat characteristic (i.e. substrate diversity, water velocity) and water chemistry (i.e. nutrients and organic pollutants) in the surface water, habitat characteristics contributed to most of the variation in the macroinvertebrate community. All environmental and biotic factors evaluated contributed synergistically to the variance in leaf decomposition. The role of macroinvertebrates, mainly shredders appeared to be particularly important in leaf litter decomposition, followed by habitat characteristics (e.g. substrate diversity, water velocity), physico-chemical variables (e.g. nutrient and organic pollutants) and biofilm bacteria.

The mesocosm experiment demonstrated that both drying events and flow resumption induced a shift of bacterial community compositions. In medium-level habitat streams, the bacterial diversity and some type of microbial metabolism activities were recovered to comparable status with permanent ones after an increase in biodiversity and a reduction in chitin degradation under drying perturbation, though not comparable with the recover capacity with those in high heterogeneous habitat streams. Controversially, low-level habitat streams possessed greater bacterial diversity and lower microbial metabolism process even after flow resumption.

Our research indicates that ecological restoration provides rivers with greater habitat heterogeneity, which is an efficient approach to restore the aquatic community, enhance the health and resilience of river ecosystem for freshwater sustainable development. This study advances our understanding of the restoration process of aquatic community and ecosystem functioning, as well as the critical factors that attribute to these processes, which offers water managers an important guidance for future planning of ecological restoration and management strategies.

Keywords: ecological restoration, habitat, bacterial community, macroinvertebrate, ecosystem function, leaf breakdown, ecosystem resilience, urban river

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List of Publications

This thesis is based on the following publications, which are referred throughout the text:

I. **Lin QY**, Sekar R, Marrs RH, Zhang YX (2019) Effect of River Ecological Restoration on Biofilm Microbial Community Composition. Water 11, 6 https://doi.org/10.3390/w11061244 (Chapter 2)

II. **Lin QY**, Zhang YX, Marrs RH, Sekar R, Luo X, Wu NC (2020) Evaluating ecosystem functioning following river restoration: the role of hydromorphology, bacteria, and macroinvertebrates. Science of the Total Environment 743, 140583 https://doi.org/10.1016/j.scitotenv.2020.140583 (Chapter 4)

III. **Lin QY**, Zhang YX, Marrs RH, Sekar R, Luo X, Wu NC (2020) The effect of habitat restoration on macroinvertebrate communities in urban rivers. PeerJ *Revised version under review*. (Chapter 3)

IV. **Lin QY**, Zhang YX, Marrs RH, Sekar R, Luo X, Wu NC (2020) Resilience of stream biofilm bacterial communities to drying perturbation in stream ecosystems: The effect of habitat heterogeneity. *To be submitted*. (Chapter 5)

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Chapter 1 General introduction: river ecological restoration and assessment

River ecosystems are one of the most dynamic systems in the natural environment, with complex biotic interactions amongst plants, animals, and micro-organisms, as well as abiotic interactions (Angelier 2003). Healthy, self-sustaining river ecosystems provide important goods and services upon which human life depends (Postel & Richter 2003; Palmer et al. 2005), such as provision of clean water, food from aquatic organisms, transportation, electricity generation, and leisure (Wilson & Carpenter 1999; Jackson et al. 2001; Jansson et al. 2007). However, with the development of global urbanization, an increasing numbers of stream ecosystems are especially susceptible to degradation directly and indirectly worldwide (Jesús-Crespo & Ramírez 2011). By occupying the lower-lying portions of landscapes, riverine ecosystems are impacted by pollutants and excessive nutrients from agriculture, industry and domestic sources (Naiman et al. 2002). The hydrology of many rivers has also been altered by hydraulic engineering such as dams, weirs and water diversions for hydropower and other industrial purposes, irrigation and domestic uses (Jackson et al. 2001; Arthington & Pusey 2003; Dudgeon et al. 2006). Additionally, rivers and adjoining riparian zones have been transformed by wetland reclamation, dredging, canalization, and clearing of riparian zones (Malmqvist & Rundle 2002). River and land-use change activities have degraded the physical structure of habitats and floodplains (Beechie et al. 1994; Hohensinner et al. 2005), disrupt the fluxes of water, sediment, and nutrients (Ward et al. 1999; Syvitski et al. 2005), and contaminated water via loading of nutrients and pollutants (Tilman et al. 2001). These activities have greatly changed riverine ecosystems (Poff et al. 2007), dramatically altered the processes that drive ecosystem structure and function (Poff et al. 1997; Jansson et al. 2000), and reduce the value of ecosystem service provide to human society. With climate change and increasing human demands for water and land, stress on riverine ecosystems will be exacerbated (Barnett et al. 2005).

1.1 Ecological restoration of river ecosystems

To mitigate the effects of long-term degradation, there has been increasing interest in the ecological restoration of freshwater systems worldwide (Matthews et al. 2010), with restoration actions that primarily tackle the source of ecosystem degradation (Kondolf et al. 2006, Roni et al. 2008). Restoration is sometimes termed as rehabilitation, reclamation, or mitigation. Defined as 'the programme of assisting the recovery of a degraded/damaged ecosystems' (SER 2004), ecological restoration aims to recreate certain physical, chemical or biological conditions, or various combinations of the three (Nilsson et al. 2015), through a gradient of activities from creating new habitats, to mitigate for lost habitat, with the expectation of returning the aquatic ecosystem to their original, undisturbed state (Roni & Beechie 2013), or develop natural structures and functions in response to the new conditions (Palmer et al. 2005; Beechie et al. 2008; Nilsson et al. 2015). There are two main types of ecological restoration, active and passive. With active restoration, actions are taken to restore or improve degraded ecosystem conditions and processes, whereas, passive restoration focuses more on political management, for instance, using regulations, laws, land-use practices, habitat protections to prevent or eliminate the impact of anthropogenic disturbance on freshwater ecosystems, and allowing the natural recovery of degraded ecosystems following interference (Roni & Beechie 2013). Both restoration approaches form complementary actions for the recovery and protection of freshwater system, providing water managers with cost-effective strategies for the management of aquatic ecosystems.

1.2 Process-based ecological restoration: principle and restoration actions

In the early 21st century, most aquatic ecosystem restoration projects were implemented with a focus on physical structures rather than on ecological processes (Wortley et al. 2013). More recently, process-based restoration that focuses on correcting anthropogenic disruptions to driving processes, and hence leading to the recovery of habitats and biota, emerged as a new direction for ecological restoration (Beechie et al. 2012). Riverine ecosystems are controlled by a suite of hierarchically-nested physical, chemical, and biological processes operating at widely-varying spatial and temporal scales (Beechie & Bolton 1999; Beechie et al. 2010). Focusing on correcting anthropogenic disruptions to these processes, such that the river-floodplain ecosystem progresses along a recovery trajectory with minimal corrective intervention (Wohl et al. 2005), process-based restoration aims to re-establish normative rates and magnitudes of physical, chemical, and biological processes that create river and floodplain ecosystems and sustain the ecosystems over future perturbations (e.g., climate change, stochastic process; Beechie et al. 2010).

Process-based ecological restoration is directed by four fundamental principles (Beechie et al. 2010):

(1) targeting the root causes of habitat and ecosystem change;

(2) tailoring restoration actions to local potential;

(3) matching the scale of restoration to the scale of physical and biological processes;

(4) clearly determining about the expected outcomes, including recovery time.

These four principles guide the design of restoration strategies (Brierley et al. 2002; Beechie et al. 2010), for the purpose of restoring the dynamics of rivers, ensuring the effective of restoration actions over the long term, hence allowing freshwater ecosystem to respond smoothly to future perturbation (Roni & Beechie 2013).

The first principle guides us to identify disruptions to driving processes which help design appropriate restoration actions and identify the anticipated physical or biological conditions in a restored system. The second principle directs us to design restoration strategies and techniques in line with local physical and biological potential caused by human constraints. The third principle inspires us to clearly distinguish the precise scale of restoration (i.e. watershed-scale, reach-scale, or in-stream-scale), and set restoration solutions accordingly. The fourth principle guides us to form realistic expectations for potential restoration consequence and the time-frame demand (Pollock et al. 2007; Beechie et al. 2008b), which is important for designing monitoring programs.

1.3 Ecological restoration approaches and strategies

These four principles of process-based restoration can be applied to three classical restoration classes, full restoration, partial restoration (rehabilitation), and habitat creation or improvement (enhancement) (Beechie et al. 2010). Within each restoration class, numerous methods have been emerged along with the development of water management. Selecting appropriate techniques to restore particular habitat types or ecosystem process that address the cause of ecosystem degradation and meet the specific restoration objectives would help the sufficient restoration of degraded freshwater ecosystems.

1.3.1 Connectivity

The connectivity of stream habitats is essential for the flux of water, sediment, nutrients, organic matter, and the movement of aquatic biotas (Fulleton et al. 2010). Longitude connectivity (upstream-downstream connectivity) facilitates the sediment transport, material cycling, energy flow, and biota dispersal between upstream and downstream parts of the river (Hooke 2003; Roll et al. 2012); lateral connectivity (connection of rivers to the floodplain and riparian areas) supports two-way transfer of sediment, energy and various organisms between the main channel and hydraulic-linked aquatic habitat on the floodplain and along the riparian zone (Paetzold et al. 2006); vertical connectivity (connection to the hyporheic zone and subsurface area) eases the water exchange between the surface channel and sediment in the hyporheic zone, support the flux of nutrient and oxygenation process in the hyporheic zone (Datry & Larned 2008; Boulton et al. 2010).

Anthropogenic disturbances and climate change have impacted on these ecological processes interfering with the connectivity of rivers (Grill et al. 2019). For example, many rivers became segmented due to urbanization, creation of dams, weirs, pipeline crossings, bridges, culverts, road or stream crossings etc., interrupting the longitudinal transportation processes, and halting migration of aquatic biota (Roni & Beechie 2013). To solve these problems, common approaches in the restoration of longitude connectivity include the removal and modification of dams, culverts, stream-crossings, or the construction of fish passages (Roni & Beechie 2013), which help maintain and restore natural riverine processes thus creating and maintaining the living habitat of aquatic biota (Pess et al. 2005). Laterally, many channels have become isolated from their historic floodplains due to land-use changes, agricultural practices. Thereafter, restoring connections between the main channel and its floodplain, and hence include levee removal or setbacks. These approaches reconnect rivers with isolated floodplains, wetlands, sloughs, and other habitats. Together, they support the recovery of riverine functions, such as retention and natural exchange of water, wood, sediment and nutrients between a floodplain and mainstream, fine sediment deposition, channel migration, the development of a greater diversity of riparian conditions, seed dispersal, and a greater diversity of habitat types (Pess et al. 2005; Roni et al. 2008). The vertical connectivity ceased in many urban rivers because of lining the streambed with concrete, channel simplification, deposition of fine sediment over formerly permeable beds, and flow intermittent due to climate change and excessive water demands (Roni & Beechie 2013). Accordingly, this can be restored by excavating fine sediment, restoring channel complexity by increasing geomorphic complexity and sinuosity (Hester & Gooseff 2010; Lawrence et al. 2013), establishing a pool-riffle sequence (Kasahara & Hill 2006), installing boulders and large woody debris (LWD) in channelized rivers (Boulton 2007; Krause et al. 2014; Mayer et al. 2010). Removing impermeable channel lining (Bernhardt & Palmer 2007), repairing streamside riparian vegetation (Dosskey et al. 2010) will also promote instream hydraulic diversity which in turn promotes the vertical exchange of water, and hence restoring the biogeochemical transformation and microbial, invertebrates activities between the surface water and underground water (Boulton 2007).

1.3.2 Hydrology

Hydraulics, formed by the interaction between the discharge and the channel geometry, regulates the particles that are deposited into the stream, the forming of different habitats in different areas of streams, and subsequently controlling the physico-chemical condition, community composition of aquatic biota, and processes within stream ecosystems (Elosegi et al. 2019). Due to anthropogenic changes on water discharge, such as water abstraction by weirs or dams, water release from reservoirs, a flow is modified in affected streams (Elosegi et al. 2019).

Hydrological stressors, therefore, have a large impact on the flow pattern of streams, which threatens ecosystem quality, stream biodiversity (Nõges et al. 2016), and ecosystem processes across the world (Poff & Zimmerman 2010; Reich & Lake 2015). The effects of flow regime changes on the ecosystem structure and functioning are often aggravated when interacting with morphological degradation, and other factors, such as acidification, pollution or biological invasions (Turunen et al. 2016).

To remediate the impact of human activities on the hydrologic process, a number of approaches have been developed to restore the natural level of flow regime (Roni et al. 2013). Catchment retention and riparian restoration help restore natural hydrological processes by reducing material inputs (nutrients, pollutants, sediments) into streams (Reich & Lake 2015). Restoration of hydrological connectivity through barrier removal (i.e. of dams, weirs, culverts), flow regimes can be adjusted to support the reinstatement of in-stream habitat which is critical for the recovery of ecosystem structure and functioning (Paillex et al. 2013). Restoration of instream flows (i.e. restore base flows and flood pulses, reduce water withdrawal) in highly regulated streams (Roni et al. 2013; Reich & Lake 2015) helping to maintain aquatic and riparian habitat and production of aquatic ecosystem and biota (Arthington & Pusey 2003). It also serves as a basis for other restoration practice, including habitat improvement and riparian zone restoration (Roni et al. 2013).

1.3.3 Habitat restoration

Aquatic habitats including flood plain, in-stream habitat, wood supply and aquatic vegetation are crucial parts of the aquatic ecosystem. Healthy aquatic habitats not only form the basis habitat for the living of micro-organisms, aquatic flora (e.g. algae, aquatic plants) and fauna (macroinvertebrates, fishes), but also serve as important sites for reducing heavy pollutants (including nutrients, organic matter and heavy metals) by a series of ecological mechanisms, and supporting the ecological process and self-resilience of aquatic ecosystem.

Due to the importance of healthy aquatic habitat for improving water quality, supporting aquatic biota, and biota associated ecosystem function, habitat restoration forms a main part of ecological restoration approaches for channelized and simplified streams. Habitat improvement methods include instream habitat techniques (i.e. create riffles, pools and cover to improve habitat complexity; Roni et al. 2006), floodplain rebuilding (i.e. construct side channels, backwaters, off-channel ponds, and wetlands; Roni et al. 2005), reshaping the sinuosity or meandering of channels (Pess et al. 2005; Vought & Locoursiere 2010). These are important methodologies to recover the complexity of aquatic habitats, inducing relatively rapid improvement in habitat quality and quantity (Roni et al. 2008), enhancing organic matter retention and flow heterogeneity (Pretty et al. 2003), hence increasing aquatic biotas like fish within a few years (Roni et al. 2002, 2008).

1.3.4 Riparian zone restoration

Riparian areas are land-water transitional zones with distinguished biophysical gradients to link aquatic ecosystems to land through hydrology connection and subsidy flux exchanges of energy, materials, and nutrients (Gregory et al. 1991; Naiman & Décamps 1997). Riparian areas with distinctive vegetation are ecologically important life-support hotspots in many landscapes for maintaining habitat heterogeneity, biodiversity, productivity (Naiman et al. 2005), and ecosystem functioning (Decamps 2011). Through interactions between vegetation, animals, water, soil and people, riparian areas at watershed landscape provide essential functions for healthy streams and enhance the supply of freshwater resources by maintaining hydrologic cycles, increasing water filtration, purification, and erosion control services, storing and cycling nutrients, and minimizing fertilizer and pesticide runoff (Groffman et al. 2003). Riparian zones and stream ecosystems are often impacted by land use change, especially in urban areas (Walsh et al. 2005). In urban catchments, the capacity of riparian areas to reduce nutrient and contaminant loads to streams is limited by anthropogenic disturbance, such as clear-cutting of streamside vegetation, creation of large impervious areas, altered hydrology, and engineered channels. Increased nutrient and pollution from impervious catchment with reduced removal capability can induce stream eutrophication, and significantly impact aquatic ecosystems and biodiversity.

Ecological restoration projects aim at reversing the impacts of urbanization on stream riparian zones are focused on re-establishing riparian vegetation (Bernhardt et al. 2005, Palmer et al. 2005), by applying silviculture techniques such as seeding, planting, removal of trees, competing understory, or invasive plants (Pollock et al. 2005). Riparian restoration has become an increasing popular strategy to stabilize banks and block nutrient flows from adjacent field for reducing the downstream flux of nutrients in many urban streams (Craig et al. 2008; Jones & Swan 2016). In China, many cities have invested billions of RMB to riparian corridor restoration projects. Riparian management should enhance nutrient processing, restore ecological and geomorphologic integrity, and maintain riparian community functional traits to ensure ecosystem resilience to environmental change (Kominoski et al. 2013). It is reported that riparian biotas are impacted positively by restoration of European rivers through increasing species richness and abundance of riparian carabid beetles (Januschke & Verdonschot 2016), and increasing the connections in the riparian food web (Kupilas 2017).

1.3.5 Vegetation management

Aquatic plants, including submerged macrophytes, emergent macrophytes and phytoplankton are important part of freshwater ecosystems. Serving as primary producers, they not only provide food and nutrients for aquatic organisms, but regulate the material and energy cycling through aquatic ecosystems. Aquatic macrophytes influence the physicochemical condition and biological organisms in shallow waters (Carpenter & Lodge 1986) and increase the water transparency (Scheffer & Jeppesen 1998) through a number of mechanisms, such as reducing sediment suspension (Horppila & Nurminen 2003), removing pollutants efficiently (Zhang et al. 2016), and suppressing algal growth via competition for nutrients (Kosten et al. 2009), light and space (Zhang et al. 2015) and releasing allelochemicals (Nakai et al. 2000).

However, with the simplification of stream channels and the clearance of aquatic plants for navigation, the physico-chemical environment, structure and function of stream ecosystem are impacted severely. Biomanipulation, such as transplantation of submerged macrophytes, emergent macrophytes, or a combination of various aquatic plants as artificial forests have been widely applied as a useful method to restore eutrophic shallow waters (Yu et al. 2016). Biological control aiming at recovering the ecosystem health through food web restoration are effective ways to improve water quality and ecosystem structure (Søndergaard et al. 2008; Yu et al. 2016).

Insecticide concentrations in surface waters can be decreased dramatically by transplanting aquatic plants (Moore et al. 2011; Brogan & Relyea 2017). Submerged macrophytes provide a refuge for zooplankton (Lauridsen et al. 1996), benefitting foraging piscivorous fish by providing habitats (Casselman & Lewis 1996) and improved light conditions (Salonen & Engström-Öst 2010; Yu et al. 2016). By providing special niches for biofilms growth, submerged macrophytes also determine the community of microorganisms in biofilms (Zhang et al. 2016).

1.4 Monitoring and evaluation of river ecological restoration programme

1.4.1 Definition of M&E (monitoring and evaluation)

Monitoring and evaluation are the processes of gathering qualitative or quantitative data to identify programme progress, the changes in physical, chemical, or biological parameters towards project or programme objectives, and follow-up option in water resource management (Roni 2005; Roni et al. 2013). Common types of monitoring include baseline, status, trend monitoring in the prioritization process, implementation monitoring during the project implementation, and effectiveness and validation monitoring post restoration project (MacDonald et al. 1991; Roni 2005). M&E provide important evidence on restoration effectiveness, covering how restoration impact on physico-chemical and biological variables, which is essential to guide future water management efforts by understanding the individual and synergistic influence of restoration project. Hence, M&E is a key part of restoration process (Roni et al. 2013).

1.4.2 Purpose of M&E

Monitoring, evaluation and adaptive management play crucial roles in supporting effective river restoration (Bernhardt et al. 2007; Naiman et al. 2012). Because of the complexity and changing context in different river systems, most restoration measures are, in essence, experiments. Monitoring and evaluation are crucial to help researchers to identify what approaches have, and have not, been successful, determine which techniques are effective, worthwhile investments, and why. Evaluation of the restoration process not only monitors the progress of the restoration process, but the experience gained can be used as a basis to form more systematic and efficient restoration strategies (Zan et al. 2017) for adaptive management or future endeavors (Downs & Kondolf 2002).

1.4.3 How to M&E restoration actions/ component of M&E

Although monitoring and reporting on the results of stream restoration programmes have typically been executed poorly (Bernhardt et al. 2007), many evaluations do not definitively answer if the restoration has succeeded, hence monitoring and evaluation has become increasingly common. Many metrics have been adopted to monitor stream restoration, with the universal insight to understand the complexity of stream systems and their potential responses to restoration (Zan et al. 2017). River restoration involves changes to the physical, chemical, biological and hydrological components of the system (Speed et al. 2016). It is important to include both abiotic and biotic factors, including the structural and functional variables when evaluating the response of ecosystem condition to restoration activities in line with restoration objectives (Gessner & Chauvet 2002; Pascoal et al. 2005).

1.4.3.1 Abiotic factors (hydromorphology/ water chemistry)

Currently, water quality and hydromorphological aspects of study received most attention when monitoring restoration progress. Monitoring of habitat and water quality were applied widely throughout the United States since the introduction of monitoring guidelines by the Environmental Protection Agency (Larsen et al. 2004; USEPA 2004). Methods were also developed to test the water quality in rivers throughout the 20th century in European (Moss 2010). In China, water quality monitoring was introduced for aquatic restoration projects in the last few years (Qu & Fan 2010; Wang et al. 2016). The hydromorphology and habitat composition, i.e. the biological and functional variation, has been included in the methodology for monitoring restoration progress.

Generally, stream restoration should lead to an increase in habitat heterogeneity, an increase in water currency, an enhanced dissolved oxygen, and a reduction in nutrient and organic pollutants in previously contaminated rivers. By applying ecological engineering restoration in a non-point source polluted river, it was shown that there was a significant reduction in ammonium (NH_4-N), chemical oxygen demand (COD), and the five-day biochemical oxygen demand (BOD_5) (Mi et al. 2015). Lake restoration by biomanipulation were reported to decline the concentrations of total nitrogen (TN), total phosphorus (TP), total suspended solids (TSS), and chlorophyll *a* (Chl-*a*), while increased the water transparency (Yu et al. 2016). Habitat restoration led to a remarkable increase in dissolved oxygen (DO) and a reduction in total organic carbon (TOC) in the surface water of the restored River Zenne in Belgium (Atashgahi et al. 2015). However, the responses were not always consistent in streams undergoing different management approaches.

1.4.3.2 Ecosystem structure

Aquatic organisms are very useful for assessing the acute and chronic effects of pollution and environmental gradients (Loeb & Spacie 1994). Bioassessment, methods to evaluate the diversity of aquatic organisms (i.e. algae, invertebrates, fish), are increasingly developed and applied by water managers for monitoring water quality and ecosystem health (Dodds & Whiles 2010). Some studies have measured biological indicators (i.e. microbes, algae, invertebrates, and fish) to assess the structural integrity and ecosystem health (Frainer et al. 2017; Schmutz et al. 2016). Multi-metric indices, such as the index biotic integrity (IBI) have been used in recent researches to evaluate the restoration success (Zitek et al. 2008).

As the basis of the food web (Battin et al. 2016), biofilms in streams that includes bacteria, archaea, algae, fungi, protozoa and even metazoan are key sites of enzymatic activity, including nutrient and organic matter cycling, ecosystem respiration and primary production (Fischer et al. 2003; Romaní et al. 2008). Given the vital importance of biofilms in promoting river ecosystem process and functioning, as well as the sensitivity of biofilms to complex environmental challenges within short life cycle (Fechner et al. 2012; Cai et al. 2016), biofilms are considered as a good bioindicator of environmental health (Lear et al. 2012).

As a middle link of the food chain within river ecosystems, macroinvertebrate communities composed of species that tolerate a wide range of environmental conditions (Plafkin et al. 1989) play a key role in ecosystem processes such as organic material cycling and energy flow (Strayer 2006; Duan, Wang & Xu 2010). It is recognized as another biological indicator of water quality

(Hilsenhoff 1988), ecosystem health (Karr 1999), and restoration effectiveness (Besacier-Monbertrand et al. 2014), for the reason that they are available in most freshwater ecosystems; they are sensitive to environmental disturbance, deterioration, and improvement (Li et al. 2015); macroinvertebrates can reflect the relative long-term temporal and spatial changes of river ecosystems and can be used to predict future problems for their relative long lifecycles and weak migration ability (Shao et al. 2006; Dos et al. 2011). As primary producers and top predators, algae and fish serve as two main bioindicators of aquatic ecosystem health are also studied under multiple stressors and sometimes under stream management. Due to limited time of this research programme, these two indicators were excluded. Evaluating how aquatic organisms success and stimulate the integrity of river ecosystem will guide us with the basic knowledge of restoration mechanism that leading to the health of river ecosystems.

Although restoration should have a positive effect on aquatic communities with increasing habitat heterogeneity (Miller et al. 2010), observed changes have been inconsistent with the scale and specific metrics assessed (Palmer et al. 2010; Ernst et al. 2012). By reviewing 104 stream or river restoration projects that focus on enhancing habitat heterogeneity to restore the reach-scale biota, only two evaluated projects showed a significant recovery of macroinvertebrate biodiversity (Jähnig et al. 2010; Palmer et al. 2010). Habitat restoration by reintroducing coarse sediment (cobbles and boulders) and large wood to previously channelized rivers also showed a weak response for most species (i.e. macroinvertebrates, fish, riparian plants; Nilsson et al. 2015).

Among the reports generated based on stream restoration programmes, most monitoring reports were for one or two specific restoration techniques, few reports were results from the monitoring a combination of restoration approaches as a whole strategy. Furthermore, although more researches were conducted in U.S. and European countries than China, no consistent results were obtained when evaluating different biological indicators for the variance of stream condition applied for study, the recovery mechanisms of aquatic organisms in restored streams is still unclear. Hence, evaluating how aquatic biota reflect to stream ecological restoration, particularly in areas that have rarely been monitored, would hence advance our knowledge of the restoration process and restoration mechanisms.

1.4.3.3 Ecosystem function

Ecological function has been increasing applied to identify the ecological health of freshwater systems, particularly litter decomposition (Gessner & Chauvet 2002), and ecosystem metabolism (Young et al. 2008), although few studies have been performed to access the functional ecosystem response to freshwater management by examining processes such as primary production, ecosystem respiration (Niyogi et al. 2002; Colangelo 2007; Aldridge et al. 2009), or leaf litter decomposition (Wenger et al. 2009; Flores et al. 2011).

Organic matter breakdown has been proposed as a good indicator of ecosystem integrity (Pascoal et al. 2005; McKie & Malmqvist 2009), and an alternative measure of stream health (Young et al. 2008; Niyogi et al. 2013) for its importance in nutrient cycling and energy flow in freshwater ecosystem (McKie et al. 2006; Tiegs et al. 2019). Organic matter breakdown is controlled by both abiotic (i.e. hydromorphology, water chemistry) and abiotic factors (Pascoal & Cassio 2004), hence, restoration mediated changes in physico-chemical and biological factors would definitely influence the organic matter breakdown rate consequently. However, the relative importance of environmental and aquatic organisms on litter decomposition has rarely been studied in managed streams (Encalada et al. 2010).

1.5 Scope of this thesis

Monitoring and evaluation of restoration programmes is critical in increasing our knowledge of ecological restoration, indeed it should be a key component of the restoration process. Currently, most attention is placed on hydromorphological and water chemistry for monitoring restoration progress, although a few studies have included biological indicators such as measures of microbes (Coe et al. 2009), algae (Frainer et al. 2017), invertebrates (Verdonschot et al. 2016; Frainer et al. 2017), and fish (Haase et al. 2013; Schmutz et al. 2016). Yet, the responses of benthic community composition to ecological restoration approaches are varied and unclear. Little is known about the effect of habitat restoration on the ecosystem function, such as primary production, ecosystem respiration (Niyogi et al. 2002; Colangelo 2007), or leaf litter decomposition (Dangles et al. 2004; Wenger et al. 2009; Flores et al. 2011). Moreover, the resilience of freshwater ecosystems to future climate and anthropogenic disturbance following river ecological restoration has rarely been considered, particularly for restoration projects implemented in China.

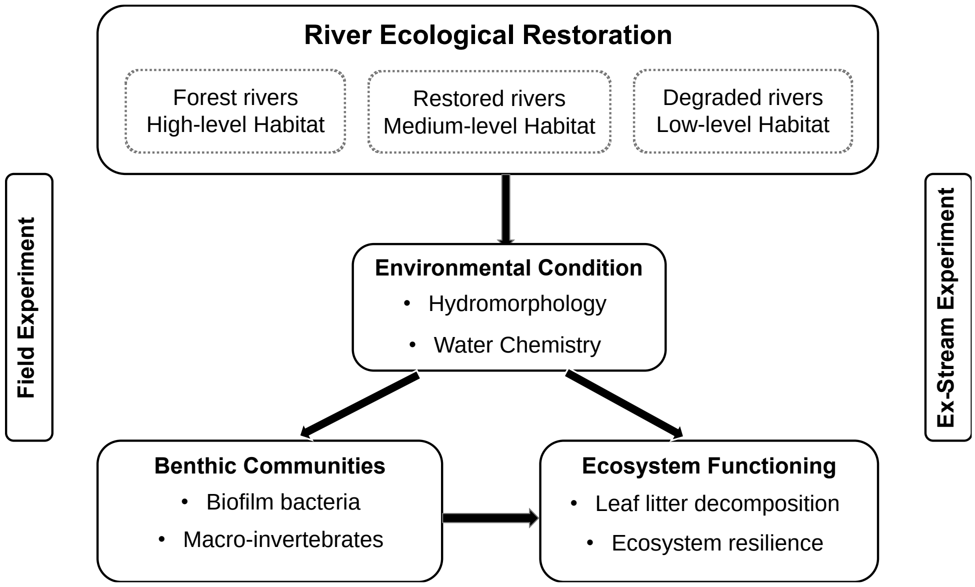

Figure 1.1 Conceptual model of this PhD programme. The research programme was performed by a field experiment and an Ex-Stream experiment. Generally, river ecological restoration induced the variance of hydromorphology conditions and water quality conditions, which in turn influenced the ecosystem structure (i.e. biofilm bacteria, macroinvertebrates) and ecosystem function (i.e. leaf litter decomposition, ecosystem resilience) of the river ecosystem.

In order to test the alteration of benthic ecosystem structures and ecosystem function by urbanization and habitat restoration, hence determine the extent to which river restoration can mitigate the urbanization impact, provide evidence for the development of efficient ecological restoration strategies for adaptive management and future endeavors, this research programme included biofilm bacteria, macroinvertebrates as bioindicators, and leaf litter decomposition and ecosystem resilience as indicators of ecosystem health. By comparing the bacterial, macroinvertebrate community composition, and leaf breakdown rates in urban restored rivers with that in degraded urban rivers and rivers in forested areas (i.e., reference conditions), linking the community composition and leaf decomposition with abiotic and biotic factors through a field study, and contrasting the biofilm bacteria community in intermittent streams with that in permanent streams under different habitat characteristic through an Ex-Stream experiment (Figure 1.1), this thesis tests the four research gaps identified in this introduction (Figure 1.2):

- *- The effect of river ecological restoration on biofilm microbial community composition (Chapter 2)*

  In this chapter, 16S rRNA genes targeted high-throughput Illumina Miseq sequencing was used to characterise the difference in biofilm bacterial communities in forest rivers (i.e. reference sites), urban degraded rivers and urban rivers undergoing habitat restoration from the same watershed, with the aim to determine the shift pattern of biofilm bacterial community and linked environmental variables in rivers following habitat restoration. The hypothesis tested was that habitat restoration would alter the biofilm bacterial community composition in the previous degraded rivers and shifted the bacterial community toward a near-natural state, due to an increase in dissolved oxygen, and a reduce in nutrients and organic pollutants in the restored rivers.

- *- The effect of habitat restoration on macroinvertebrate communities in urban rivers (Chapter 3)*

  In this chapter, the macroinvertebrate community composition was compared in three types of rivers within the same watershed, forest rivers (i.e. reference sites), urban degraded rivers and urban rivers undergoing habitat restoration. The aim was to determine how macroinvertebrate community composition and taxonomic diversity differed in restored rivers relative to degraded and reference sites, the environmental factors shaping macroinvertebrate communities across the three river types. The hypothesis tested was that habitat restoration would increase the macroinvertebrate richness, Shannon diversity, and improve the macroinvertebrate community composition by replacing dominant tolerant species with EPT (Ephemeroptera, Plecoptera, and Trichoptera) species which are sensitive to external disturbance. Substrate composition, water velocity and organic matter might be the major factors that leading the changes of the macroinvertebrate communities.

- - *Evaluating ecosystem function following river restoration: the role of hydromorphology, bacteria, and macroinvertebrates (Chapter 4)*

  In this chapter, leaf litter decomposition was used as an indicator of ecosystem integrity to assess the ecosystem function of restored rivers in China. By comparing the leaf breakdown rates in urban rivers undergoing habitat restoration with that in degraded urban rivers and rivers in forested areas (i.e. reference conditions), and linking the leaf decomposition to abiotic and biotic factors, the impact of habitat restoration on leaf litter decomposition could be measured, and the contributing factors that cause the variance in leaf litter breakdown rates assessed. The hypothesis tested was that stream habitat restoration would enhance the leaf breakdown rate due to the variance of both abiotic factors (i.e. enhanced substrate diversity, water velocity, dissolved oxygen) and biological factors (i.e. improved microbial and macroinvertebrate community composition and activity).

- - *Resilience of stream biofilm bacterial communities to drying perturbation in stream ecosystems: the effect of habitat heterogeneity (Chapter 5)*

  To understand the resilience of freshwater ecosystems, especially the restored rivers to future climate and human disturbance, an Ex-Stream experiment was conducted in Anhui Jiulongfeng Nature Reserve to investigate the resilience of aquatic community structure to different flows (intermittent/ drying perturbations) in streams with different habitats, using benthic biofilm bacteria as bioindicators. With the aim of assessing the shift pattern of benthic bacterial community composition under flow intermittence, and the resilience of benthic bacterial community to drying condition in streams of different habitats. The hypothesis tested was that heterogeneity habitat in restored rivers would support more diverse benthic bacteria and promote specialization of organisms by providing various hydrology condition, a mosaic of habitat patches. In addition, stream with more heterogeneity habitat would provide numbers of strategies and refuges for living organisms encountering drying conditions, possessing greater resilience than streams with low-level habitat.

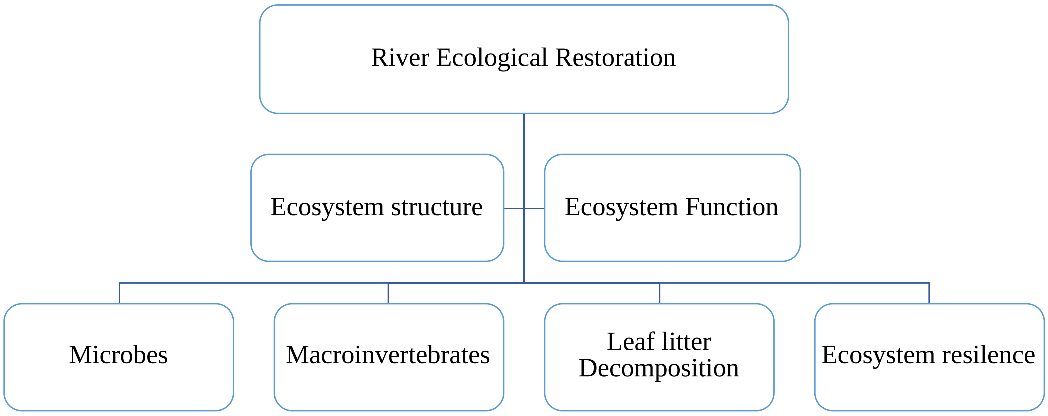

Figure 1.2 Flowchart of this PhD thesis. This research aims to determine how river ecological restoration influences the ecosystem structure (i.e. biofilm microbes, macroinvertebrates) and ecosystem function (i.e. leaf litter decomposition, ecosystem resilience) of the river ecosystem.

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Chapter 2 Effect of river ecological restoration on biofilm microbial community composition

2.1 Abstract

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

Keywords: bacterial community; biofilm; Illumina Miseq sequencing; habitat restoration; river ecosystem

2.2 Introduction

One of the current aims in riverine ecology is to use ecological restoration techniques to improve the quality of river ecosystem health, especially in urban areas where rivers have often been degraded severely (Bernhardt et al. 2007). Degraded rivers are normally formed by water pollution, land reclamation, dredging, channelization, altered hydrology and the clearing of riparian zones (Malmqvist & Rundle 2002; Naiman et al. 2003). Ecological restoration approach aims to recover river habitat quality by increasing river habitat complexity and heterogeneity; this is achieved by reconfiguring the river channel, increasing flood plain areas, adding in-stream islands, and aquatic vegetation (Bernhardt et al. 2007); all designed to enhance the hydraulic and substrate heterogeneity and macrophyte colonization. In combination, these treatments should increase food availability within the ecosystem (Laasonen et al. 1998; Lepori et al. 2005), and eventually, a complexity of aquatic habitats (e.g., riffle, run, pool, and debris dam classifications) will develop in these restored rivers (Miller et al. 2010).

Healthy river habitats not only allow the living micro-organisms, aquatic flora (e.g., algae, aquatic plants) and fauna (e.g., macroinvertebrates, fishes) to persist, but they can also provide important ecosystems services, for example by reducing pollutants, such as organic matter, nutrients and heavy metals (Palmer et al. 2014). Riverine habitats are known to influence the diversity and composition of aquatic biotas through river morphology, hydrology, sedimentation, and by changing environmental variables at the reach scale, the latter important for larger stream organisms such as fish and macroinvertebrates (Kail et al. 2015). For example, the surface features of the stream may influence detritus accumulation (Douglas & Lake 1994), and hence form ‘refuges’ for predators (Palmer et al. 1996; Lake 2000). Moreover, the habitat complexity generated by surface irregularities exerts a significant impact on the abundance and diversity of benthic invertebrates in stream systems (Miller et al. 2010; Louhi et al. 2011; Simaika et al. 2015; Flores et al. 2017). In a meta-analysis, in-stream habitat heterogeneity restoration (including wood, boulder additions and channel reconfigurations) enhanced macroinvertebrate richness (Miller et al.

2010). Nuttle et al. (2017) also found that cutting gates, restoring substrates, and enhancing in-stream and riparian habitats, significantly enhanced (i) the taxon richness of macroinvertebrates, and (ii) the richness and abundance of fish in 18 mitigation sites (Nuttle et al. 2017). In spite of this, very little is known about the effects of river habitat restoration on the composition of biofilm microbial communities.

Biofilms are a complex assemblage of microbial communities composed of bacteria, archaea, fungi, algae, and exopolysaccharides produced by the microorganisms. They are important components of stream ecosystems and are considered a good bioindicator of environmental health (Lear et al. 2012), not only because of their high abundance in most natural environments but also because of their sensitivity to environmental changes with short life cycle. Biofilms are a basic component of freshwater food webs; they adhere to the surfaces of rock particles and aquatic plants and are influenced by many environmental factors including temperature, light, shear forces, nutrients and contaminants (Gantzer et al. 1991; Lawrence et al. 2004; Lear et al. 2008). They fix energy and carbon by photosynthesis and chemosynthesis and some can also fix nitrogen (Battin et al. 2016). They also recycle organic nitrogen, impact on dissolved organic matter, and play key roles in nutrient cycling, organic compound degradation, water quality remediation and suspended sediment removal (Fischer et al. 2003). Effectively, altering any environmental factor can affect stream biofilm communities, and this may, in turn, alter their function of the whole stream ecosystem (Sheldon & Walker 1997). Bacteria are an indispensable part of the epilithic biofilm, usually occupying 1%–5% of the epilithic biofilm, and playing key roles in nutrient cycling, metabolic processes and many other biogeochemical processes and ecosystem functions (Cotner & Biddanda 2002; Battin et al. 2003; Zeglin 2015). The rates of bacterial-mediated nitrification, denitrification, and heterotrophic nitrogen (N) uptake in small streams have been shown to affect downstream water quality (Zeglin 2015; Valett et al. 2008; Mulholland et al. 2008). However, the impact of habitat restoration on biofilm bacterial community composition is still unclear.

To address this lack of information about biofilms during riverine restoration, I compared microbial populations in three different river types along a disturbance gradient. The most disturbed sites in this study were in urban areas, and the least disturbed sites were in forested catchments. In between, were rivers in urban areas where the habitat had been restored within the last seven years as part of an ecological restoration strategy. I measured a range of environmental factors and assessed the microbial community using a standardized field procedure followed by 16S rRNA Illumina MiSeq. Through comparing the relationship among habitat status, environmental parameters and bacterial community composition, I aimed to determine: (i) the biofilm bacterial community composition affected by habitat restoration (ii) the difference in bacterial diversity in restored rivers and urban degraded rivers, and (iii) any correlations between bacterial community composition and selected environmental variables. It is hypothesized that habitat restoration would alter the biofilm bacterial community composition in these restored rivers compared to the degraded ones and that they would become similar to the reference forest rivers. The bacterial diversity would be shifted toward a near-natural state where habitat had been restored. The substrate composition and physico-chemical variables like dissolved oxygen, nutrients and organic pollutants might be leading factors affecting the bacterial community composition in river groups.

2.3 Materials and methods

2.3.1 Study sites

This study compared three stream types in the winter of 2017: (i) degraded rivers in urban areas, (ii) restored rivers, where an aquatic habitat restoration scheme had been implemented within the last seven years for each river; (iii) rivers in forested catchments as reference conditions. Nine streams with similar-sized watersheds within the Anji City Region, Zhejiang Province PRC were selected for this study (Figure 2.1, Supplementary material Table S2.1). There were three replicates of each stream type, all located in different places in Anji City. The average day/night temperatures of the region were 12 °C/5 °C in winter, and average precipitation of 50 mm.

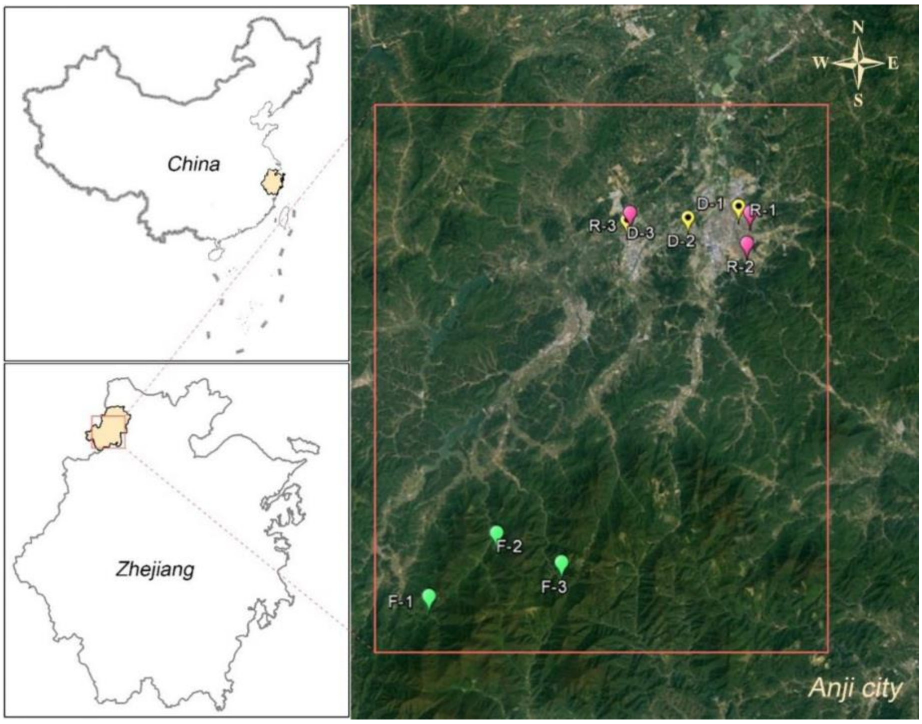

Figure 2.1 Location of the sampling sites within the Anji City Region, People's Republic of China (PRC), containing three degraded urban rivers (D), three restored rivers (R) and three forested rivers (F). The three forest streams (F) were upstream from Anji City; the three restored rivers (R) and the three degraded urban rivers (D) were downstream of the forest ones.

The three urban degraded sites (denoted D) were similar to the pre-restoration status of our restored rivers, Tongxin River is located in the city center, and the other two are located in the suburban districts. The three restored rivers (denoted R) have been restored for up to seven years using a mixture of ecological restoration techniques to reconstruct a natural river form. The techniques used included channel re-meandering, creation of riffles, pools and run areas, construction of floating islands, aquatic plant re-introduction, and riparian zone afforestation. A subsidiary aim was to provide ecosystems that could be used for ecological research, education and entertainment. Three forest streams (denoted F) were in the Tianmu Mountains (maximum elevation 590 m), 40-km upstream from Anji City were set as our "reference" conditions because pristine rivers were not available in the city area. There has been relatively little human interference on these forest streams, and they represent pre-urban landscape form where the urban rivers have derived (Violin et al. 2011).

2.3.2 Habitat survey and physico-chemical parameters of stream water

Habitat surveys were performed in December 2017 and January 2018. Reach canopy cover was estimated visually and the presence of various mesohabitat counted (island, pool, riffle). To estimate the variation of sediment grain size within each reach studied, 100 sediment particles were selected randomly on the river bed and proportions of boulders (>256 mm in diameter), cobbles (64–256 mm), pebbles (4–64 mm) and sand grains (2–4 mm) were counted (Kondolf 1997). The substrate diversity was calculated using the percentage cover of all substrate classes using the Shannon diversity index H' (Shannon 1997) for each study site.

Thereafter, within each river, the river width was measured using a 100 m tape. Water velocity and river depth were measured at five evenly-spaced points across the channel using Teledyne flow meters (ISCO, Lincoln, NE, USA) and a steel ruler. Water quality in each river was monitored at three different points with 3 m interval at the maximum by *in situ* measurements of temperature, pH, both using a HACH pH/temperature meter (LA-pH 10, HACH, Loveland, CO, USA), dissolved oxygen (DO), using a YSI Professional Plus probe (YSI Pro Plus, YSI, Yellow Springs, OH, USA), and turbidity, using a turbidity meter (DR2100Q, HACH, Loveland, CO, USA). One liter of water sample was collected from each stream and filtered in the field through 0.45 μm Jingteng syringe tip filters and preserved at 4 °C before sending to the laboratory. These water samples were analyzed within 48 h for (i) total nitrogen (TN) and total organic carbon (TOC), measured using a total organic carbon analyzer with a total nitrogen module (Multi N/C3100, Analytik Jena, Jena, Germany), (ii) ammonium nitrogen (NH_4-N), nitrate-nitrogen (NO_3-N), and total phosphorus (TP), measured using a QuickChem® Flow Injection Analysis system (Lachat Instrument, HACH, Loveland, CO, USA), and (iii) chemical oxygen demand (COD), measured using a DR1010 COD analyzer (HACH, Loveland, CO, USA).

2.3.3 Biofilm sampling procedure

Biofilm was sampled by placing four 10 cm × 10 cm autoclaved unglazed tiles, at 0.3 m water depth in each river for 39 days; thereafter the biofilms were collected by scraping the accumulated materials from the tiles into 50 mL tubes covered with aluminum foil, and transported in a cool box to the laboratory. The material in each 50 mL tube was then separated into two, one part was filtered through 0.45 μm membrane filter (Jingteng) to measure chlorophyll *a* (Chl-*a*) using a fluorimeter (10AU, Turner Designs, Sunnyvale, CA, USA) after acetone extraction (Elizabeth & Arar 1997), and the other part was filtered on 0.22 μm pore size polycarbonate membrane filters (Millipore, MA, USA) using a vacuum pump; these filtrates were stored in sterile Petri dishes at −20 °C until DNA extraction.

2.3.4 DNA extraction and analysis of bacterial community composition

The genomic DNA of all the biofilm samples was extracted using DNA extraction Kit (MO BIO PowerBiofilm® DNA Isolation Kit, MO BIO Laboratories, Carlsbad, CA, USA) based on a standard protocol. The DNA concentration was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the ratio of absorbance at 260 nm and 280 nm was checked to ensure the quality of DNA obtained. All DNA samples were then preserved at −80 °C before processing for bacterial community analysis.

The bacterial diversity and community composition of all biofilm samples were measured using the Illumina Miseq sequencing at Suzhou Genewiz Company. Using 30–50 ng DNA as the template, the 16S rRNA genes covering the V3-V4 regions were first amplified from the DNA extracts using the forward primer 347F "CCTACGGRRBGCASCAGKVRVGAAT", and the reverse primer 802R "GGACTACNVGGGTWTCTAATCC". PCR amplification was conducted in triplicate for each sample using 25 μL PCR reactions mixture containing 2.5 μL TransStart Buffer, 2 μL dNTPs, 2 μL of each primer, 0.2 μL BSA, 0.4 μL FastPfu DNA polymerase, 20 ng DNA template and ddH_2O. PCR was performed using the following conditions: initial denaturation at 95 °C for 3 min, 24 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 90 s, and extension at 72 °C for 10 s. The PCR amplicons were checked by 2% agarose gel electrophoresis and purified using MagPure Gel Pure DNA Mini Kit (Magen, Guangzhou, China). The purified amplicons were pooled and paired-end sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) at a read length of 2 × 300 bp.

After 16S rRNA sequencing, the reads were sorted to the samples according to barcodes, and the barcodes and primers were then removed. The low-quality reads were discarded, including the reads which did not exactly match the primer, the reads containing ambiguous character (N), a sequence length <200 bp, and reads with an average quality score <20. Then, chimeric sequences were detected and removed by comparing the sequences with the reference database (RDP Gold database) (Wang et al. 2007) using UCHIME algorithm (Edgar et al. 2011). The high-quality sequences were clustered into operational taxonomic units (OTUs) using the clustering program VSEARCH9 (1.9.6) against the Silva 128 16S rRNA database with 97% sequence identity threshold. The Ribosomal Database Program (RDP) classifier was used to assign a taxonomic category to all OTUs at a confidence threshold of 0.8. The 16S rRNA gene sequences were submitted to the National Centre for Biotechnological Information (NCBI) Sequence Read Archive database under the accession numbers MH889163-MH890450.

2.3.5 Statistical analysis

I evaluated differences in habitat characteristics, physico-chemical features, bacterial diversity and richness in different stream types (forest, urban restored and degraded) using one-way analysis of variance (Torres-Mellado et al. 2012), followed by the Tukey's HSD post-hoc test for comparison of means. Pearson correlation coefficients were used to explore relationships between environmental parameters and all microbial variables. Differences were accepted as significant at the $p = 0.05$ level. These statistical analyses were performed in the R statistical environment (R Core Team 2017).

Based on the results of the operational taxonomic units (OTUs) analysis, α-diversity indices (Shannon-Weiner index; Chao1 richness) were calculated in QIIME1.9.1 (Wang et al. 2018). Non-metric multi-dimensional scaling (NMDS) plot was performed to display β-diversity based on Euclidean dissimilarities between each samples using the 'vegan' package (Oksanen et al. 2018) within the R statistical Environment (R Core Team 2017). Analysis of similarities (ANOSIM) was then performed to evaluate the bacterial community similarity among three river types using the vegan package. Venn diagrams were drawn to analyze overlapped and unique OTUs of each sample based on cluster analysis of OTUs. Metastats (White et al. 2009) was performed to detect the differentially abundant taxonomic groups at phylum and genus levels between different river types. The relationships between the bacterial community and environmental parameters (pH, turbidity, DO, TN, TP, TOC, NH_4-N, NO_3-N and COD) were assessed using redundancy analysis (RDA) within Canoco 4.5 for Windows (Ter Braak 1988).

2.4 Results

2.4.1 Habitat characteristics

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

2.4.2 Effects of habitat restoration on physico-chemical properties of stream water

Physico-chemical values (Table 2.1) revealed no significant differences among river types for river width ($F_{2,6} = 0.336$) and mean depth ($F_{2,6} = 0.791$), and no difference in the surface water for pH ($F_{2,6} = 1.815$), NH_4-N ($F_{2,6} = 1.533$), NO_3-N ($F_{2,6} = 0.374$), TN ($F_{2,6} = 2.708$), TP ($F_{2,6} = 0.042$) and COD ($F_{2,6} = 5.069$). However, significant differences were observed in surface water properties among the stream types for DO ($F_{2,6} = 7.398$, $p = 0.024$), turbidity ($F_{2,6} = 7.69$, $p = 0.022$), TOC ($F_{2,6} = 17.86$, $p = 0.003$) and Chl-*a* ($F_{2,6} = 8.94$, $p = 0.016$). The forest and restored rivers had similar concentrations of DO, and both had significantly greater DO concentrations than the degraded rivers ($p < 0.05$) (Figure 2.2A). The turbidity in degraded rivers was much greater than forest rivers ($p = 0.018$), while no differences were observed between forest rivers and restored rivers, restored rivers and degraded rivers ($p > 0.1$) (Figure 2.2B). Degraded rivers and restored rivers had greater TOC concentrations than forest rivers ($p = 0.002$ and $p = 0.027$, respectively). Although no significant difference was detected when comparing restored rivers with degraded rivers ($p > 0.1$), a reduction in TOC concentration was observed (Figure 2.2C). In terms of Chl-*a*, no differences were detected when comparing forest rivers with restored rivers and degraded rivers ($p > 0.1$), whereas rivers under restoration had a much higher Chl-*a* concentration than degraded rivers ($p = 0.013$) (Figure 2.2D).

Table 2.1 Mean values of physico-chemical variables in different types of rivers within the Anji City Region, PRC. The values represent the mean ± standard error of three replicate samples.

River Type	Width (m)	Mean Depth (cm)	Dissolved Oxygen (mg/L)	pH	Turbidity	NH_4-N (mg/L)	NO_3-N (mg/L)	TN (mg/L)	TP (mg/L)	Chemical Oxygen Demand (mg/L)	Total Organic C (mg/L)	Chlorophyll *a* (mg/L)
Forest	8.83 ± 1.64	35.87 ± 7.97	14.16 ± 0.80	7.33 ± 0.11	0.62 ± 0.14	0.02 ± 0.01	1.06 ± 0.13	1.99 ± 0.21	0.18 ± 0.02	2.44 ± 0.15	0.48 ± 0.16	0.61 ± 0.23
Restored	13.17 ± 3.09	28.13 ± 7.22	13.14 ± 0.65	7.64 ± 0.14	3.52 ± 0.85	0.08 ± 0.02	1.13 ± 0.40	2.74 ± 0.77	0.17 ± 0.02	3.35 ± 0.76	2.81 ± 0.32	1.22 ± 0.19
Degraded	11.57 ± 5.72	22.87 ± 3.86	7.91 ± 1.52	7.38 ± 0.11	22.81 ± 14.93	1.37 ± 1.19	0.79 ± 0.40	4.01 ± 0.76	0.18 ± 0.05	8.82 ± 3.40	6.70 ± 2.21	0.20 ± 0.09

Table 2.2 Mean values of microbial diversity in different types of rivers within the Anji City Region, PRC. The values represent the mean ± standard error of three replicate samples.

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

2.4.3 Effects of habitat restoration on bacterial community composition

A total of 3,300,566 reads were obtained from the 27 samples. After filtering, denoising, and chimera removal, 1,650,283 high-quality 16S rRNA gene-reads were obtained, ranging from 48,473 to 69,662 reads per sample. Mean OTUs and α-diversity values (Table 2.2) showed that bacterial diversity measured by Shannon diversity index (H') was different between the river types ($F_{2,6}$ = 14.067, p = 0.005), being significantly greater in degraded rivers ($F_{2,6}$ = 6.98, p = 0.004) than restored rivers, whereas no distinct difference was found between restored rivers and forest rivers with respect to bacterial diversity (Figure 2.2F). Bacterial richness (Chao 1 Index) varied from 629 to 874, however, no significant differences were detected among river types for bacterial richness (Figure 2.2E).

The NMDS analysis produced a stress value <0.094, indicating that the ordination produced a good summary of the observed distances between samples with obvious clustering (Figure 2.3). The bacterial community structures among all three river types were distinct from each other (R = 0.508, p = 0.001) as shown by analysis of similarities (ANOSIM) (Table 2.3). Although there was some overlap between restored and degraded rivers, the bacterial community composition was significantly different (R = 0.256, p = 0.008) and there was a clear shift in bacterial community composition along the first axes from degraded to restored rivers, and from restored to forest rivers.

Table 2.3 Analysis of similarities (ANOSIM) of biofilm bacterial communities in contrasting river types within the Anji City Region, PRC.

River-Type Comparison	ANOSIM	
	R	*p*
Forest vs. Degraded	0.645	0.001
Forest vs. Restored	0.733	0.001
Restored vs. Degraded	0.256	0.008

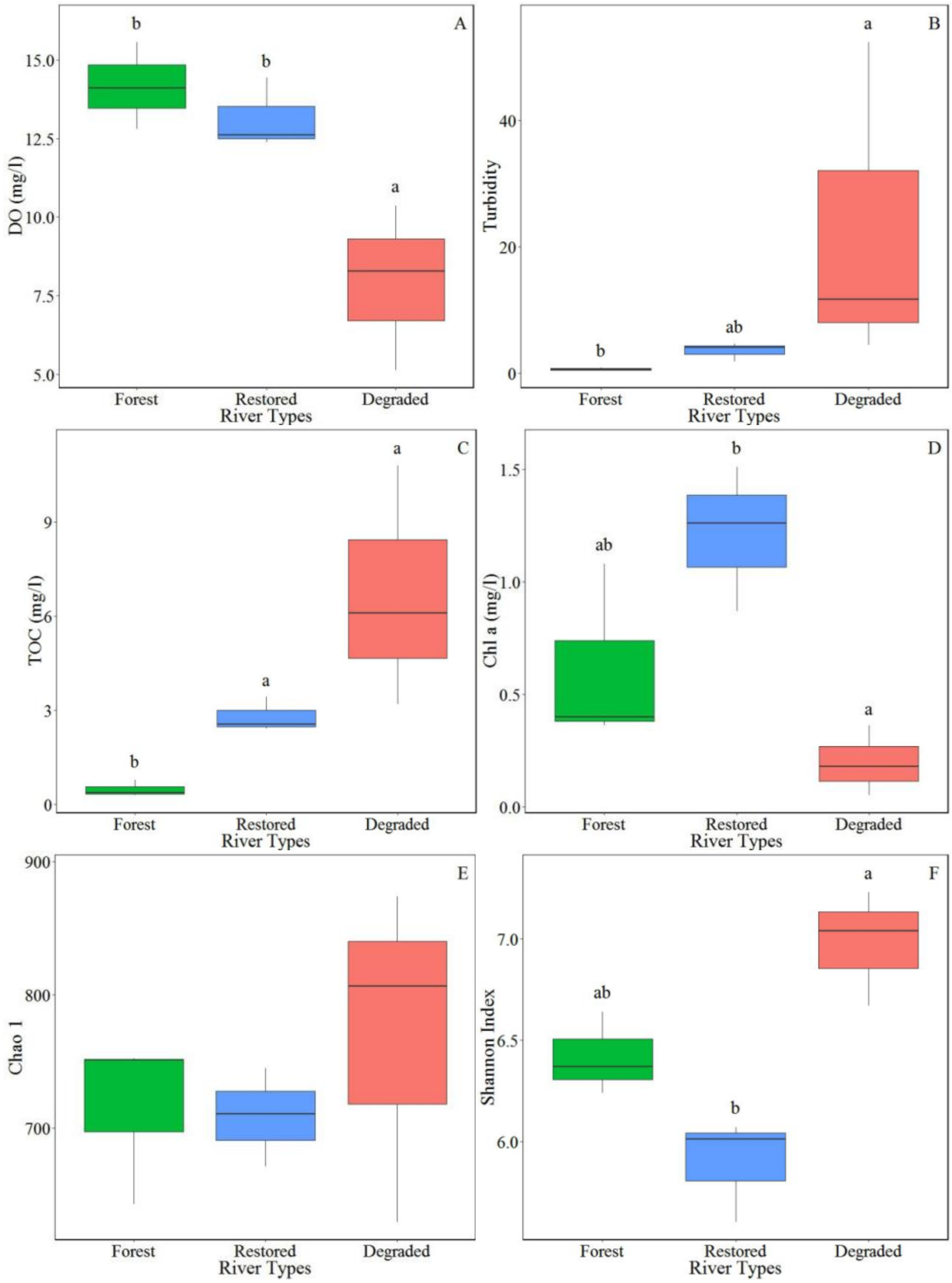

Figure 2.2 Boxplots representing the variance of physico-chemical parameters (A) dissolved oxygen (DO), (B) turbidity, (C) total organic carbon (TOC), (D) Chl-*a* and bacterial α-diversity (E) bacterial richness (Chao 1 Index), (F) bacterial diversity (Shannon Index) in forested, restored and degraded rivers within the Anji City Region, PRC. Black line: median value; box: quartile interval; whiskers: minimum and maximum value. Different lowercase letters indicate the significant difference observed at the p = 0.05 level.

In total, 383 OTUs were detected, 232 OTUs (61%) of which were universally present from biofilms in all rivers, and the three types of rivers contained 11.5% (forested), 4% (restored) and 23% (degraded) unique OTUs, respectively (Figure 2.4). The degraded rivers had a greater percentage of unique OTUs, including the members of the orders

Rhodocyclales, Cytophagales, Sphingobacteriales, however, no statistical differences were detected among river types for unique OTUs ($F_{2,6} = 2.81$).

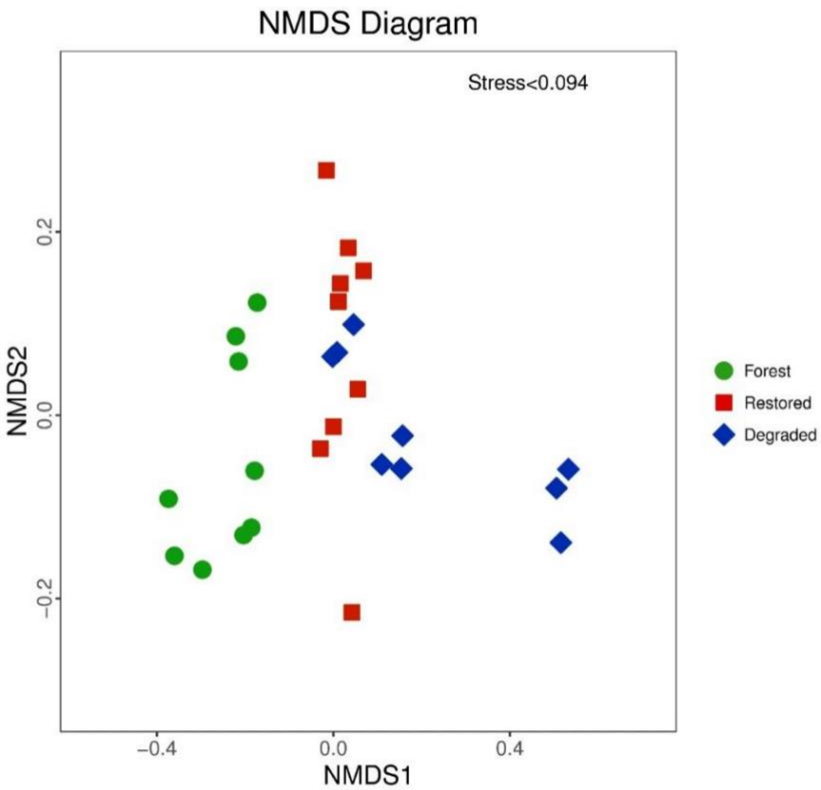

Figure 2.3 Non-metric multi-dimensional scaling (NMDS, stress < 0.094) ordination of biofilm bacterial communities in forested, restored and degraded rivers within the Anji City Region, PRC within the Anji City Region, PRC.

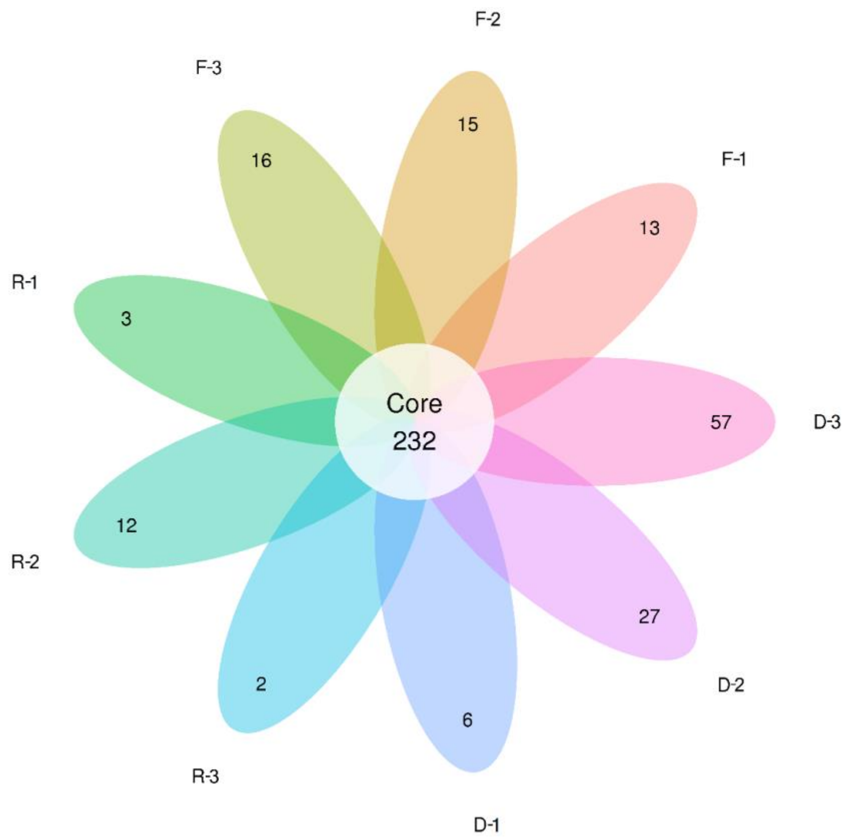

Figure 2.4 Venn diagram showing the number of unique and shared operational taxonomic units (OTUs) among biofilms in forested (F), restored (R) and degraded (D) rivers within the Anji City Region, PRC.

The relative abundance of the bacterial community was calculated respectively both at the phylum and genus levels. At the phylum level (Figure 2.5A), Proteobacteria was the most abundant phylum in all rivers, followed by Bacteroidetes, Firmicutes, Cyanobacteria, Verrucomicrobia, Acidobacteria and Actinobacteria. Rivers in the forest and after restoration had a greater Proteobacteria abundance than degraded rivers (p = 0.050, p = 0.049, respectively), while no difference was detected between forest and restored rivers ($p > 0.05$). The relative abundance of bacteria in the phylum Bacteroidetes, a taxa commonly assumed to be specialized in degrading high molecular weight (HMW) compounds (Fernandez-Gomez et al. 2013), was slightly greater in degraded rivers than forest rivers (p = 0.064), while, no differences of Bacteroidetes were observed when comparing forest rivers with restored rivers, and restored rivers with degraded rivers ($p > 0.01$).

In terms of relative abundance at the genus level, *Flavobacterium*, *Duganella*, *Pseudomonas*, *Undibacterium* and *Arenimonas* were commonly distributed in all studied rivers (Figure 2.5B). Degraded rivers showed significant numbers of reads allocated to *Flavobacterium* (p = 0.001), *Arenimonas* (p = 0.026) and *Acinetobacter* (p = 0.001). Forest rivers had a higher relative abundance of *Duganella* (p = 0.022), *Indobacter* (p = 0.010), *Clostridium_sensu_stricto_13* (p = 0.006), *Methylotenera* (p = 0.001) and *Rhodoferax* (p = 0.007) than degraded rivers. Among restored rivers, a greater relative abundance of *Flavobacterium*, *Pseudomonas*, *Acinetobacter* and a lower relative abundance of *Indobacter*, *Clostridium_sensu_stricto_13*, *Methylotenera* and *Rhodoferax* ($p < 0.05$) was found when comparing restored rivers with forest rivers. Restored rivers had a greater relative abundance of *Duganella* (p = 0.023) than degraded rivers. No difference in genus abundance was found between restored and degraded rivers for other taxa.

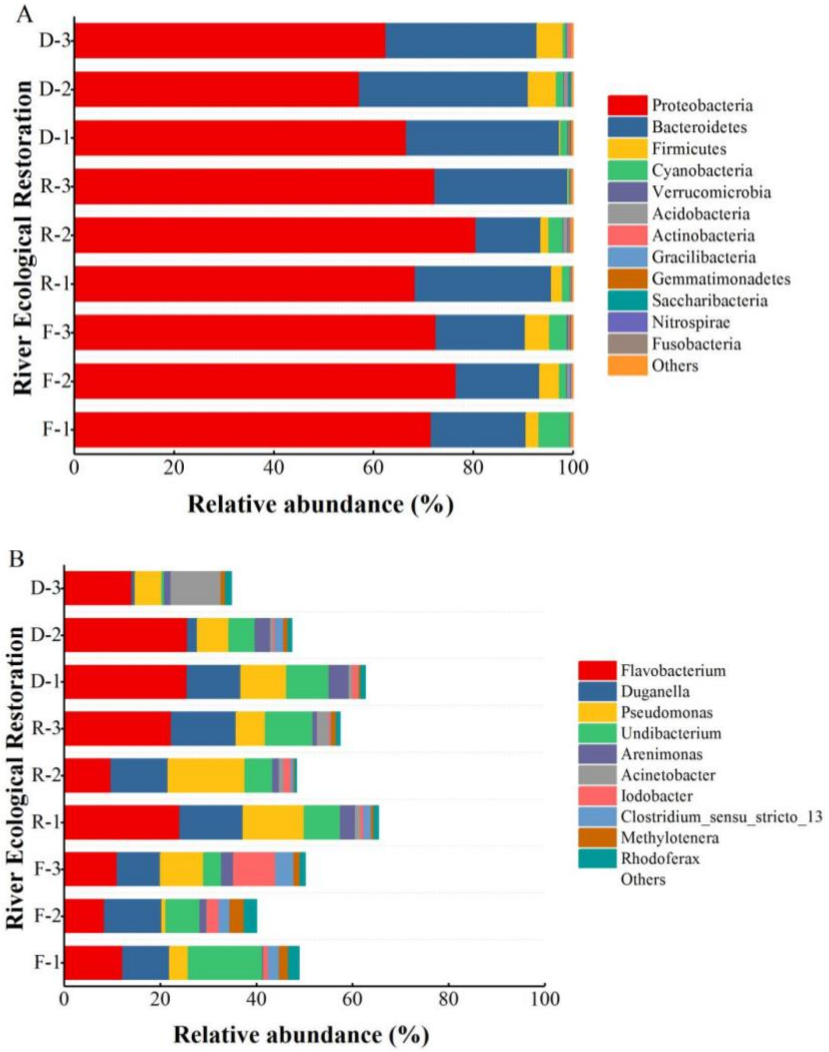

Figure 2.5 Relative abundance of bacterial community at Phylum (A) and Genus level (B) in forested (F), restored (R) and degraded (D) rivers within the Anji City Region.

2.4.4 Correlation between bacterial community composition and environmental variables

Bacterial richness (OTUs) showed a positive correlation with water turbidity and a negative correlation with TP concentration (p = 0.049, p = 0.032, respectively). Bacterial diversity showed a strong positive correlation with water turbidity (p = 0.006), COD (p = 0.023), and TOC concentration (p = 0.019), and was negatively correlated with substrate diversity (p = 0.033). The relationship between environmental parameters and the total bacterial community composition was further evaluated by constrained redundancy analysis (RDA), which produced eigenvalues for the first two axes of 0.322 and 0.159, respectively (Figure 2.6). The environmental variables explained 48.1% of bacterial community structure variance. The biofilm bacterial assemblages in forest rivers were positively correlated with substrate diversity (r = 0.156), and Chl-*a* concentrations (r = 0.828), and were negatively affected by NH_4-N (r = −0.621) and COD (r = −0.629) of surface water. The reverse pattern was found for biofilms in the degraded rivers, COD (r = 0.999), TOC (r = 0.984), NH_4-N (r = 0.738) and TN (r = 0.635) in the surface water presented as major factors linking to the bacterial structure in degraded rivers. For the restored rivers, the bacterial samples showed positive correlations with DO (r = 0.571) and substrate diversity (r = 0.652) and was affected negatively by COD (r = −0.522) and NH_4-N (r = −0.526), though the correlations were not as strong as the forest rivers.

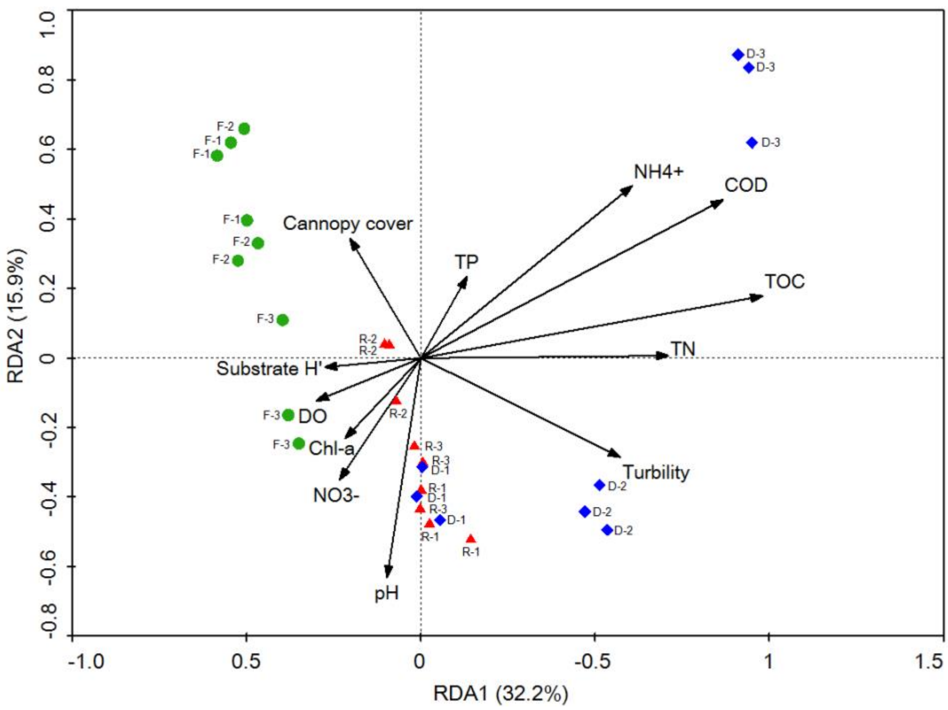

Figure 2.6 Relationship between the biofilm bacterial community and environmental variables in forested (F, circles), restored (R, triangles) and degraded (D, diamonds) rivers within the Anji City Region, PRC.

2.5 Discussion

Rehabilitation of aquatic biota, through habitat restoration, is now being implemented around the world to prevent further damage and mitigate existing freshwater degradation (Geist & Hawkins 2016). Accumulating evidence has linked aquatic rehabilitation to reducing nitrogen, phosphorus and organic matter concentrations, and thereafter to improved conditions for macroinvertebrate and fish populations (Miller et al. 2010; Nuttle et al. 2017; Shrestha et al. 2017). Microbial communities are often ignored in stream restoration studies yet they are crucial for supporting aquatic ecosystem processes and functions with key roles in driving organic matter and nutrient cycling (Fisher 1995). It is, therefore, imperative that we obtain a better understanding of the underlying mechanisms of microbe-mediated processes. In this study, therefore, I described the bacterial community composition including those involved in important ecological functions in restored rivers, and compared them with both degraded urban sites and "pristine" reference forest sites; to do this I used high-throughput 16S rRNA gene amplicon sequencing method. The results showed clear differences in the structure of biofilm microbial communities among these three main river ecosystems, and these differences were strongly correlated to the changes in habitat and physico-chemical characteristics in these river groups. This finding is consistent with the results of surveys in New Zealand and the USA, showing that local environmental conditions, rather than spatial factors, such as latitude or elevation, best predicted the variance of community composition and diversity (Fierer et al. 2007; Lear et al. 2013). Although the differences in the bacterial community here were mainly led by the variance in habitat and environmental characteristic in the rivers, the longitudinal natural changes in rivers may account for some of the environmental and biological variations observed (Vannote et al. 1980).

2.5.1 Habitat restoration impact on physico-chemical properties of stream water

The consistent input of pollutants from both point and diffuse sources in the urban (pre-restored) rivers caused high enrichment of TOC. Habitat restoration led to a reduction in TOC, and a significant increase in DO in the surface water of the restored rivers. These results are consistent with habitat restoration experiments in the Zenne River in Belgium (Atashgahi et al. 2015). Essentially, habitat restoration improved conditions by reducing TOC and increasing DO, suggesting that organic pollutants entering the degraded river were removed through habitat restoration. There was no difference in DO concentration between restored and reference forest rivers, suggesting that habitat restoration improved the physico-chemical environment of restored rivers.

2.5.2 Impact of habitat restoration on the bacterial community

The diversity and composition of bacterial communities change according to habitat characteristics (Levi et al. 2016), hence, rehabilitation methods and the intensity of application should affect both the composition and diversity of microbial communities. Here, no differences were detected among river types for bacterial richness, and a significant decline in bacterial diversity was detected in restored rivers compared to degraded rivers. This is consistent with studies in wastewater treatment plant (WWTP) effluent in both urban and rural areas where a reduced diversity of biofilm bacteria has been detected (Drury et al. 2013; Lu et al. 2014). The difference in bacterial diversity might reflect the physico-chemical variables of surface water in the different river types. Dissolved inorganic nitrogen, dissolved organic carbon and hydrological variability has been demonstrated to be the most important environmental factors affecting biofilm responses (Ponsatí et al. 2016). In this study, the increase of DO concentration caused by habitat restoration might lead to the development of aerobic microbial community and higher efficiencies of chemical oxygen demand (COD) removal through oxidative decomposition (Gu et al. 2015). The decline in organic carbon quality could also influence the abundance of biofilm bacteria (Olapade & Leff 2005; Ponsatí et al. 2016), which might have led to the decrease in heterotrophic anaerobic microorganisms that rely on organic resources, which lead to the decline of bacterial diversity in rivers after habitat restoration. Epilithic bacterial populations can also be affected indirectly by inorganic nutrients via the influence of nutrients on algal biomass (Tank & Webster 1998; Rier et al. 2002).

Distinct bacterial communities were detected in each of the river types, a dissimilar composition was found between (i) forest rivers and degraded rivers, (ii) forest rivers and restored rivers, and (iii) restored rivers and degraded rivers. These differences were strongly correlated with the changes in habitat substrate diversity, and physico-chemical characteristics (DO, TOC and COD) of these river types. The results from this study suggest that the differences in bacterial community compositions were mainly caused by the variations in habitat and habitat-specific physico-chemical characteristics (Hempel et al. 2010; Levi. et al. 2016). Rivers with diverse substrates may provide more dynamic surface and a higher degree of resource heterogeneity within the microhabitats for biofilms, shaping distinct bacterial communities in forest and restored rivers from the microbiome in degraded rivers. The variations in physico-chemical attributes (e.g., TOC) in the forest and restored rivers might lead to the difference in bacterial community composition between these two river types. Moreover, the bacteria clustered in the restored rivers were distributed between the bacteria in the degraded and forest rivers, indicating that they were moving in the correct direction, i.e., towards the reference forest rivers. There was, however, some overlap between the restored and degraded rivers, indicating that there was still a legacy effect of the previous degraded state. Overall, the degraded rivers possessed significantly greater bacterial diversity than the restored rivers. Hence, restoration to “pristine” conditions will take longer than seven years, and further studies are needed to determine exactly how long.

Compared with forest rivers, degraded rivers had a slightly greater abundance of Bacteroidetes, a member of phylum specialized in degrading high molecular weight (HMW) compounds, and possessed significantly higher relative abundance of *Flavobacterium*, *Arenimonas* and *Acinetobacter*, which are capable of metabolizing/mineralizing organic compounds (Verma & Rathore 2015; Chen et al. 2015; Garcia-Garcera et al. 2017), and a remarkably low abundance of *Duganella*, *Indobacter*, *Methylotenera*, *Rhodoferax* and *Clostridium_sensu_stricto_13*; these genera are major players in cycling of carbon compounds in the environment (Risso et al. 2009; Vorobev et al. 2013), and organic matter utilization (Zhao et al. 2017). This suggests that the degraded rivers with a high TOC load and limited DO have a distinct impact on the microbial community, shaping the microbiome with a greater ability to degrade/mineralize high molecular weight (HMW) compounds in degraded rivers; this ability differentiates these degraded rivers from the forest ones.

The restored rivers, however, had a greater relative Proteobacteria abundance than degraded rivers; this phylum is often found in nutrient-poor conditions with a low TOC (Atashgahi et al. 2015). Moreover, *Duganella* genus, which utilized organic compounds, but required oxygen to survive (Kämpfer et al. 2012), was greater in restored rivers compared to the degraded ones. This may imply that along with the establishment of more diverse substrates and aerobic and sub-aerobic system in the restored rivers, habitat restoration shifted the dominant components of the bacterial community that mineralize and degrade organic matter to bacteria that utilize organic matter for growth. At the same time, there is also a shift from species that occur in predominantly anaerobic conditions to aerobic conditions. This is consistent with the RDA results, where the bacterial community in the degraded rivers was strongly correlated to organic pollutants TOC and COD, whereas, for restored rivers, the bacterial community only showed weak positive correlations with substrate diversity and DO in the surface water.

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

2.6 Conclusions

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

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Chapter 3 The effect of habitat restoration on macroinvertebrate communities in urban rivers

3.1 Abstract

In recent decades, the biodiversity of freshwater environments has decreased sharply due to anthropogenic disturbances that damaged ecosystem structures and functions. Habitat restoration has emerged as an important method to mitigate the degradation of river ecosystems. Post-project monitoring has been promoted to access the progress of the restoration, however, it is still unclear how ecosystem structure (e.g. aquatic community) changes following river habitat restoration in China. Macroinvertebrate communities intermediately positioned within ecosystem food webs play a key role in ecosystem processes within river ecosystem, driving energy flow and nutrient cycling. Here, benthic macroinvertebrate community is used as bioindicators to assess the ecosystem health of degraded urban rivers, urban rivers undergoing habitat restoration and rivers in forested areas (i.e., reference conditions). This study aims to determine: (i) how habitat restoration impacts on benthic macroinvertebrates and how this compared to degraded and reference conditions; (ii) how did macroinvertebrate community compositions differ in restored relative to degraded and reference sites; (iii) the environmental factors shaping macroinvertebrate communities across the three river groups. A developed macroinvertebrate community in restored rivers was detected. Habitat restoration significantly increased the diversity and richness of macroinvertebrate community and intolerant species. The habitat characteristics and water chemistry, including substrate diversity, water velocity and both nutrients and organic pollutants in the surface water, appeared to shape the turnover of these communities. Habitat characteristics contributed to most of the variation of the entire macroinvertebrate community. Our research indicated that habitat restoration is an efficient approach to restore the aquatic community. It is a beneficial manner to recover the aquatic biodiversity in the degraded urban rivers and enhance river ecosystem health for freshwater conservation and sustainable management. This study strengthens our understanding of the changes of macroinvertebrate community after habitat restoration and important controlling variables that attributing to these changes, which provides an important guidance for future planning of ecological restoration strategies of degraded freshwater ecosystems.

Keywords: habitat restoration, bioindicator, macroinvertebrate, indicator species, river ecosystems

3.2 Introduction

Anthropogenic disturbances, such as urbanization, damming, water withdrawal and pollution, have sharply increased in the past centuries, which markedly damaged freshwater ecosystem structure and decreased biodiversity (Zhang et al. 2019). To mitigate and prevent the degradation of river ecosystems, habitat restoration has emerged as a key activity around the world (Geist & Hawkins 2016). The aim of habitat restoration is to improve the ecosystem health of freshwater systems through enhancing habitat complexity and heterogeneity. To this end, river channel re-meandering, riverbed reconstruction, adding both in-stream islands and aquatic vegetation, and increasing flood plain areas are most widely included in the restoration strategy (Bernhardt et al. 2007). In combination, these treatments should enhance the substrate and hydraulic heterogeneity, increasing both macrophyte colonization and food availability (Laasonen et al. 1998; Lepori et al. 2005), eventually developing a complex and heterogeneous aquatic habitat (Milleret al. 2010).

Different types of riverine habitats are known to influence the community composition of aquatic organisms such as fish and macroinvertebrates, attributing to the variance of river morpho-hydrology, substrate composition and environmental condition at the reach scale (Zhang et al. 2009; Kail et al. 2015). A few studies measured benthic biological indicators (i.e. microbes, algae, invertebrates) to assess the structural integrity and ecosystem health following habitat restoration (Coe et al. 2009; Frainer et al. 2017; Schmutz et al. 2016). Evidence accumulated indicated that aquatic rehabilitation would improve habitat condition and water quality for aquatic biotas through restructuring heterogeneous habitat, re-introducing aquatic plants, riparian zone re-forestation, etc. (Miller et al. 2010; Kail et al. 2015). However, evidence of ecological improvements associated with habitat restoration have been highly varied, the response of benthic aquatic communities to habitat restoration remain unclear in China. Therefore, it is imperative to obtain a better understanding of the restoration effect and the underlying ecological mechanisms. Some information could be gained to better understand this restoration progress by comparing the effectiveness of restoration schemes relative to the pre-restoration state and near-natural targets, hence provide sufficient evidence for post river management and improvement of future endeavors.

Macroinvertebrate communities are composed of a range of species that tolerate a wide range of environmental conditions (Plafkin et al. 1989). As a middle link of the food chain within river ecosystems, macroinvertebrate play a key role in ecosystem processes such as organic material cycling and energy flow (Zhang et al. 2004; Strayer 2006; Duan et al. 2010). Stream macroinvertebrates are generally recognized as good biological indicators of water quality (Hilsenhoff 1988) and ecosystem health (Karr 1999), because of their availability in most freshwater ecosystems, and their sensitivity to environmental changes such as disturbance, deterioration, and improvement (Zhang et al. 2010; Li et al. 2015). They can reflect the relative long-term temporal and spatial changes of river ecosystems and can be early warning indicators of environmental pressures given that they are such a diverse group containing a high number of species with a large variability in ecological requirements (Smith et al. 1999; Shao et al. 2006; Dos et al. 2011). Hence, macroinvertebrates are frequently used as indicators of restoration efficiency (Spänhoff & Arle 2007; Besacier-Monbertrand et al. 2014).

The use of macroinvertebrates as bioindicators for restoration have been studied in Europe and North America (Kail et al. 2015; Zan, Kondolf & Riostouma 2017), but there have been few assessments of restoration in Asia and, in particular China. Although the restoration-related effect on macroinvertebrate communities should be theoretically positive with the increase of habitat heterogeneity (Miller et al. 2010), as surface features of stream habitat may influence detritus (Douglas & Lake 1994), epiphytic algae (Dudley et al. 1986), and form 'refuges' from high flow conditions for predators (Lake 2000), observed changes have been inconsistent with the scale and specific metrics assessed (Palmer et al. 2010; Ernst et al. 2012). The results may also differ when investigating rivers with diverse and complex conditions, especially in China.

In this study, the macroinvertebrate communities of three river groups were compared, (1) degraded urban rivers, (2) urban rivers undergoing habitat restoration and (3) rivers in forested areas (i.e., reference conditions), essentially providing a gradient from severely damaged to near-natural. Within each river, a range of habitat features, physico-chemical factors, spatial factors were measured, and the macroinvertebrate communities were sampled. Through comparing the relationship between macroinvertebrate community composition and environmental variables along this simple gradient, this study intends to determine: (i) how habitat restoration impacts on benthic macroinvertebrates and how this compared to degraded and reference conditions; (ii) how did macroinvertebrate community compositions differ in restored relative to degraded and reference sites; (iii) the environmental factors shaping macroinvertebrate communities across the three river groups. I hypothesized habitat restoration would improve the benthic macroinvertebrate community, the macroinvertebrate diversity and richness would increase, and there would be an improvement in both water quality and availability of living habitat following the restructuring of heterogeneous habitat, re-introducing of aquatic macrophytes and riparian zone re-forestation. Moreover, some tolerant species that are dominants in degraded urban rivers will be replaced by Ephemeroptera, Plecoptera, and Trichoptera species (EPT) that are sensitive to external disturbance. Substrate composition, water flow velocity and physico-chemical variables were hypothesized to be the main factors affecting any change in macroinvertebrate community composition (Figure 3.1).

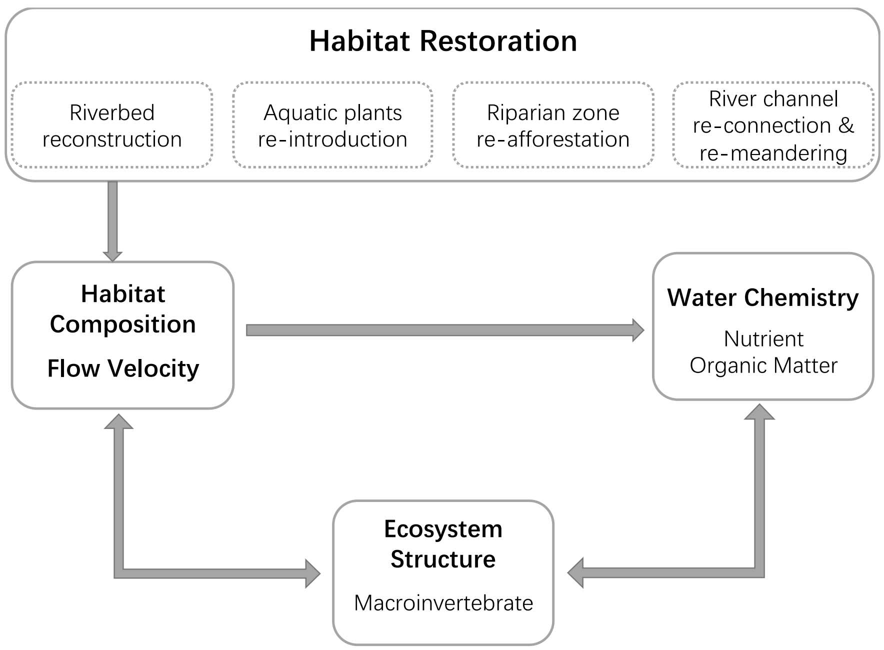

Figure 3.1 Conceptual model of the experiment.

River ecological restoration induced the variance of habitat and flow conditions, which in turn influenced the water chemistry and ecosystem structure (macroinvertebrate) of the river ecosystem.

3.3 Materials and methods

3.3.1 Study sites

Three groups of streams selected from the same catchment (Shaoxi River) in Anji, Zhejiang Province PRC were investigated, each group with three different rivers. Three stream groups (Figure 3.2, Table S3.1.) include (i) undisturbed forest rivers (reference sites, denoted F), (ii) urban rivers undergoing habitat restoration in the last seven years (denoted R); and (iii) degraded urban rivers (denoted D). In summer 2018, the average day/night temperatures of the region were 29℃/ 21℃ and the average precipitation was 133 mm.

Similar conditions existed in the degraded rivers and pre-restored urban rivers (Lin et al. 2019). Straitened and hardened with concrete, these three degraded rivers were covered with mud and were listed as rivers to be restored in the future by the local water conservancy bureau. Two of the degraded rivers are surrounded by suburban areas, and another one is located in the city center. The three restored rivers located in urban areas were at the same elevation with those degraded rivers. These rivers had been restored using a similar ecological restoration strategy for up to seven years. This involved natural reconstruction of the riverbed using diverse substrates (e.g. boulders, cobbles and pebbles), the channel was re-connected and re-meandered, floating islands were constructed, aquatic plants including submerged macrophytes and emergent plants were re-introduced into rivers, and the riparian zone was re-afforested in an attempt to recover a more natural river form. Three forest streams were 40-km upstream of these urban rivers within the same catchment, and these undisturbed rivers were considered as approximations to reference sites.

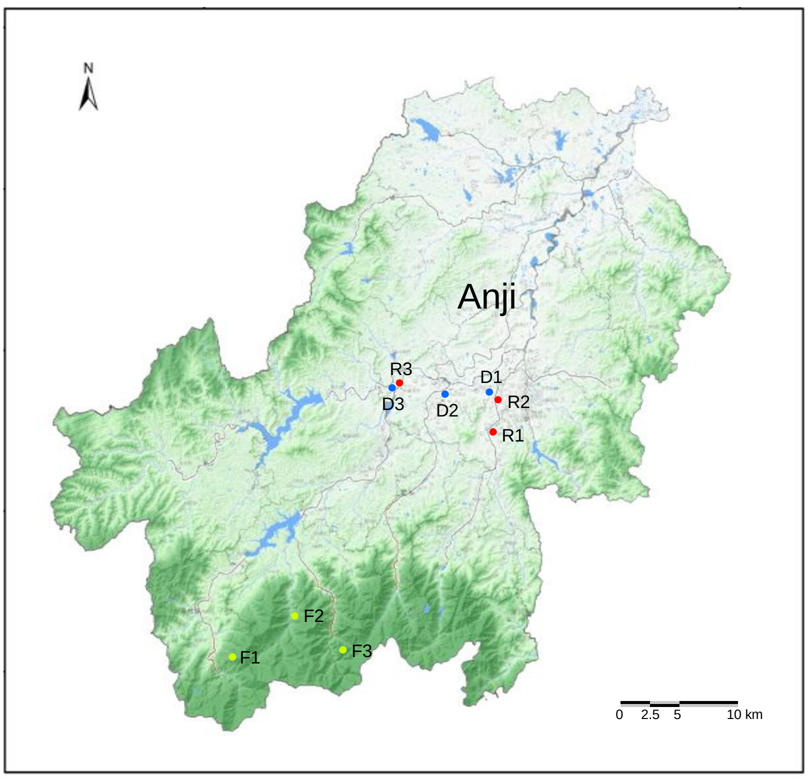

Figure 3.2 The sampling sites within the Anji City Region, PRC showing the locations of the three degraded urban rivers (D), three restored rivers (R) and three forested rivers (F).

3.3.2 Habitat characteristics

Habitat surveys were performed in July and August 2018. At each river, habitat characteristics (denoted Habitat) were measured within a 50 m sampling reach as described in Lin et al. (2019). After visually estimated the reach canopy cover, the water velocity across the channel was measured by Teledyne flow meters (ISCO, Lincoln, NE, USA), the river-bed types were counted, the substrate composition was tested by random-selecting 100 sediment particles on the riverbed and counting the ratio of substrate classes (boulders, cobbles, pebbles, sand grains) according to Kondolf (1997). The substrate diversity was then calculated by means of the Shannon diversity index H' (Shannon, 1997) for each site.

3.3.3 Physico-chemical variables in surface water

The river width and the river depth were measured at five-evenly spaced points across the channel. Three sampling positions were selected in each river and physico-chemical variables (denoted ENV) was monitored by standard methods (Lin et al. 2019). Briefly, (1) temperature, pH, dissolved oxygen (DO), and turbidity were measured *in situ* using handheld water quality analyzers, and (2) a one litre water sample was taken, filtered through a 0.45 µm filter and tested within 48 hours for ammonium nitrogen (NH_4-N), nitrate-nitrogen (NO_3-N), total nitrogen (TN), total phosphorus (TP), total organic carbon (TOC) and chemical oxygen demand (COD).

3.3.4 Spatial factors

Principal Coordinates of Neighborhood Matrices (PCNM) was applied to assess the geographical position and dispersal across the rivers (Guo et al. 2019). Geographic coordinates (latitude and longitude) were used to calculate the Euclidean distance matrix with the 'earth.dist' function in the 'fossil' R package. Seven PCNM matrices were then generated by performing the 'pcnm' function in the 'vegan' R package (Jyrkänkallio-Mikkola et al. 2017). Four positive eigenvalues (PCNM2-5) combined with latitude and longitude were used as spatial factors (denoted Spatial; Guo et al. 2019).

3.3.5 Macroinvertebrates sampling procedure

In each river, benthic macroinvertebrates were sampled from July to August 2018 in three sampling sites in each river using a 1 m x 1 m quadrat distributed randomly along a 30 m stretch.

Within each quadrat macroinvertebrates were sampled using a kick net (opening: 9.5 cm x 14.5 cm; mesh size: 500 mm) by disturbing vegetation and substrates; the samples were then preserved in 70% ethanol for storage, sorted and all macroinvertebrates then identified to family level using Merritt et al. (2008), and classified into groups according to their ability to water pollution using the Family Tolerance Value (Mandaville 2002).

Differences in the structure of benthic macroinvertebrate communities were then assessed by calculating total abundance, total richness, Shannon's diversity (H'), Pielou's evenness (Shannon 1997), the abundance and richness of EPT (Ephemeroptera, Plecoptera, and Trichoptera) and richness of intolerant taxa for each river group. Indicator species for each group of river was selected using Multilevel pattern analysis at significance level of $p < 0.05$.

3.3.6 Statistical analysis

Differences in habitat features, physio-chemical parameters, and macroinvertebrate alpha (α) diversity properties in three stream groups were evaluated through analysis of variance with post hoc Tukey–Kramer test (Torres-Mellado et al. 2012). Environmental factors and α-diversity indexes were ln (x + 1) transformed if the residuals deviated from normality. The similarity in macroinvertebrate community among three river groups was then assessed by analysis of similarities using the 'anosim' function in 'vegan'. A *p*-value of 0.05 was used as the cutoff for significance.

To explore relationships between habitat characteristics, physio-chemical features and α-diversity of macroinvertebrate, respectively, Spearman's correlation coefficients were calculated, explanatory variable that indicates significant multi-collinearity (Spearman correlation coefficient ≥0.70) was excluded from further analysis (Cai et al. 2017). The macroinvertebrate abundance matrices were Hellinger-transformed and detrended correspondence analysis (DCA) was then carried out using 'decorana' function in R package vegan to choose response model (linear or unimodal) for the macroinvertebrate community data. The length of the first DCA ordination axis was less than four, which indicated that RDA was suitable for taxonomic composition. Accordingly, RDA was performed, and the significance was tested using the 'anova.cca' function in 'vegan'. Explanatory variables were selected by performing forward selection using function 'forward.sel' in the 'packfor' R package. Monte Carlo permutation tests was then applied to test the contribution significance of each variables. Finally, variation partitioning was performed to explore the pure contribution of each group (i.e. habitat, environmental data, and spatial factors) to the variation of macroinvertebrate community using the ‘varpart’ function in the ‘vegan’ R package (R Core Team 2017). Multivariate analysis including DCA, RDA, forward selection and variation partitioning were performed according to Borcard et al. (2018).

3.4 Results

3.4.1 Habitat characteristics

Significant differences in water velocity ($F_{2,6} = 6.661$, $p = 0.030$), substrate diversity ($F_{2,6} = 57.37$, $p < 0.001$) and canopy cover ($F_{2,6} = 16.37$, $p = 0.004$) were detected between the three river groups; restored rivers have a faster water velocity and lower canopy cover than both degraded rivers and forest rivers (Figure 3.3E); the substrate diversity in the forest and restored rivers was remarkably greater than degraded rivers ($p < 0.001$) (Figure 3.3F). Four types of sediment particles (boulder, cobble, peddle, granule) formed the riverbed of restored and forest rivers, whereas degraded rivers have only one kind of particles (granule). The habitat diversity in forests and restored rivers was also much greater than that in degraded rivers. Riffles, pools, and islands constituted the habitat structure of the forest and restored rivers, whereas degraded rivers were formed by pools and a few islands.

3.4.2 Physico-chemical properties of surface water

Analysis of variance indicated no significant differences among three river groups in river width ($F_{2,6} = 0.336$), and mean river depth ($F_{2,6} = 0.791$), and no difference in water variables such as pH ($F_{2,6} = 0.325$), DO ($F_{2,6} = 1.716$), NH_4-N ($F_{2,6} = 2.619$), NO_3-N ($F_{2,6} = 2.498$), TP ($F_{2,6} = 1.609$) and Chl-*a* concentration ($F_{2,6} = 0.579$). However, surface water variables exhibited significant differences in water turbidity ($F_{2,6} = 11.75$, $p = 0.008$), TN ($F_{2,6} = 16.17$, $p = 0.004$), COD ($F_{2,6} = 5.965$, $p = 0.038$) in different stream groups. Forest rivers had significantly lower concentrations of TN, TOC and COD and turbidity than the degraded rivers ($p = 0.003$, $p = 0.047$ $p = 0.032$, and $p = 0.014$, respectively; Figure 3.3A-D). Restored rivers possessed a greater turbidity ($p = 0.013$) and a slightly increased TN concentrations ($p = 0.060$) than forest rivers (Figure 3.3A, Figure 3.3B), whereas, a weak reduction in TN was found in restored rivers compared to degraded rivers ($p = 0.073$) (Figure 3.3B).

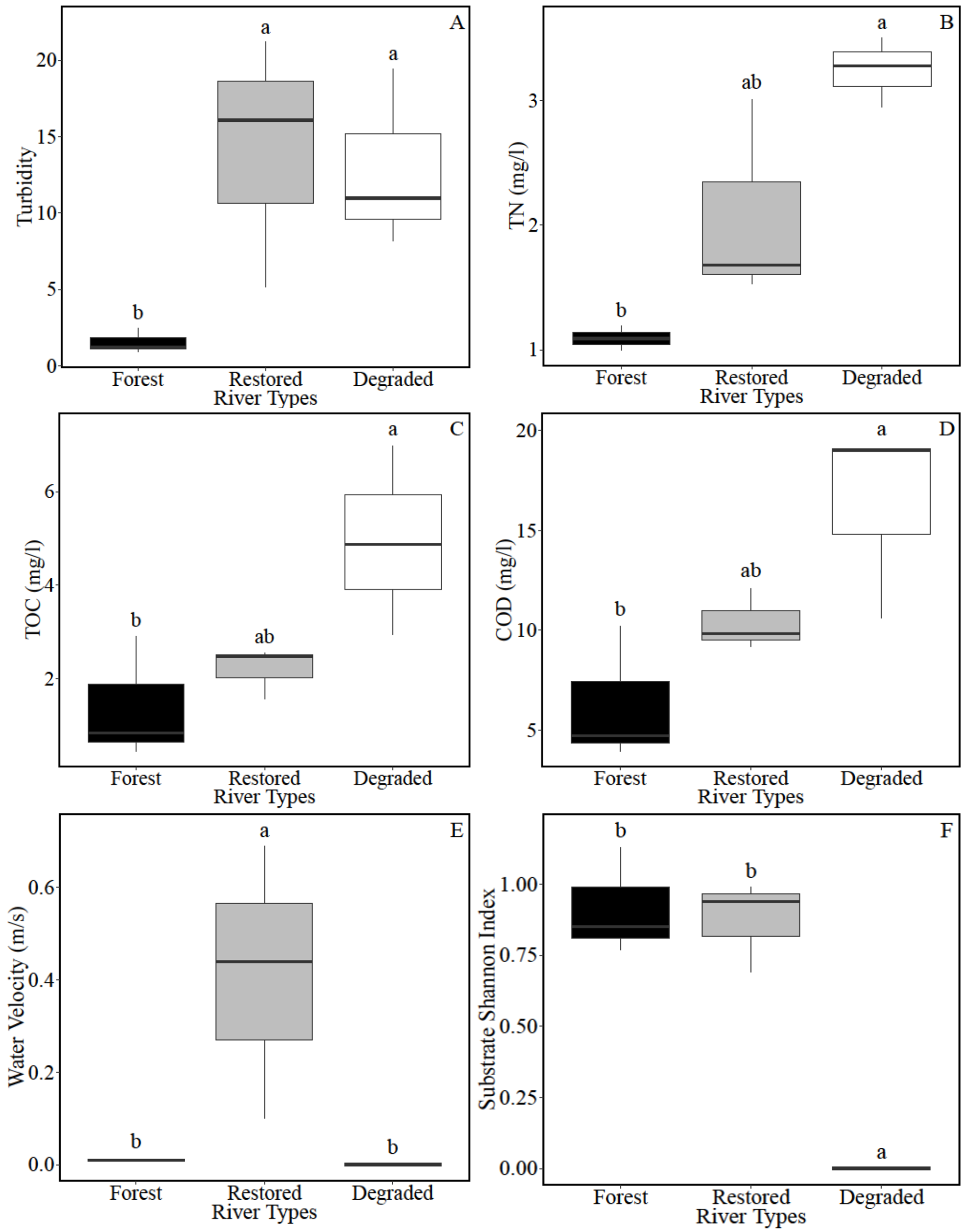

Figure 3.3 Comparison of the (A) turbidity, (B) total nitrogen (TN), (C) total organic carbon (TOC), (D) chemical oxygen demand (COD), (E) water velocity and (F) substrate Shannon index in three contrasting river types within Anji City Region, PRC. Mean values (± SE, n = 3) are presented; different lowercase letters indicate a significant difference observed at p = 0.05 level.

3.4.3 Benthic macroinvertebrate community

9,990 macroinvertebrates were identified across all rivers studied, 4,006 individuals in forest rivers, 5,792 in restored rivers, and 192 macroinvertebrates in degraded rivers. Macroinvertebrate α-diversity values (Table 3.1) showed that there were significant differences among river types for total abundance ($F_{2,6} = 37.32$, $p < 0.001$), total richness ($F_{2,6} = 222.20$, $p < 0.001$), EPT abundance ($F_{2,6} = 90.40$, $p < 0.001$), EPT richness ($F_{2,6} = 67.41$, $p < 0.001$), intolerant species richness ($F_{2,6} = 122.10$, $p < 0.001$) and Shannon diversity index ($F_{2,6} = 49.00$, $p < 0.001$). Both forest sites and restored sites had significantly greater total abundance, total richness, EPT abundance, EPT richness, Shannon-Wiener diversity and intolerant taxa richness than degraded rivers ($p < 0.001$) (Table 3.1, Figure 3.4), whereas no significant difference of taxonomic diversity was detected between forest rivers and restored rivers ($p > 0.05$). No difference was found among three river groups for the evenness of macroinvertebrate ($F_{2,6} = 0.532$).

Table 3.1 Mean values of macroinvertebrate taxonomic metrics in different groups of rivers summer within the Anji City Region, PRC. The values represent the mean ± standard error of three replicate samples.

River Type	Total abundance	Total richness	EPT abundance	EPT richness	Intolerant richness	Pielou's Evenness	Shannon-Weiner Index
Forest	445.11±98.60	23.00±2.53	251.89±56.13	10.56±0.99	7.67±0.69	0.74±0.01	2.29±0.05
Restored	643.55±117.44	19.78±0.22	394.11±82.46	7.33±0.38	4.89±0.67	0.65±0.07	1.95±0.21
Degraded	21.33±10.48	2.67±0.19	1.0±1.00	0.33±0.33	0.11±0.11	0.61±0.14	0.57±0.11

The analysis of similarities (ANOSIM) based on the macroinvertebrate samples showed a significant difference of macroinvertebrate community compositions among the three river groups ($R = 0.845$, $p = 0.001$). Among the 46 families of macroinvertebrates identified in this survey, thirteen taxa were selected as indicator species (Table 3.2). Eight species were highly associated with forest rivers, including dominant species Leptophlebiidae (22.35%), Perlidae (7.43%), and some other species like Dytiscidae, Scirtidae, Coenagriidae, Hydrophilidae, Leptoceridae, Tipulidae. Leptophlebiidae, Perlidae, Leptoceridae, Dytiscidae and Coenagriidae were significantly more distributed in the forest rivers than both urban river groups ($p < 0.05$ in all cases), no difference of these taxa was found between restored rivers and urban degraded rivers ($p > 0.05$). Five indicator families (Corbiculidae, Glossiphoniidae, Hepobellidae, Lymnaeidae and Heptageniidae) were found in restored rivers, dominant species were the Caenidae (31.21%), Chironomidae (14.95%) and Baetidae (12.39%). Of the EPT taxa sampled, Caenidae was the most dominant family in the restored sites, and was significantly greater than that in degraded urban rivers ($p = 0.05$) and comparable to forest rivers ($p > 0.05$), Baetidae and Heptageniidae were also presented in the restored rivers in greater numbers than in degraded rivers ($p = 0.088$, $p = 0.066$, respectively), although these trends were not significant. Two of the tolerant taxa (Corbiculidae and Glossiphoniidae), however, were significantly greater in restored rivers compared to both degraded and forest rivers ($p < 0.05$). No indicator species was allocated to degraded rivers, but degraded rivers possessed greater abundance of Tubificidae (46.92%), Chironomidae (32.36%) and Viviparidae (12.26%) (Table S3.2).

Table 3.2 Indicator species of macroinvertebrate communities in three contrasting river types within the Anji City Region, PRC. IV = Indicator value.

River Type	Taxa	IV	*p* - value
Forest	Dytiscidae	1.000	0.035*
	Scirtidae	1.000	0.035*
	Perlidae	1.000	0.035*
	Coenagriidae	0.991	0.035*
	Hydrophilidae	0.982	0.035*
	Leptoceridae	0.974	0.035*
	Tipulidae	0.964	0.035*
	Leptophlebiidae	0.941	0.035*
Restored	Corbiculidae	1.000	0.039*
	Gossiphonidae	1.000	0.039*
	Hepobellidae	0.985	0.039*
	Lymnaeidae	0.977	0.039*
	Heptageniidae	0.871	0.039*

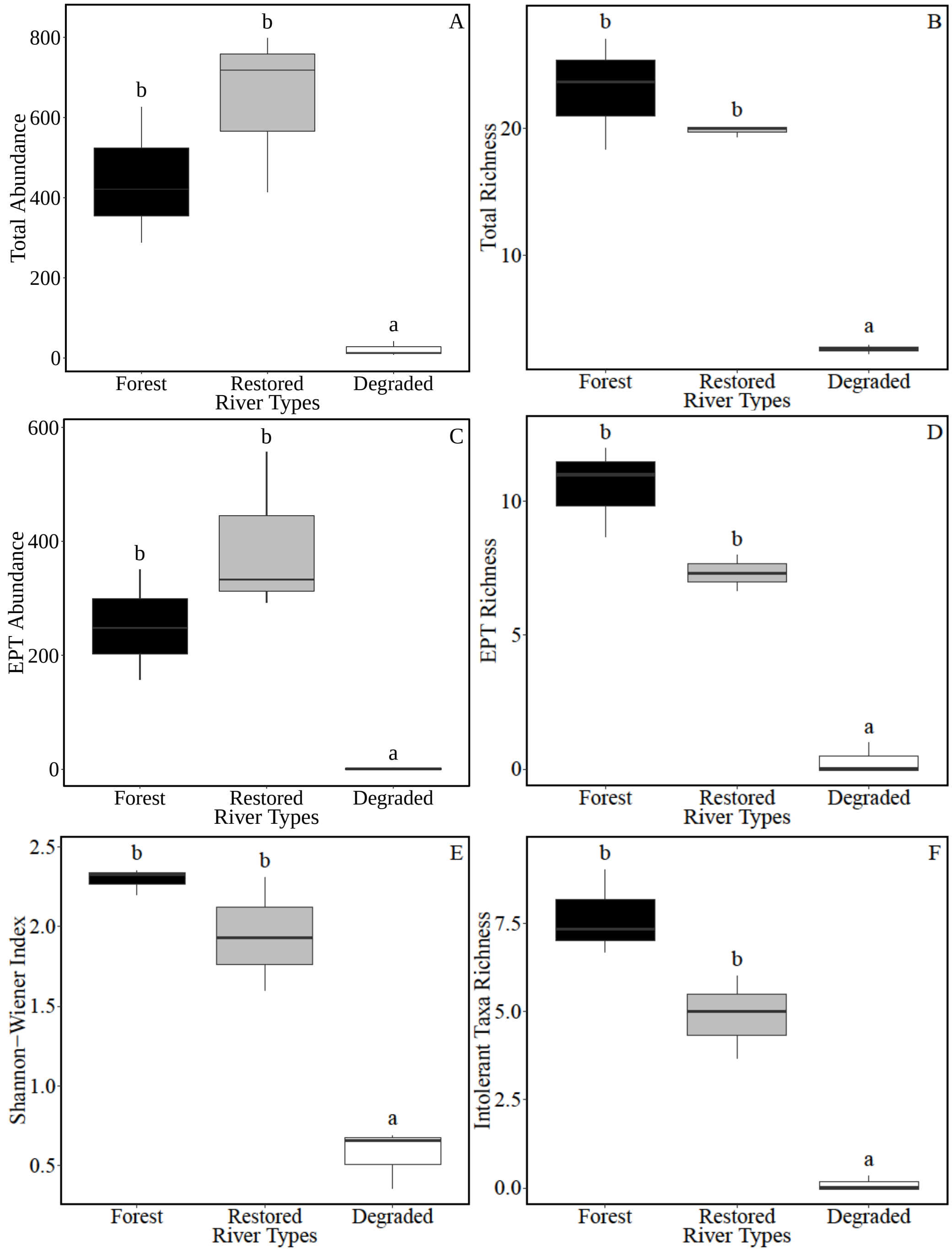

Figure 3.4 Comparison of macroinvertebrate alpha diversity (A) total abundance, (B) total richness, (C) EPT taxa abundance, (D) EPT taxa richness, (E) macroinvertebrate diversity (Shannon Index) and (F) intolerant taxa richness in forested, restored and degraded rivers within the Anji City Region, PRC. Mean values (± SE, n = 3) are presented; different lowercase letters indicate a significant difference observed at $p = 0.05$ level.

3.4.4 Correlation between environmental variables and macroinvertebrate community

The correlation between macroinvertebrate α-diversity and environmental variables (i.e. habitat characteristics, and physico-chemical variables) produced comparable correlations (Table S3.3). The relationship among environmental variables, spatial factors and total macroinvertebrate community structure were examined by constrained redundancy analysis (RDA), eigenvalues of 0.500 and 0.249, respectively for axis one and two were generated (Figure 3.5). The environmental variables including habitat characteristic, physico-chemical variables and spatial variables, explained 74.9% of the variance in macroinvertebrate community structure. Monte Carlo permutation tests revealed that substrate diversity, water velocity, COD and longitude significantly affected the macroinvertebrate community (p < 0.05 in all cases). The macroinvertebrate assemblages in forest rivers were mainly structured by diverse substrates ($F_{2,6}$ = 3.472, p = 0.004) and low COD concentration ($F_{2,6}$ = 2.285, p = 0.022). COD in the surface water ($F_{2,6}$ = 25.599, p = 0.006) was also a major factor influencing macroinvertebrate community in degraded rivers. In restored rivers, the macroinvertebrate communities showed a strong correlation with water velocity ($F_{2,6}$ = 3.801, p = 0.014), substrate diversity ($F_{2,6}$ = 9.843, p = 0.018) and longitude ($F_{2,6}$ = 5.687, p = 0.026).

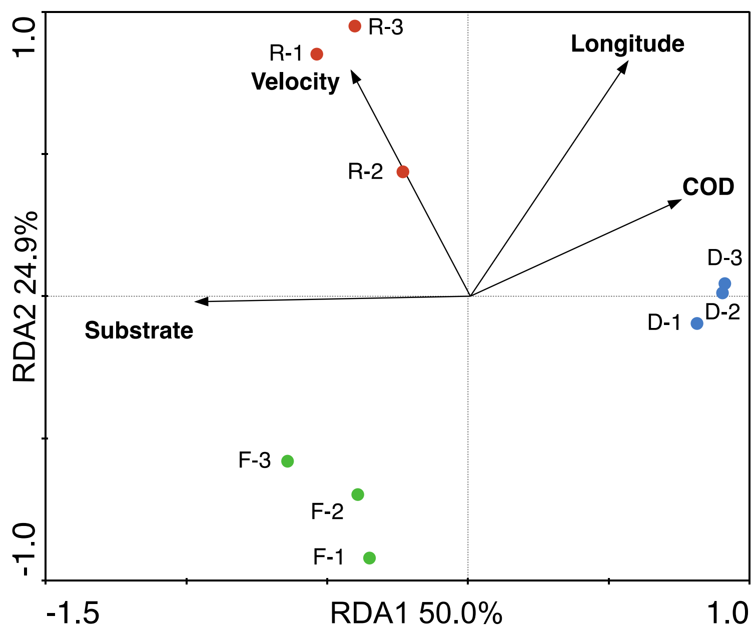

Figure 3.5 Relationships between the benthic macroinvertebrate communities and environmental variables in forested (F, green circles), restored (R, red circles) and degraded (D, blue circles) rivers within the Anji City Region, PRC.

3.4.5 Relative importance of environmental, spatial and habitat factors

Variation partitioning showed that 44% of the community taxonomic composition was explained by three sets of environmental variables; habitat factors explained 22%, followed by physico-chemical variables (ENV, 5%) and spatial factors (4%); 12% of the variation was shared by all three sets, 4% between habitat and ENV and 2% between ENV and spatial factors (Figure 3.6A). No shared effect was found between habitat and spatial factors (Figure 3.6A). In terms of indicator species, 36% of the total variation was explained by the three explanatory sets of variables. Habitat features was still the main factor explaining 10%, spatial factors explained 2% and physico-chemical variables explained nothing; 4% of the variation was shared by all three sets, 11% between ENV and spatial factors, 9% between spatial factors and ENV and 5% between habitat and ENV (Figure 3.6B).

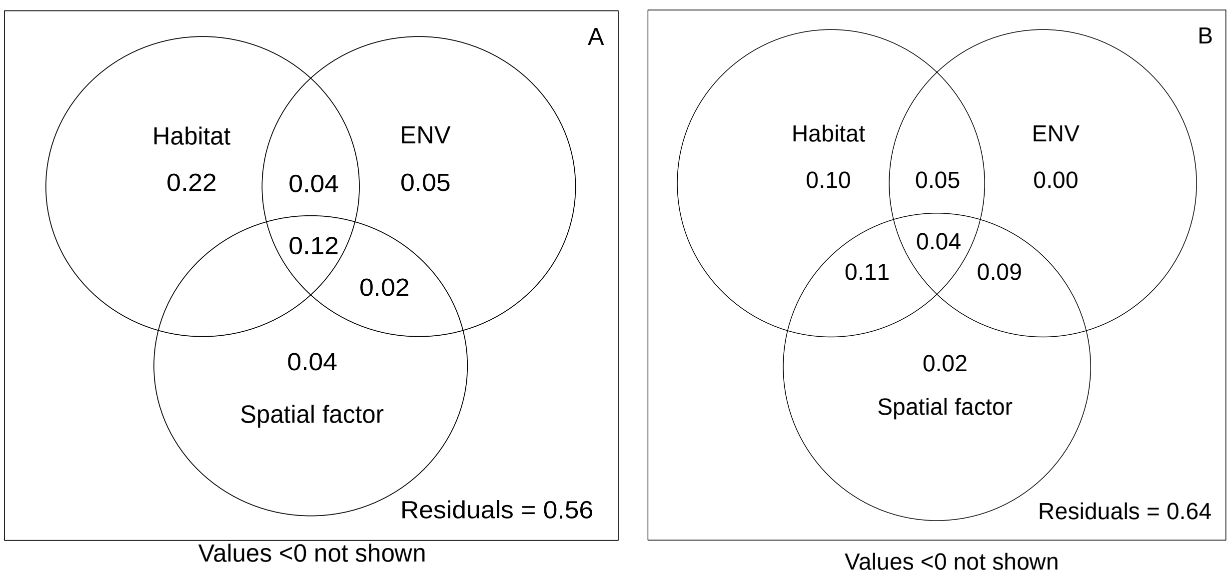

Figure 3.6 Venn diagrams illustrating the variation partitioning analysis for (A) taxonomic composition and (B) indicator species. Habitat, ENV, and Spatial factor are sets of variables representing habitat variables, physico-chemical variables, and spatial factors, respectively. Residuals are shown in the lower right corner. All fractions based on adjusted R_2 are shown as percentages of total variation.

3.5 Discussion

3.5.1 Taxonomic diversity of macroinvertebrate community

Overall, there were significant differences in macroinvertebrate community composition between the forest, restored and degraded rivers. The taxonomic diversity and composition of macroinvertebrate community in restored rivers were distinct from degraded rivers and strongly associate with habitat characteristic substrate diversity and water velocity, indicating that habitat restoration had impacted the structure of the communities. Compared with degraded rivers, there was a significant increase in macroinvertebrate diversity and total richness in restored rivers, meanwhile, EPT richness and intolerant taxa richness also increased under habitat restoration. These results are in accordance with the stated hypothesis and in line with previous studies in northern Poland, indicating that habitat heterogeneity had significant, positive effects on macroinvertebrate richness and diversity (Matthaei & Diehl 2005; Miller et al. 2010; Obolewski et al. 2016). In-stream habitat restoration enhanced the macroinvertebrate richness and diversity (Flores et al. 2017).

The difference in macroinvertebrate diversity might reflect the variation of habitat characteristics and physico-chemical variables in the surface water (Shi et al. 2019). As demonstrated previously, increased depth and frequency of pools should increase species richness through greater habitat heterogeneity (Brasher 2003). Obolewski et al. (2016) also suggested that rehabilitation induced hydrological connectivity improved water quality and increased the diversity and abundance of macrozoobenthos. Here, substrate composition, organic carbon TOC and nutrient TN in the surface water were important in influencing macroinvertebrate diversity. Riverbed reconstruction and aquatic macrophytes re-introduction applied to the restored rivers enhanced the substrate diversity, diverse substrate with large particle size (e.g., cobbles) in the riverbed can enhance the stability of habitats and form abundant interstitial spaces for macroinvertebrates (Luo et al. 2018). Macroinvertebrates are very sensitive to organic pollutants and water degradation (Kalyoncu & Gülboy 2009; Patang et al. 2018). The decline in organic carbon and nutrient quality in restored rivers induced by faster flow and greater hyporheic exchange facilitated denitrification in re-connected channel (Craig et al. 2008), nutrient uptake by macrophytes (Preiner et al. 2020), developing microbial activity in heterogeneous habitat (Jarno et al. 2018), may improve the water quality and stimulate the growth and development of macroinvertebrate of low tolerance value. This finding differs with many habitat restoration schemes which resulted in modest /unsuccessful ecological responses for the persist of water quality problem (Palmer et al. 2010).

Relative abundance of EPT and intolerant species also increased in restored rivers compared to degraded rivers. Many pollution-intolerant taxa belong to the EPT insect orders Ephemeroptera, Plectoptera and Trichoptera. The observed increase in sensitive EPT taxa agree with earlier observations in field studies and mesocosm experiments, suggesting that EPT taxa are sensitive to environmental degradation (Cabria et al. 2011), EPT taxa often decline where there is a reduction in flow velocity accompanied by fine sediment deposition which reduced food availability (Ryan 1991) and physically damages gills and filter-feeding apparatus by abrasion, clogging (Beermann et al. 2018; Jones et al. 2012; Piggott et al. 2015). On the contrary, EPT taxa may increase upon deposited coarse substrate and fast flow.

3.5.2 Macroinvertebrate community composition and their leading factors

Distinct macroinvertebrate communities were found among river types. These differences were closely related to the changes in water velocity and substrate diversity, COD, and longitude of the rivers. These results support the hypothesis that macroinvertebrate community composition was driven by habitat characteristics, physico-chemical variables and spatial factors, and in line with a summarized concept that benthic macroinvertebrate species are sensitive to both hadromorpholgy and water quality factors in their environment (Mandaville 2002; Shi et al. 2019).

Habitat characteristics contributed to most of the variation of the entire macroinvertebrate community, followed by ENV and spatial factors (Englund et al. 1997). This supports the view of Jahnig & Lorenz (2008) and Luo et al. (2018), that habitat specific habitat variables explained the major variation in macroinvertebrate community composition.

Macroinvertebrate fauna can be classified into flow exposure groups (obligate, facultative, and avoiders) and habit groups (clinger, burrowers, sprawlers, and swimmers) in accordance with their preference towards hydraulic conditions that guided by their flow exposure preferences and behavioral activities (Merritt et al. 2008). Rivers with diverse substrates can provide an abundance of micro-habitats and heterogeneous food resources for macroinvertebrates (Mandaville 2002), especially as water velocity varies at different seasons; hence a diverse species assemblage, adapted to various natural flows can be maintained. Here, the changes in substrate diversity and flow velocity induced by habitat restoration was important in shaping the macroinvertebrate communities in restored rivers compared to those in degraded rivers.

Differences in physico-chemical variables (e.g., TN and TOC) also contributed to the shifts in macroinvertebrate community composition among three river groups, though the influence is not as strong as habitat characteristics. Given that water quality conditions are a product of catchment-wide processes which act as large scale filter of the regional species pool (Poff 1997), but habitat-scale variation drives differences in macroinvertebrate communities within the species pool, which yield a greater statistical influence (White et al. 2019). These results are similar to those reported for the river Danube (Rico et al. 2016) and an indoor experiment (Corcoll et al. 2015). The shared effects of hydromorphological and water chemical factors (ENV vs. Habitat vs. Spatial factor), however, had greater influences on macroinvertebrate communities than single effect of physico-chemical or spatial factors. Consistent with Rico et al. (2016), who indicated that chemical pollution had a lower contribution to invertebrate community than shared effect of habitat characteristics and physico-chemical conditions.

Spatial factors have a lower contribution on the macroinvertebrate community variance than physico-chemical and habitat variables. The biological communities in rivers may change along the variation of spatial factors (Vannote et al. 1980). However, habitat and water quality conditions, rather than spatial factors, best explained the variance of invertebrate community and diversity (Rico et al. 2016). Overall, the macroinvertebrate clustered in the restored rivers possessed greater community diversity and richness, the community composition distinct from macroinvertebrates in the degraded and forest rivers, and these changes were caused mainly by improved habitat characteristics, followed by physico-chemical variables and lastly spatial factors.

3.5.3 Indicator species of macroinvertebrate and their deterministic factors

Groups of indicator species were observed in each of the three river groups. Habitat characteristics contributed the most to the structure variation of indicator species, followed by physico-chemical and spatial variables. First, no indicator species was allocated to degraded rivers, but degraded rivers possess greater abundance of tolerant species Tubificidae, Chironomidae and Viviparidae. Tubificidae and Chironomidae have been demonstrated to be abundant in streams with heavy organic pollution and low oxygen conditions (Al-Shami et al. 2011; Arimoro 2009). This is in agreement with our results, which suggested that organic pollutants played important roles in shaping the macroinvertebrate community in the degraded rivers.

Forest rivers possessed eight indicator species, including Leptophlebiidae and Perlidae. Compared with both urban river groups, forest rivers had greater abundance of EPT taxa (Leptophlebiidae, Perlidae, and Leptoceridae), which are sensitive indicators of habitat conditions (Boehme et al. 2016), and Coenagriidae, which is sensitive to water quality degradation and tolerant to low levels of organic pollution (Patang et al. 2018). Overall, our results suggested that forest rivers with high substrate diversity and good water quality have a distinct macroinvertebrate community compared to urban rivers.

Restored rivers possess five indicator species Corbiculidae, Glossiphoniidae, Hepobellidae, Lymnaeidae and Heptageniidae, and had greater relative abundance of mayfly Caenidae and Baetidae and Heptageniidae than degraded ones. Caenidae is usually noted as an indicator of organic pollution (Hilsenhoff 1988). Occurring primarily in streams with fast currents and high levels of oxygen (Bauernfeind & Humpesch 2001), Baetidae and Heptageniidae are quite sensitive to water quality degradation and only tolerant of low organic pollution (Patang et al. 2018). This may imply that the improved habitat heterogeneity and decline in nutrient and organic pollutants in the restored rivers provide more favorable conditions for the development of sensitive EPT species, as faster water flow and substrate diversity benefitted EPT richness (Luo et al. 2018). However, species Glossiphoniidae and Corbiculidae, both tolerant of organic pollution (Luo et al. 2018) were greater in the restored rivers than either degraded or forest rivers. This may imply that habitat restoration shifted the dominant component of macroinvertebrates to sensitive EPT species with the improvement of river habitat and water quality, facilitated the establishment of some tolerant species that live in specific habitat such as sediment, aquatic plant, and exist under low level of pollution in restored rivers, and this distinguishes macroinvertebrate community in restored rivers from the community in the other two river types. Thus, to recover the restored rivers to the status of reference rivers, long-term post-project management is required to improve the degradation of excessive nutrients and organic pollutants in the restored rivers, and protect these rivers from future disturbance. Moreover, long-term ecological monitoring should be promoted to ensure that projects are hydromorphologically sustainable.

3.6 Conclusions

In this study, I examined the effect of habitat restoration on macroinvertebrate community composition in the urban rivers with and without restoration by comparing with undisturbed forest rivers. The results support our hypothesis that habitat restoration positively altered the benthic macroinvertebrate community structure in comparison to that in degraded rivers. Attributing to the increase in substrate diversity, flow velocity, and accompanying decline in total nitrogen, total organic chemical in the surface water, habitat restoration induced a greater diversity, a greater richness and abundance of macroinvertebrate, and greater richness and abundance of less tolerant EPT taxa. This study supports the finding that applying habitat restoration in river management enhances habitat heterogeneity, improve water quality, which can in turn stimulate the shift of macroinvertebrate community composition in urban rivers. Accordingly, habitat restoration could be used as an efficient approach to recover the aquatic biodiversity in the degraded urban rivers, it is a positive manner to enhance river ecosystem health for freshwater conservation and management.

3.7 References

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Chapter 4 Evaluating ecosystem function following river restoration: the role of hydromorphology, bacteria, and macroinvertebrates

4.1 Abstract

Ecological restoration of freshwater ecosystems is now being implemented around the world to prevent further damage and mitigate anthropogenic disruption. In many areas, including China, most emphasis is placed on assessing physico-chemical and hydromorphological properties to monitor restoration progress, and less is known about the structural integrity and ecosystem health of the aquatic ecosystems. In particular, little is known about how ecosystem function changes following river habitat restoration, especially in China. Leaf litter decomposition can be used as an indicator of ecosystem integrity in stream ecosystems. Therefore, the leaf breakdown rate was measured in this study to assess the ecosystem function of restored rivers. By comparing the leaf breakdown rates in urban rivers undergoing habitat restoration with that in degraded urban rivers and rivers in forested areas (i.e., reference conditions), and relating the leaf decomposition to abiotic and biotic factors, I aimed to determine: (i) how habitat restoration affected leaf litter decomposition? (ii) the relationship between leaf litter decomposition and both environmental (habitat and physico-chemical variables) and biological factors (benthic communities), and (iii) identify the factors that contribute most to the variance in leaf litter breakdown rates. The results demonstrated a significant increase in leaf breakdown rate (120% in summer and 28% in winter) in the restored rivers compared to the degraded rivers. All environmental and biotic factors evaluated in this study contributed synergistically to the differences in leaf litter decomposition among the three river types. The role of macroinvertebrates, mainly shredders, appeared to be particularly important, contributing 52% (summer) and 33% (winter) to the variance in decomposition, followed by habitat characteristics (e.g. substrate diversity, water velocity; 17% in summer, 29% in winter), physico-chemical variables (e.g. nutrient and organic pollutants; 11% in summer, 1% in winter) and biofilm bacteria (0% in summer, 15% in winter). Habitat restoration positively affected the structure and function of the previously degraded streams. Knowledge of important controlling variables and their attribution to the changes of ecosystem functioning provides guidance to assist the future planning of ecological restoration strategies.

Keywords: habitat restoration, ecosystem function, leaf litter breakdown, river ecosystems, freshwater management

4.2 Introduction

With the increasing use of habitat restoration to manage freshwater ecosystems around the world, an abundance of publications emerged about the monitoring and evaluation of restoration projects (Bernhardt et al. 2005; Palmer & Ruhi 2019). Evaluation of restoration not only monitors the progress of the restoration, but the experience gained can be used as a basis to form more systematic and efficient restoration strategies for future endeavors (Knodolf & Micheli 1995; Zan et al. 2017). Within the overall assessment process, it is important to include both structural and functional variables when evaluating the response of ecosystem condition to human activities (Matthews et al. 1982; Gessner & Chauvet 2002; Pascoal et al. 2005). Currently, water quality and hydromorphological aspects of study receive the greatest attention for monitoring the restoration progress in freshwater systems. A few studies included biological indicators such as measures of microbes, algae, invertebrates, and fish to assess the structural integrity and ecosystem health (Coe et al. 2009; Frainer et al. 2017; Schmutz et al. 2016). However, few studies have been conducted to assess the functional ecosystem response to freshwater management by examining processes such as primary production, ecosystem respiration (Niyogi et al. 2002; Colangelo 2007; Aldridge et al. 2009), or leaf litter decomposition (Dangles et al. 2004; Wenger et al. 2009; Flores et al. 2011).

Anthropogenic disturbances (such as logging and damming) impact freshwaters in many ways, including geomorphology, hydrology, water quality, riparian plant communities, aquatic communities, and many other factors (Little & Altermatt 2018; Hashemi et al. 2019; Zhang et al. 2019). River ecological restoration, in turn, may reverse this damage through restructuring heterogeneous habitat, re-introducing aquatic plants, riparian zone re-forestation, etc., all of which can directly or indirectly affect organic matter breakdown in streams.

Organic matter breakdown is important ecosystem function in aquatic system in terms of nutrient cycling and energy flow (McKie et al. 2006; Tiegs et al. 2019), driving the stream food-web interactions (Zhang et al. 2004). Organic matter breakdown has been proposed as a good indicator of ecosystem integrity (Pascoal et al. 2005; McKie & Malmqvist 2009), and an alternative measure of stream health (Young et al. 2008; Niyogi et al. 2013). Such ecosystem functioning is regulated by both physico-chemical and biological factors (Pascoal & Cassio 2004). Environmental factors such as pH (Dangles et al. 2004), temperature (Ferreira & Chauvet 2011; Martínez et al. 2014), current velocity (Martínez et al. 2015), organic matter input (Graça et al. 2015), and leaf nutrient status (Greenwood et al. 2007; Pérez et al. 2012) can play important roles in influencing leaf litter decomposition. Elevated temperature and dissolved nutrients (N and P) in streams speed up leaf decomposition through stimulating microbial activity (Gulis et al. 2006; Hladyz et al. 2010; Ferreira & Chauvet 2011) and increase the abundance and biomass of shredders (Robinson & Gessner 2000). High inputs of nutrients and organic matter, however, slow down leaf decay rate by reducing the activity of microbial and invertebrate decomposers as a result of a reduction in dissolved oxygen (Medeiros et al. 2008). Faster flow velocity may enhance leaf decomposition through increasing shear force on leaf litters (Paul et al. 2006). In contrast, acidification can slow leaf breakdown by affecting the diversity and activity of aquatic organisms (Dangles & Chauvet 2003) and pollution with heavy metals can harm both microbes and macroinvertebrates (Niyogi et al. 2001).

Leaf litter decomposition may also be influenced by the interactions of those aquatic organisms that convert leaf litter mass to fine particulate organic matter (FPOM), dissolved organic matter, and CO_2 (Gessner et al. 1999; Zhang et al. 2003). Starting from microbial colonization, micro-organisms spread over the leaf surface, then penetrate the leaf interior, reducing leaf toughness through hydrolytic processes, and contribute to leaf litter mineralization (Hieber & Gessner 2002; Gessner et al. 2010). Both softened leaves and colonized microbes enhances the food quality, provides important nutrients for invertebrates (Graça 2001). Macroinvertebrates can be classified into five functional feeding groups (FFGs; Mandaville 2002), collector-gatherers (C-G), collector–filterers (C-F), scrapers (Scr), shredders (Shr), and predators (Prd). Among these FFGs, the feeding activity of leaf-shredding insects were thought to be most important in accounting for differences in leaf breakdown rates between streams (Benfield et al. 1991). Shredding invertebrates speed up leaf decomposition by breaking coarse particulate organic matter into smaller fragments (Suberkropp 1998; Gulis & Suberkropp 2003; Martínez et al. 2015). The enhanced surface area of recalcitrant compounds, in turn, stimulates the colonization of microbial species favoring the metabolism of such compounds (Gessner et al. 2010; Noël et al. 2020), contributing to the subsequent decomposition and overall mineralization (Palmer & Ruhi 2019).

In summary, environmental and biological factors contribute synergistically to leaf decay in the aquatic ecosystems. However, the relative importance of environmental and aquatic organisms on leaf breakdown has rarely been studied (Encalada et al. 2010), particularly in streams of shifting habitat status. In this study, I compared leaf litter breakdown rates, as a measure of ecosystem function in three contrasting river types: i.e. (1) degraded urban rivers, (2) urban rivers undergoing habitat restoration and (3) rivers in forested areas (i.e., reference conditions) (Figure 4.1). In each, the importance of the habitat composition, water chemistry and both benthic bacterial and macroinvertebrate communities in two seasons (winter and summer) were assessed. I aimed to determine: (i) how habitat restoration affects leaf litter decomposition? (ii) the relationship between leaf decomposition to both habitat factors (substrate diversity, water velocity) and physico-chemical variables, (iii) the relationship between leaf litter breakdown and benthic organisms, and (iv) which factors contribute to most of the variance in leaf litter breakdown rate within these three river types. Our first hypothesis is that stream habitat restoration would enhance the leaf breakdown rate and be a useful indicator of success. Our second hypothesis is that leaf decomposition would be affected by both abiotic and biological factors. Habitat restoration will lead to faster current velocity that speed the leaf decay through physical process. Improved substrate diversity, dissolved oxygen and living space would shift the community composition and stimulate the microbial and macroinvertebrate activities in decomposing leaf litters. Our third hypothesis is that microbes and macroinvertebrate will contribute more on leaf mass loss than abiotic factors through microbial degradation and feeding activity of shredders.

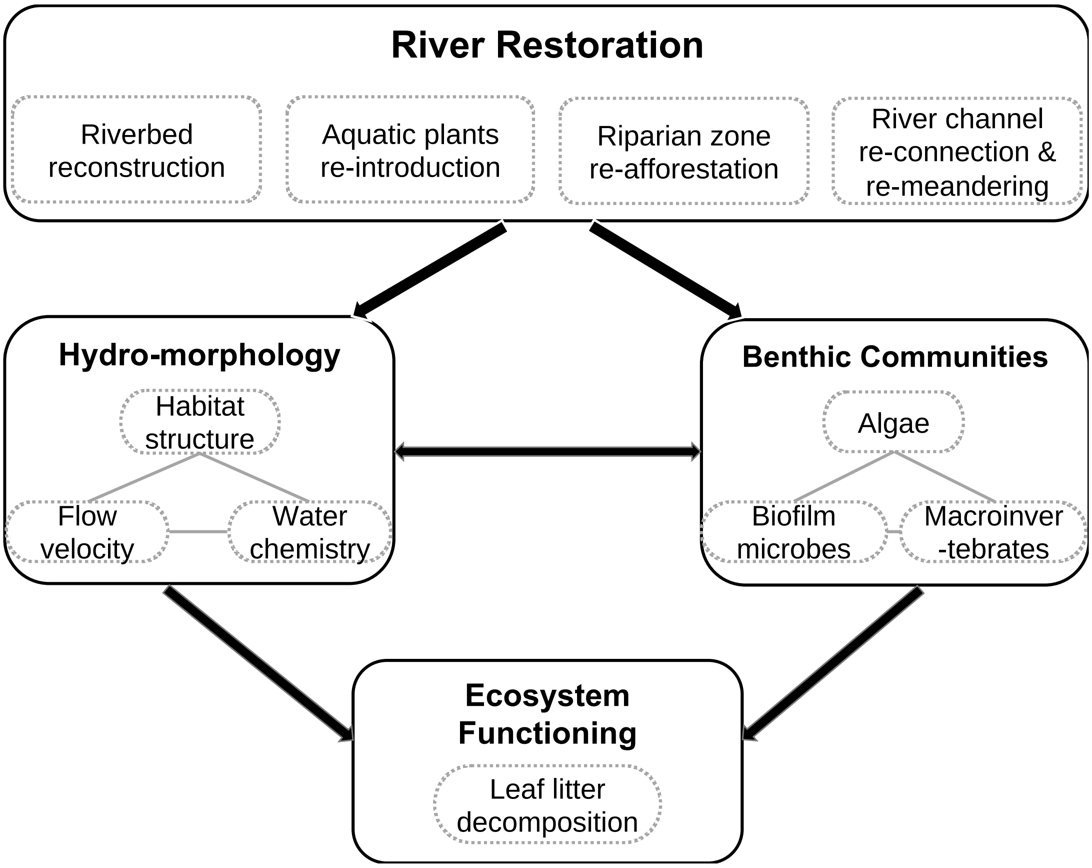

Figure 4.1 Conceptual model of the experiment. River ecological restoration induced the variance of a host of environmental conditions (habitat structure, flow velocity and water chemistry), which in turn influence the ecosystem structure (benthic communities) and ecosystem functioning (leaf litter decomposition) of the river ecosystem.

Practically, knowledge derived from this study will enrich our understanding on the response of ecosystem function to river ecological restoration and linked important controlling variables, which will be useful for policymakers and water managers in future planning of ecological restoration strategies for degraded freshwater streams (Solangi et al. 2019).

4.3 Methods

4.3.1 Study sites

This study investigated three stream types (Figure S4.1), each with three replicates in both winter (December 2017 to January 2018) and summer (June to August 2018). The stream types were a reference forest stream, a restored urban stream, and a degraded urban stream. The nine streams are located in the same watershed (the Shaoxi River), Zhejiang Province PRC within the Anji City Region.

The degraded rivers possessed similar conditions to those in the pre-restored urban rivers (Lin et al. 2019). The degraded urban rivers were canalized with concrete, had high cover of mud, and high pollutant loads and were classified recently as "rivers to be restored" by the local water conservancy bureau. The three restored urban rivers had been restored for up to seven years using an ecological restoration strategy in an attempt to recover a more natural river form. This involved re-connection and re-meandering the river channels, natural reconstruction of the riverbed using diverse substrates (e.g. boulders, cobbles, and pebbles), construction of floating islands, transplant of submerged macrophytes and emergent plants, and riparian zone re-afforestation. The three undisturbed forest streams were 40-km upstream of these urban rivers within the Tianmu Mountains and were viewed as approximations to reference sites, for the pre-urban landscape form they represented (Violin et al. 2011).

4.3.2 Habitat characteristics (denoted Habitat)

Habitat surveys were performed in both winter and summer. Within each river, I visually estimated the reach canopy cover, counted the river-bed types, measured the water velocity across the channel by Teledyne flow meters (ISCO, Lincoln, NE, USA), and tested the substrate composition by selecting 100 sediment particles on the riverbed randomly and counting the percentage of substrate classes (boulders, cobbles, pebbles, sand grains), according to Kondolf (1997). The substrate diversity was calculated for each site by means of the Shannon-Weiner diversity index H' (Shannon 1997).

4.3.3 Physico-chemical parameters of stream water (denoted ENV)

Physico-chemical characteristics of surface water were measured in three sampling spots in each stream in both experimental seasons. pH, dissolved oxygen (DO) and turbidity were *in situ* measured with a HACH pH/temperature meter (LA-pH 10, HACH, Loveland, CO, USA), a YSI (Professional Plus, YSI Incorporated, Yellow Springs, OH, USA), and a turbidity meter (DR2100Q, HACH, Loveland, CO, USA) respectively. One liter of water sample was collected from each location, filtered through 0.45 μm filters, and analysed within 48 hour for a range of chemical measures, these included ammonium nitrogen (NH_4-N), nitrate-nitrogen (NO_3-N), total phosphorus (TP) with a Lachat flow injection analyzer (QuickChem 8500, Hach, USA), total organic carbon (TOC), total nitrogen (TN) with a total organic carbon analyzer (Multi N/C3100, Jena, Germany), and chemical oxygen demand (COD) with a COD analyzer (DR1010, HACH, Loveland, CO, USA).

4.3.4 Spatial factors (denoted Spatial factor)

Geographical position and dispersal across the rivers were assessed using Principal Coordinates of Neighborhood Matrices (PCNM) (Guo et al. 2019). An euclidean distance matrix was calculated using geographic coordinates (latitude and longitude) with the 'earth.dist' function in the 'fossil' R package. PCNM matrices were then derived using the 'pcnm' function in the 'vegan' R package (Jyrkänkallio-Mikkola et al. 2017). Seven PCNMs were generated, and those with positive eigenvalues (PCNM2-5) together with latitude and longitude were used as spatial factors (Guo et al. 2019).

4.3.5 Macroinvertebrates

Macroinvertebrates were sampled in three randomly-distributed sampling quadrats (1m x 1m) close to leaf bags in each river using a kick net (opening: 9.5 cm x 14.5 cm; mesh size: 500 mm) in both winter (January 2018) and summer (August 2018) to coincide with the end of the litter breakdown studies. After disturbing substrates for around ten minutes, macroinvertebrate samples were collected and *in situ* stored in 70% ethanol. Macroinvertebrates were then sorted and identified to family level according to Merritt et al.

(2008). Alpha diversity indices (α-diversity, i.e. total abundance, total richness, Shannon-Wiener diversity) were calculated; all macroinvertebrates except Chironomidae were classified into functional feeding groups (FFGs) at family level according to Mandaville (2002), i.e. shredder, collector-gatherer, predator, scraper, collector-filterer. The relative abundance of each FFG was calculated and analyzed.

4.3.6 Biofilm bacteria

Biofilm colonized on three 10 cm × 10 cm autoclaved unglazed ceramic tiles at 0.3 m water depth of rivers were collected from each river after 39 days experiment in both seasons. After scraping and filtering on 0.22 μm pore size polycarbonate membrane filters (Millipore, MA, USA), DNA was extracted (MO BIO PowerBiofilm® DNA Isolation Kit, MO BIO Laboratories, Carlsbad, CA, USA) for each sample based on these filtrates, the V3-V4 region of bacterial 16S rRNA genes were amplified using PCR primer pairs 237F/802R according to protocol described in Lin et al. 2019, purified via MagPure Gel Pure DNA Mini Kit (Magen, Guangzhou, China) and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) at Suzhou Genewiz Company.

Sequences were treated and analysed via QIIME 1.8.0. Following removal of the primer, all low-quality reads that containing ambiguous characters, a sequence length less than 200 bp, and having an average quality score < 20 were discarded. After removal of chimeras detected using the UCHIME algorithm (Edgar et al. 2011), the high-quality reads were clustered into OTUs (Operational Taxonomic Units) via USEARCH (1.9.6) with a 97% similarity (Edgar 2010). All OTUs were then assigned to taxonomic category using the Ribosomal Database Project (RDP) classifier at a confidence threshold of 0.8. Bacterial α-diversity indices (i.e. Shannon-Weiner index; Chao1 richness) were calculated based on the results of the operational taxonomic units (OTUs).

4.3.7 Leaf litter decomposition

Leaves of *Cinnamomun camphora* (Camphor), an evergreen and widely distributed tree in Southern China, were collected just after abscission around the Xi'an Jiaotong-Liverpool University campus (31°16′28″ N, 120°44′17″ E) in November 2017 and May 2018 for winter and summer experiment, respectively. After gently removing small, attached particles, intact leaves were oven dried at 60 °C for 48 hours, weighed into 5 g groups, and placed in coarse-mesh (8-mm mesh) bags (16/20 cm). Six leaf bags were prepared and distributed at the bottom of each river on the first day of the experiment in each season. Four leaf bags were retrieved from each river after 39d of leaf immersion, with the other two bags missing. The collected leaves were gently rinsed with deionized water, dried at 60 °C to constant mass (48 h), and weighed to the nearest 0.001 g. The leaf breakdown rate was calculated according to the formula:

$$\ln (W_t/W_0) = - kt + b$$

where W_t is the leaf weight remaining at time t, W_0 is the initial leaf weight, *t* is the time in d, and *b* is the y-intercept.

4.3.8 Statistical analysis

All data were analyzed using R (version 3.6.1, R Core Team 2019). Differences in habitat characteristics, water chemistry, α-diversity of bacteria, macroinvertebrate, relative abundance of macroinvertebrate FFGs, and leaf breakdown rate in three stream types as well as the temporal difference of leaf litter decomposition were analyzed using one-way analysis of variance (Torres-Mellado et al. 2012), followed by the Tukey–Kramer post hoc test for comparison of means. To explore relationships between habitat characteristics, physico-chemical features, biofilm bacterial community, macroinvertebrate community, and leaf breakdown rate, Spearman's correlation coefficients were calculated. Environmental factors and leaf decomposition rate were ln (x + 1) transformed if the residuals deviated from normality, and explanatory factor that reflects notable multi-collinearity (Spearman correlation coefficient ≥ 0.85) was excluded from further analysis (Cai et al. 2017). Stepwise multiple regression analysis was implemented to determine the best model that best explained the difference in leaf breakdown rate. Explanatory variables were selected by performing forward selection using the 'adespatial' package in R. Monte Carlo permutation tests was then used to test the response significance of litter breakdown rates to abiotic (physico-chemical and habitat variables) and biotic (bacterial and macroinvertebrate taxonomic variables) indices. Finally, variables selected by forward selection in the 'packfor' R package were assigned into three factor groups (Habitat, ENV, Spatial), all variables were grouped into four explanatory factor groups: habitat, environmental, bacteria, and macroinvertebrate, variation partitioning was performed to test the contribution of spatial factors to the variance in leaf mass loss, and to explore the contribution of abiotic and biotic factors to the variation of leaf breakdown rate using the 'varpart' function in the 'vegan' R package (Oksanen et al. 2019).

4.4 Results

4.4.1 Abiotic variables

The variations of abiotic variables in winter 2017 and summer 2018 are displayed in Table 4.1. Briefly, forest and restored rivers exhibited a substantially greater substrate diversity than degraded rivers ($p < 0.05$). In summer, rivers undergoing habitat restoration have a faster current velocity and lower canopy cover than the other two river types ($p < 0.05$). Degraded rivers had notable greater concentrations of TN, TOC, COD, and turbidity ($p = 0.003$, $p = 0.047$, $p = 0.032$, and $p = 0.014$, respectively) than the forest rivers. Restored rivers had increased turbidity and TN concentrations ($p = 0.013$ and $p = 0.060$, respectively) when compared to forest rivers and a lower concentration of TN than the degraded ones ($p = 0.073$). In winter, forest rivers had greater DO concentrations, lesser TOC, lower turbidity than that in the degraded rivers ($p = 0.029$, $p = 0.002$, $p = 0.018$, respectively), and lower TOC than the restored river ($p = 0.027$); compared with degraded rivers, restored rivers had greater DO and slightly reduced TOC concentration after habitat restoration ($p = 0.049$, $p = 0.122$, respectively).

Table 4.1 Mean values of habitat and physico-chemical variables in different types of rivers in winter and summer within the Anji City Region, PRC. The values represent the mean ± standard error of three replicate samples.

Environmental Variables	Winter			Summer		
	Forest	Restored	Degraded	Forest	Restored	Degraded
Width (m)	8.83 ± 1.64	13.17 ± 3.09	11.57 ± 5.72	13.87 ±2.31	21.87 ±6.15	16.93 ±5.14
Mean Depth (cm)	35.87 ± 7.97	28.13 ± 7.22	22.87 ± 3.86	43.44 ±2.46	36.80 ±4.22	23.93 ±2.01
Substrate	0.92 ±0.11	0.87 ±0.09	0.00 ±0.00	0.89 ±0.13	0.88 ±0.08	0.00 ±0.00
Velocity	0.01 ±0.00	0.41 ±0.17	0.00 ±0.00	0.07 ±0.02	0.61 ±0.12	0.002 ±0.002
Canopy	81.67 ±6.01	4.33 ±2.85	26.73 ±17.59	88.33 ±5.24	4.67 ±0.88	37.50 ±13.79
Dissolved Oxygen (mg/l)	14.16 ± 0.80	13.14 ± 0.65	7.91 ± 1.52	7.48 ±0.18	7.23 ±0.31	4.26 ±1.92
pH	7.33 ± 0.11	7.64 ± 0.14	7.38 ± 0.11	7.18 ±0.10	7.12 ±0.13	7.06 ±0.10
Turbidity	0.62 ± 0.14	3.52 ± 0.85	22.81 ± 14.93	1.54 ±0.46	14.13 ±4.74	12.84 ±3.38
NH_4-N (mg/l)	0.02 ± 0.01	0.08 ± 0.02	1.37 ± 1.19	0.52 ±0.09	0.61 ±0.13	2.30 ±1.29
NO_3-N (mg/l)	1.06 ± 0.13	1.13 ± 0.40	0.79 ± 0.40	0.16 ±0.01	0.69 ±0.22	0.85 ±0.40
TN (mg/l)	1.99 ± 0.21	2.74 ± 0.77	4.01 ± 0.76	1.09 ±0.05	2.07 ±0.47	3.24 ±0.16
TP (mg/l)	0.18 ± 0.02	0.17 ± 0.02	0.18 ± 0.05	0.08 ±0.04	0.07 ±0.01	0.15 ±0.05
Chemical Oxygen Demand (mg/l)	2.44 ± 0.15	3.35 ± 0.76	8.82 ± 3.40	6.29 ±1.96	10.38 ±0.88	16.22 ±2.80
Total Organic Carbon (mg/l)	0.48 ± 0.16	2.81 ± 0.32	6.70 ± 2.21	1.40 ±0.76	2.19 ±0.31	4.93 ±1.16

4.4.2 Biotic variables

As summarized in Figure 4.2a-e and Table 4.2b, the taxonomic diversity of macroinvertebrate as well as the relative abundance of shredder and collector-gatherer tested in summer 2018 were much smaller in degraded rivers, and greater in forest and restored rivers ($p < 0.05$). No difference of these indices was recorded between restored rivers and reference forest rivers ($p > 0.05$). No differences were detected among three river types with regard to biofilm bacterial taxonomic compositions (Table 4.2, Chao1 richness ($p > 0.05$). Bacterial Shannon-Wiener diversity was much greater in restored and degraded rivers than forest ones ($p < 0.05$); restored rivers had comparable bacterial diversity with degraded ones ($p > 0.05$) (Figure 4.2f).

In winter 2018, the taxonomic diversity of macroinvertebrates had a similar trend as that collected in summer investigation (Table 4.2b). Macroinvertebrate α-diversity presented considerable heterogeneity for total abundance ($F_{2,6}$ = 18.19, p = 0.0037), total richness ($F_{2,6}$ = 19.14, p = 0.0033), Shannon-Wiener diversity ($F_{2,6}$ = 17.91, p = 0.0039), relative abundance of shredder ($F_{2,6}$ = 12.9, p = 0.0088) and relative abundance of collector-gatherer ($F_{2,6}$ = 21.07, p = 0.0025). Forest and restored rivers have far more macroinvertebrate abundance, richness, and Shannon-Wiener diversity than degraded rivers ($p < 0.05$; Figure 4.2a-c), restored rivers have similar taxonomic diversity to forest rivers ($p > 0.05$). The relative abundance of shredder and collector-gatherer species were greater in restored and forest rivers ($p < 0.05$) than in degraded rivers (Figure 4.2d-e). In terms of winter bacterial α-diversity, as shown in Table 4.2a, a greater diversity of bacteria was found in degraded rivers than restored rivers, restored and forest rivers had fewer and comparable bacterial diversity (Figure 4.2f).

Table 4.2 Mean values of (a) bacterial α-diversity indices and (b) macroinvertebrate taxonomic metrics in different types of rivers in winter and summer within the Anji City Region, PRC. The values represent the mean ± standard error of three replicate samples.

(a)

Bacteria	Winter			Summer		
	Forest	Restored	Degraded	Forest	Restored	Degraded
OTU	615.22 ±41.9	585.00 ±19.86	666.89 ±69.17	338.44 ±60.8	566.00 ±217.7	574.83 ±55.7
Chao1	715.45 ±36.2	708.84 ±21.1	769.73 ±72.81	423.27 ±78.52	661.97 ±236.1	724.64 ±60.4
Shannon	6.42 ±0.12	5.89 ±0.15	6.98 ±0.16	5.89 ±0.15	6.98 ±0.16	6.42 ±0.12

(b)

Macroinvertebrate	Winter			Summer		
	Forest	Restored	Degraded	Forest	Restored	Degraded
Abundance (ind.)	387.44 ±18.42	353.78 ±28.66	21.11 ±10.26	445.11 ±98.6	643.55 ±117.44	21.33 ±10.48
Richness (taxa no.)	17.00 ±0.77	17.50 ±1.55	2.44 ±0.11	23 ±2.53	19.78 ±0.22	2.67 ±0.19
Shannon Diversity	2.00 ±0.03	1.91 ±0.23	0.55 ±0.13	2.29 ±0.05	1.95 ±0.21	0.57 ±0.11
Shredder (ind.)	4.78 ±1.31	3.78 ±1.79	0.33 ±0.33	12.55 ±2.15	56.22 ±50.9	0.44 ±0.29
Collector-gatherer (ind.)	185.22 ±35.06	156.89 ±35.32	6.11 ±1.94	199.77 ±51.49	314.67 ±115.19	6.11 ±1.94

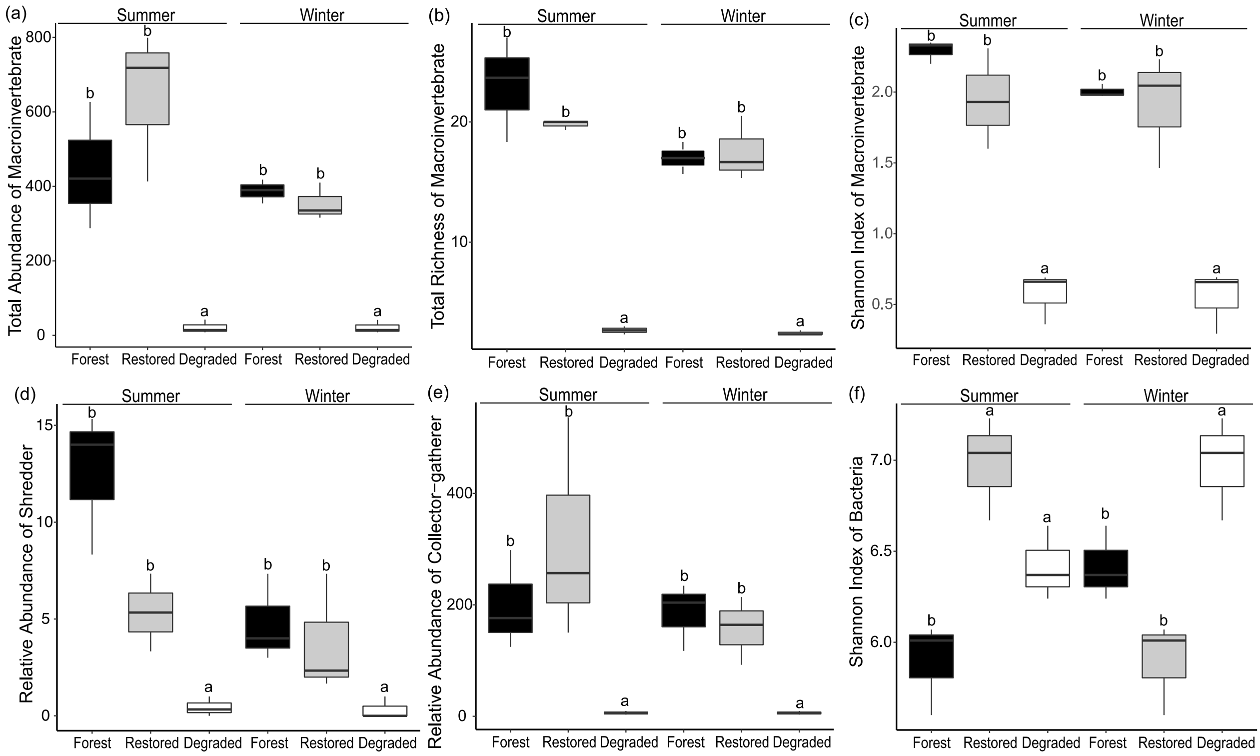

Figure 4.2 Comparison of the macroinvertebrate taxonomic diversity (a) total abundance, (b) total richness, (c) Shannon-Wiener diversity, (d) relative abundance of shredder, (e) relative abundance of collector-gatherer and bacterial diversity (f) Shannon-Wiener diversity of bacteria in three river types in summer and winter within Anji City Region, PRC. Mean values (± SE, n = 3) are presented; different lowercase letters indicate a significant difference observed at $p = 0.05$ level.

4.4.3 Leaf breakdown rate in winter and summer

In both winter and summer, significant differences of leaf breakdown rate were found among river types (Winter: $F_{2,6} = 13.58$, $p < 0.01$; Summer: $F_{2,6} = 20.79$, $p < 0.01$). Forest and restored rivers possessed faster leaf decay rates than degraded rivers in either winter or summer ($p < 0.01$; Figure 4.3). No difference in leaf decomposition rate was observed when comparing forest with restored rivers during both experiment periods ($p > 0.05$).

Temporally, leaf litter decay faster in summer than winter ($F_{5,12} = 0.001$, $p < 0.01$). In contrast to winter leaf litter decomposition, the leaf breakdown rates were greater in summer in either forest river ($p = 0.005$), or restored rivers ($p = 0.003$). No difference in leaf decomposition, however, was found in degraded rivers between winter and summer ($p > 0.05$).

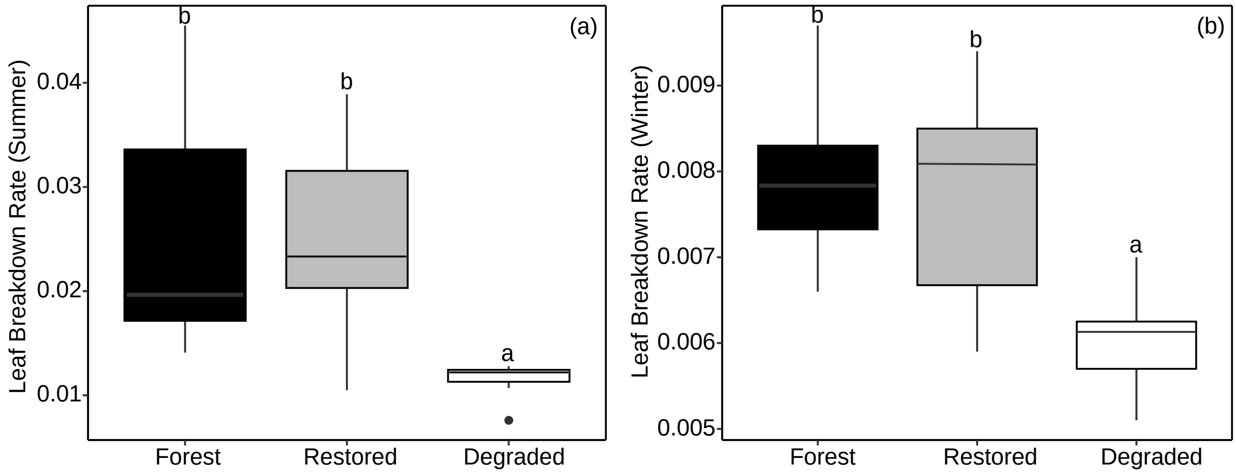

Figure 4.3 Boxplots illustrating leaf breakdown rates in summer (a) and winter (b) in forested, restored, and degraded rivers within the Anji City Region, PRC. Blackline: median value; box: quartile interval; whiskers: minimum and maximum value. Different lowercase letters indicate the significant difference observed at the $p = 0.05$ level.

Table 4.3 Correlations between environmental variables (i.e. habitat characteristics, physico-chemical variables) and leaf litter breakdown rates by days ($k.d^{-1}$) for three types of rivers within Anji City Region, PRC. Negative coefficients are specified in capturing parentheses.

ENV Variables	Summer Leaf Breakdown Rate	Winter Leaf Breakdown Rate
pH	0.3933	0.0084
Turbidity	(0.1925)	(0.6946)$^{\cdot}$
DO	0.3933	0.5523$^{\cdot}$
NH_4-N	(0.7113)$^{\cdot}$	(0.5774)
NO_3-N	(0.4435)	0.3347
TN	(0.5439)*	(0.3766)
TP	(0.1681)	(0.2343)
TOC	(0.7448)*	(0.5272)$^{\cdot}$
COD	(0.6092)	(0.8117)*
Velocity	0.7969	0.7010*
Substrate	0.6809*	0.5958*
Canopy	0.3598	0.3766

Note: The one superscript asterisks and dots show the significant level at $p < 0.05$ and 0.1, respectively.

4.4.4 Correlation between environmental factors and leaf breakdown rate

The correlation coefficients between abiotic factors (including habitat features and physico-chemical variables) and leaf litter decomposition rate in both summer and winter experiment period are displayed in Table 4.3. Leaf litter decomposition rate in summer periods had strong, positive correlations with habitat characteristics (substrate diversity) and negative correlations with surface water chemical variables (TOC, TN, and NH_4-N). In winter 2018, leaf litter decompositions were correlated positively with DO, water velocity, substrate diversity, and negatively with water turbidity, TOC, and COD concentrations.

Stepwise regression analysis indicated a greater correlation with substrate diversity (r^2 = 0.567, $p < 0.05$) than physico-chemical variable TOC (r^2 = 0.489, $p < 0.05$) in summer

(Figure 4.4a-b). In winter, leaf decomposition rates were highly correlated with substrate diversity (r_2 = 0.456, p < 0.05) and COD (r_2 = 0.711, p < 0.01; Figure 4.4c-d).

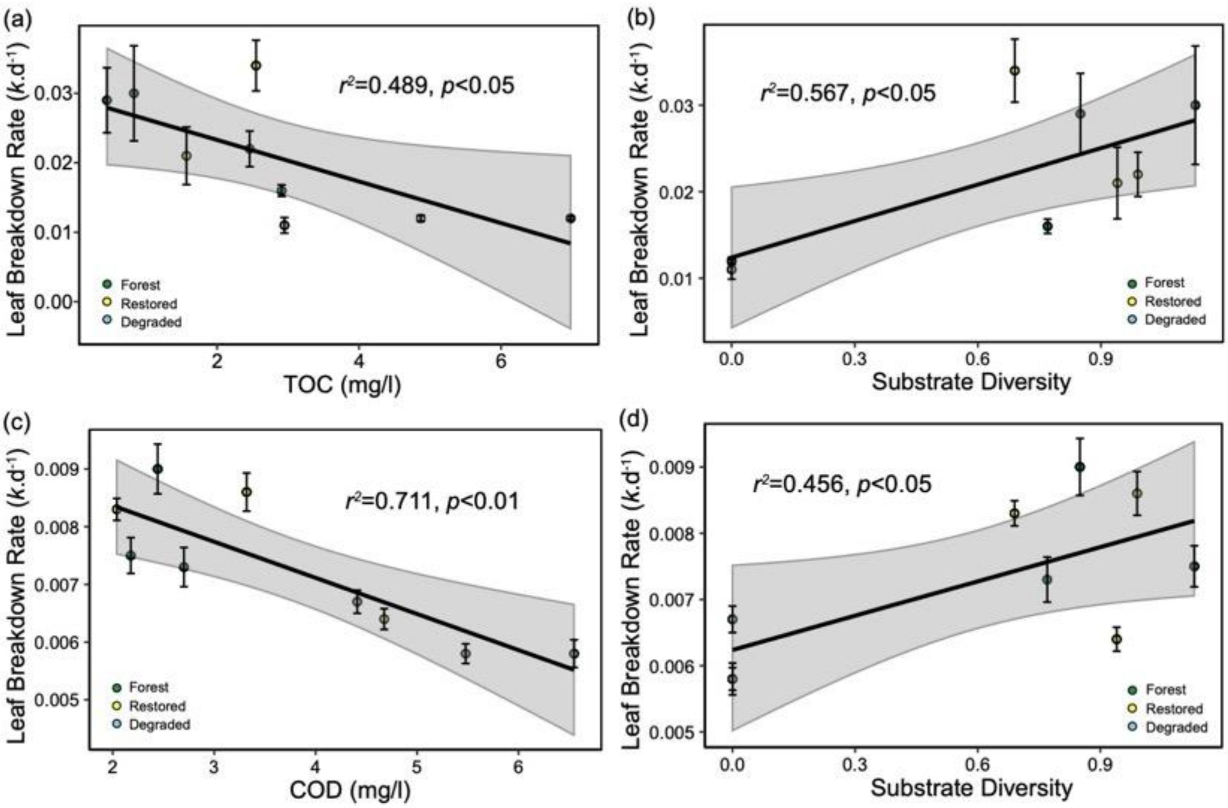

Figure 4.4 Stepwise multiple regression analysis to identify the relationship between leaf litter breakdown rates by days (k.d$_{-1}$) and physicochemical variable TOC (a), habitat factor Substrate diversity (b) in summer and Physico-chemical variable COD (c), habitat factor Substrate diversity (d) in winter. The coefficients of determination (r_2) and p are shown in each panel. Each data point represents the mean value of each treatment in each stream.

4.4.5 Correlation between benthic organisms and leaf breakdown rate

Leaf decay rate was positively related to the abundance, richness, Shannon-Wiener diversity index of macroinvertebrate, and relative abundance of functional feeding groups such as shredder in both winter and summer (Table 4.4). Though the leaf breakdown rate was more related to macroinvertebrate richness in summer and macroinvertebrate abundance in winter, stepwise regression indicated that the summer litter decay rate was multi-linearly linked to total abundance, total richness and relative abundance of collector-gatherer. The predicted values generated based on the model (k = 0.00003*Abundance+0.00046*Richness-0.00004*cg+0.00954) showed a strong fit (r_2 = 0.925, p < 0.01; Figure 4.5a). In terms of winter decomposition, it was strongly related to macroinvertebrate richness ($r^2 = 0.543$, $p < 0.05$; Figure 4.5b).

Table 4.4 Spearman correlation coefficients between biotic factor (i.e. bacterial diversity, macroinvertebrate alpha diversity, and the relative abundance of shredders, collector-gatherers) and leaf litter breakdown rates by days ($k.d^{-1}$) for different types of rivers within Anji City Region, PRC. Negative coefficients are specified in capturing parentheses.

Biotic Indices	Summer Leaf Breakdown Rate	Winter Leaf Breakdown Rate
Bacterial Richness	(0.0167)	(0.1674)
Bacterial Diversity	(0.1674)	(0.3766)
Invertebrate Abundance	0.8285*	0.8619*
Invertebrate Richness	0.8992*	0.6513*
Invertebrate Diversity	0.8536*	0.6778*
Shredder	0.8787*	0.7468·
Collector-gatherer	0.6862	0.5774·

Note: The one superscript asterisks and dots show the significant level at $p < 0.05$ and 0.1, respectively.

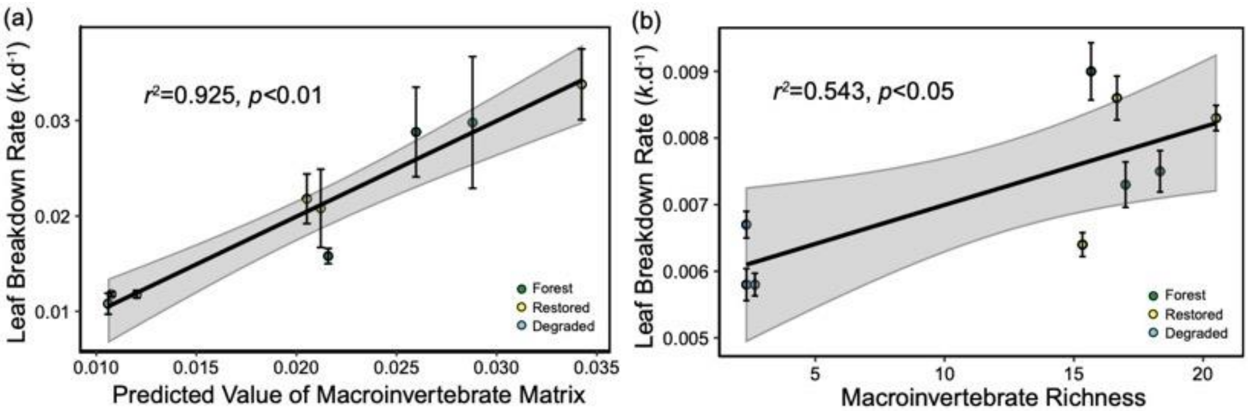

Figure 4.5 Stepwise multiple regression analysis to identify the relationship between leaf litter breakdown rates by days ($k.d^{-1}$) and (a) predicted value of macroinvertebrate matrix in summer and (b) macroinvertebrate richness in winter. The coefficients of determination (r^2) and p are shown in each panel. Each data point represents the mean value of each treatment in each stream.

4.4.6 Contribution of abiotic and biotic factors in leaf decomposition

To determine the influence of environmental factors on leaf breakdown rate, spatial factors in particular, abiotic factors were assigned to three factor groups: Habitat, ENV, and Spatial. The results demonstrated that environmental factors explained 68% of variance in summer leaf decomposition and 33% of variance in winter leaf decay, respectively (Figure 4.6ab). Most of the variation were explained by habitat variables (44% in summer, 15% in winter), spatial factors explained the lest of variation (6% in summer, 0% in winter).

To explore the driver of leaf decomposition in freshwater ecosystems, abiotic and biotic variables tested were assigned into four sets of explanatory factor groups: habitat characteristics (denoted Habitat), physico-chemical variables (denoted ENV), macroinvertebrate matrix (denoted Macroinvertebrate) and bacterial alpha diversity (denoted Bacteria). Variation partitioning revealed that 99% of the variation of the summer leaf breakdown rate was explained; macroinvertebrate taxonomic matrix accounted for most of the variance of decomposition (52%), followed by habitat factors (17%) and physico-chemical variables (11%) (Figure 4.6a). 59% of the total variation was shared by ENV, Habitat, and Macroinvertebrate, additionally, Habitat and Macroinvertebrate accounted for 4% of the decomposition variance. Bacteria explained nothing on its own, however, 11% of the variation was shared by ENV, Macroinvertebrate, and Bacteria, 5% shared by ENV, Habitat, and Bacteria and 5% shared by Habitat, Macroinvertebrate, and Bacteria. No shared effect was found among four sets of factor groups (Figure 4.6c).

In terms of winter litter breakdown, 80% of the variation was explained by the four-factor groups. Macroinvertebrates still contributed most to leaf decomposition among river types (33%), Habitat accounted for comparable variance (29%), followed by Bacteria (15%) and ENV (1%). Moreover, 55% of the total variance was shared by all four factors, 34% shared by ENV and Bacteria, 30% shared by Habitat, Macroinvertebrates and Bacteria, 20% by ENV, Habitat, and Macroinvertebrates, 11% by ENV and Habitat, and 1% by ENV and Macroinvertebrates (Figure 4.6d).

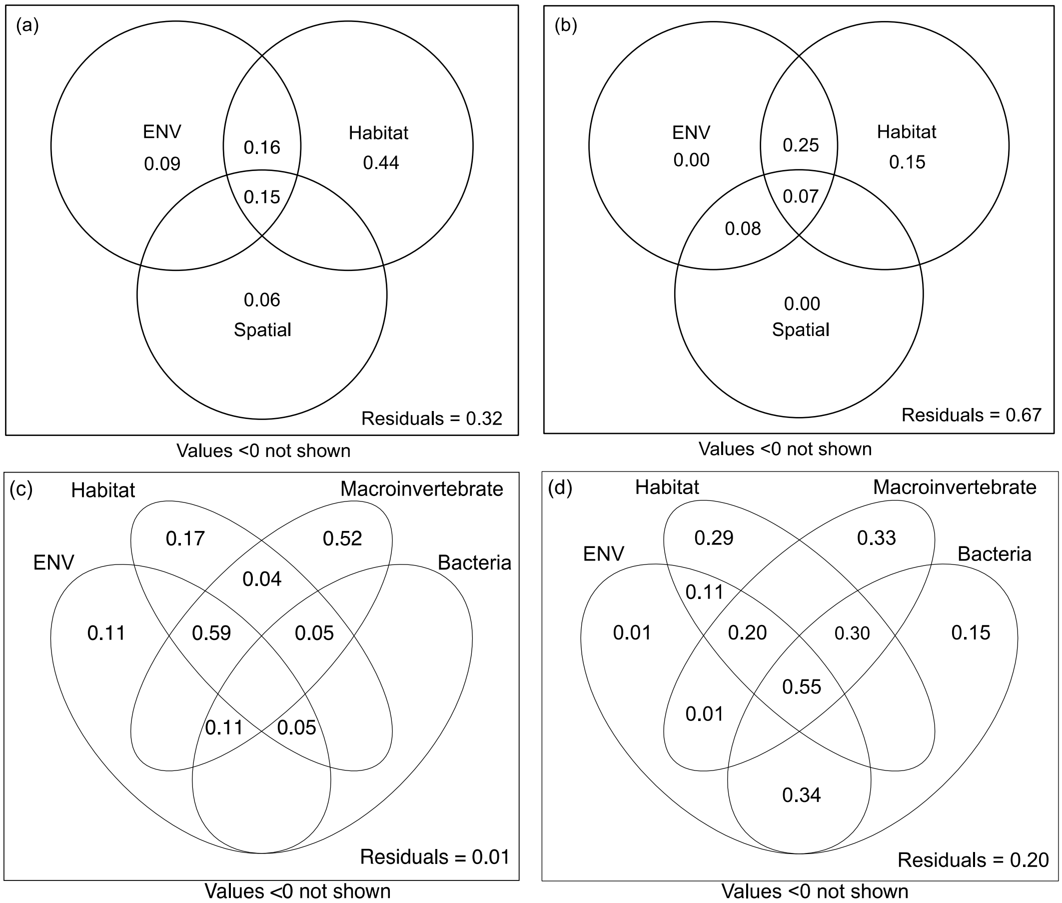

Figure 4.6 Venn diagrams illustrating the variation partitioning analysis for leaf litter breakdown rates by days (k.d^{-1}) in (a,c) summer and (b,d) winter. Habitat, ENV, Spatial, Macroinvertebrate, and Bacteria are sets of explanatory factor groups representing habitat variables, physico-chemical variables, spatial factors, taxonomic diversity of macroinvertebrate, and taxonomic diversity of biofilm bacteria, respectively. Residuals are shown in the lower right corner. All fractions based on adjusted R^2 are shown as percentages of the total variation.

4.5 Discussion

4.5.1 Leaf decomposition in degraded-restored-forest streams

Our overarching result that significant differences in leaf breakdown rate were found among the three river types in both winter and summer support our first hypothesis that stream habitat restoration would enhance the leaf breakdown rate significantly. Indeed, leaf breakdown happened much faster in the restored rivers than the degraded ones, in accordance with previous research that increasing habitat heterogeneity following habitat restoration drove elevated litter decomposition rates (Frainer et al. 2014, 2017). This suggests that habitat restoration can assist in reversing river degradation by enhancing habitat heterogeneity and improving the ecosystem function. Leaf litter decomposed at comparable speeds in the restored and the forest rivers indicated that the ecosystem function has been recovered to natural status under river management. A further important result was that environmental factors, including habitat characteristics, physico-chemical variables in the surface water and spatial factors, contributed to the differences in leaf decomposition rates among the river types. Habitat factors appeared to be more important in controlling leaf decomposition than physico-chemical variables. These results are in line with Frainer et al. (2017) who showed leaf decomposition was positively related to habitat heterogeneity. Spatial factors had the least contribution in both experiment periods, indicating that the spatial variation in sampling sites has little influence on our experiment, rather than spatial factors (i.e. longitude or latitude), local environmental conditions best determined the variance of leaf mass loss.

In the winter, the restored rivers had a more diverse substrate mix and faster leaf mass loss rate than degraded rivers, results similar to those of Rasmussen et al. (2012), indicating that streams with more heterogeneous physical habitats had faster litter decomposition rates than streams with uniform physical habitats. Riverbed reconstruction and aquatic macrophytes re-introduction implemented in the restored rivers enhanced the habitat heterogeneity (Taniguchi et al. 2003), providing living habitat for periphyton, which in turn increased the activity of microbes and the abundance of shredding invertebrates (Ledger & Hildrew 2005; Jarno et al. 2018) on leaf decomposition. Moreover, restored rivers possessed higher DO and lower TOC concentration than the degraded urban rivers, which also led to faster decomposition in restored rivers (Medeiros et al. 2008; Graça et al. 2015). With saturated DO induced by hydraulic connection and the re-introduction of aquatic plants in the restored rivers, a reduce of previous concentrated organic matter provides energy and nutrients resources for both microbes and macroinvertebrates, hence stimulating leaf litter decomposition (Graça 2001). However, very high concentration of organic matter including complex pollutants caused by urbanization depletes DO (Allan 2004), which in turn reduces the activity of microorganisms (fungal, bacteria) and shredder abundance, both of which affect leaf decomposition in degraded rivers (Wantzen & Wagner 2006; Lujan et al. 2013; Graça et al. 2015).

In the summer, the restored rivers had a greater substrate diversity and faster flow velocity than degraded ones. The faster flow caused by channel reconnection increases the shear force on leaf litters (Paul et al. 2006), and along with the enhanced substrates produced during riverbed reconstruction stimulates the growth of abundant microbial and shredding decomposers (Shi et al. 2019), which all combine to produce faster leaf litter decomposition in the restored rivers. Moreover, due to increased flow and developed nutrient cycling, the TN concentration in the restored rivers was lower than that in degraded rivers, but greater than the TN concentration in forest rivers. These moderate dissolved nutrient concentrations in rehabilitated streams provide aquatic biotas with abundant food resources, which in turn promote the metabolism activities (including organic matter breakdown) of biotas in the form of microbial decomposition (Hladyz et al. 2010; Ferreira & Chauvet 2011) and invertebrate decomposition (Gulis et al. 2006). On the contrary, leaf decomposition was reduced in the degraded rivers where habitat diversity was low and eutrophication was present, presumably by the depletion of dissolved oxygen (Allan 2004), and reduced abundance and activity of leaf associated aquatic organisms (Couceiro et al. 2006), here measured as total macroinvertebrate abundance and leaf-shredding species, such as shredders and collector-gatherers, which have greatest decomposition capacity in the first phase of leaf litter decay (Gingerich et al. 2015; Tiegs et al. 2013).

Leaf decay much faster in summer in both forest and restored rivers, which is in line with Follstad Shah et al. (2016) who suggested that warming could result in a dramatic increase in leaf breakdown rates. This could in part be attributed to the enhanced shear force on leaf litters due to the speed flow velocity in summer in both river types, and in part let by the increased water temperatures which together stimulates the metabolism of microbial and macroinvertebrate decomposers in streams with heterogeneous habitat (Gonçalves et al. 2013; Follstad Shah et al. 2016). It is notable that, no difference in leaf breakdown rate was observed in different seasons in degraded rivers. Relative low abundance, richness and diversity of detritivores presented in the degraded rivers might diminish the litter breakdown increases with temperature (Boyero et al. 2011).

4.5.2 Bacteria on leaf decomposition

Biofilm bacteria play an important role in the initial decomposition of organic matter such as leaf litters (Bärlocher 2005) as they break down large molecules (cellulose, chitin, and lignin) within leaf litters into smaller compounds through biochemical and physiological processes (Das et al. 2007). Here, bacteria contributed less than macroinvertebrate to the variance of leaf decomposition rates. Bacterial α-diversity accounted for none in the summer and 15% in winter leaf decomposition. This result is in accordance with Baldy et al. (1995) who showed that bacteria contributed little to leaf litter breakdown in a large river and another study which indicated that bacteria contribute less (4.2 to13.9%) to overall leaf carbon loss in a polluted river (Pascoal & Ca´ssio 2004). The aerobic atmosphere in the studied rivers studied here might limit the contribution of bacteria in leaf litter decomposition as bacteria contribute more to leaf decay under anoxic or hypoxic conditions (Pascoal & Ca´ssio 2004). Biofilm samples collected from the ceramic tiles rather than leaf litters might also interpreted the less contribution of bacteria to some extent, for the difference in bacterial community compositions between epilithic biofilm and biofilm associated with plant litter, although the difference is less pronounced (Buesing et al. 2009). However, bacteria account for more variance in winter leaf decomposition than summer ones. Less diverse bacteria in winter may enhance their contribution, as bacterial diversity was linked negatively to the leaf decomposition (r = -0.1674 in summer, r = -0.3766 in winter, respectively). However, litter decomposition can be controlled by the biodiversity, biomass, and activities of bacteria (Lecerf et al. 2005), evaluating α-diversity alone in this study may obscure the contribution of bacteria in leaf mass loss (Gulis et al. 2006).

Moreover, aquatic fungi, mainly hyphomycetes, have been reported to be more important in the early stages of leaf litter decomposition than bacteria (Rasmussen et al. 2012). Although microbial leaf decomposition results from the combined actions of fungi and bacteria (Das et al. 2007), fungi are more efficient than bacteria in leaf breakdown through invasion and enzymatic hydrolysis of leaf material and lysed hyphae (Chamier 1985; Shearer 1992; Das et al. 2007). Here, unfortunately, fungi were not taken into consideration and this limits the comprehensive interpretation of leaf litter decay.

4.5.3 Role of macroinvertebrates on leaf mass loss

Apart from physical abrasion and microbial degradation, invertebrate fragmentation is one of the most important processes in leaf decomposition (Graça 2001; Zhang et al. 2003). Here, the abundance, richness, and diversity of macroinvertebrate in conserved rivers (forest and restored rivers) were greater than those in urban degraded rivers, attributing to the enhanced habitat substrate diversity, faster water current flows, and improved water quality (Iñiguez-Armijos et al. 2016; Turley et al. 2016) in the restored rivers. Among all factors tested, macroinvertebrate indices account for most of the leaf decomposition variance, 52% in summer and 33% in winter, respectively, and are similar to those of Gingerich, Panaccione & Anderson (2015). The macroinvertebrate contribute greater to leaf decay than physico-chemical and microbial factors. Invertebrates play dominant roles in the later stage of breakdown (Webster & Benfield 1986), mainly due to the increased macroinvertebrate abundance, diversity, and subsequent macroinvertebrate associated leaf-shredding activities, as leaf decomposition had significant positive correlations with macroinvertebrate α-diversity indices.

Aquatic decomposition is often driven by invertebrates known as shredders (Encalada et al. 2010; Chara-Serna et al. 2012; Iñiguez-Armijos et al. 2016). Here, leaf breakdown rate in both summer and winter were all associated positively with shredder abundance (r = 0.8787 in summer, r = 0.7468 in winter, respectively). Consistent with researches which demonstrated a weakened leaf decay due to a decreased shredder abundance (Wallace &

Webster 1996; Sponseller & Benfield 2001). The relative abundance of shredders was greater in heterogeneous habitat rivers (forest and restored rivers) than degraded rivers (Frainer et al. 2017), demonstrating the reasons for faster leaf decay in the forest and restored rivers compared to degraded rivers. However, elsewhere it has been shown that shredders play a minor role in leaf litter breakdown in neotropical streams (Mathuriau & Chauvet 2002; Goncßalves et al. 2007). Further studies might help to explore the cause of variations.

4.6 Conclusions

This study indicates that habitat restoration had an important positive effect on leaf breakdown rates in river ecosystems, hence enhancing ecosystem function. Leaf litter decayed faster in rivers under positive management (forest and restored rivers) than degraded urban rivers. Leaf decomposition rate can, therefore, be a good indicator of successful ecological restoration. All factors measured here (i.e., physico-chemical factors, habitat factors, macroinvertebrate, and bacteria) made an appreciable contribution to the leaf litter breakdown process in our study streams. Our results suggest that under habitat restoration, faster water and a more diverse substrate increased the physical abrasion of the leaf litter by stronger shear forces, enhanced the metabolism of leaf litter by active benthic biological decomposers such as macroinvertebrates and bacteria. Accelerated nutrient dilution and cycling declined excessive nutrients and organic pollutants in the surface water of the restored rivers, which in turn promoted the productivity and activity of decomposers by providing moderate nutrient and appropriate living habitat. The biofilm bacteria present can break down large molecules of leaf litter into smaller compounds for macroinvertebrates and the greater abundance of shredders can combine to produce a faster leaf decay rate in the forest and restored rivers compared to degraded rivers through feeding activities. To summarize, all factors evaluated in this study played a synergetic contribution to the change in leaf litter decomposition rates among the three river types. The role of macroinvertebrates, mainly shredders appeared to be particularly important, followed by habitat factors, physico-chemical variables, and biofilm bacteria. For the comprehensive evaluation of the stream ecosystem function, leaf-associated fungal community and microbial production should also be tested in future determinations.

Our findings show that the habitat restoration of streams can improve degraded streams by increasing habitat elements, enhancing channel connectivity, changing water chemistry and aquatic communities (e.g., microbe, macroinvertebrate), all of which combine to improve energy and nutrient cycling process, here measured using leaf litter decomposition rates. Habitat restoration positively affected the structure and function of the deteriorate stream ecosystems. The overall findings of this study contribute to our understanding of the responses of ecosystem function to habitat restoration in urban rivers, providing useful evidence that habitat restoration can be used as an effective measure of freshwater management via recovering ecosystem structure and function. For future water conservation and management, I recommend that habitat features, physico-chemical properties and aquatic organisms should be taken into consideration in ecological restoration strategies to restore the ecosystem integrity and related ecosystem process.

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Chapter 5 Resilience of stream biofilm bacterial communities to drying perturbation in stream ecosystems: The effect of habitat heterogeneity

5.1 Abstract

Climate change and anthropogenic activities induced flow intermittent becomes a big challenge that influence the aquatic ecosystems. Upon the habitat restoration of stream ecosystems to mitigate existing stream degradation and pollution, little is known how restored ecosystem could resist to future disturbance, including the flow intermittent caused. To understand the resilience of aquatic ecosystems, especially the aquatic community structure to drying perturbation in streams of different habitats, using benthic biofilm bacteria as bioindicator, an Ex-Stream experiment was conducted to investigate: (i) how heterogeneity habitat influences benthic bacterial community composition and their diversity; (ii) what pattern do benthic bacterial community composition show under flow intermittence; (iii) if benthic bacterial community could persist in drying condition; (iv) the resilience of benthic bacterial community in streams of different habitats. The results demonstrated a shift of bacterial community compositions either after drying events or flow resumption. The bacterial richness and diversity were remarkably increased in streams with low-level and medium-level habitat after a longtime drying and reached a comparable status with permanent ones in all stream types after flow resumption, except for an increased in bacterial diversity in low-level habitat streams, mitigating the functional legacy induced by flow intermittent. Longtime drying diminished the chitin degradation in streams with low-level and medium-level habitat and improved the sulfate degradation, nitrogen fixation, decreased the atrazine metabolism function in all three streams types with the shape of hypoxic or anoxic mosaic habitat. Through speed activation and recolonization of microbes, rewetting positively stimulated the microbial metabolism processes such as chitin degradation, atrazine metabolism and aromatic hydrocarbons degradation in high-level habitat streams, and chitin degradation in medium ones. Indicating that biofilm bacteria hold excellent resilience capacity towards flow intermittent, particularly in streams with

heterogeneous habitat. Consequently, habitat restoration implemented for the recovery of degraded streams can possess greater resilience capacity toward future hydrological changes and uncertainty.

Keywords: resilience, biofilm bacteria, drying, flow intermittent, habitat, stream ecosystem

5.2 Introduction

Enhancing habitat heterogeneity is a major focus of habitat restoration of freshwater rivers. This is often achieved at the reach scale with the aim of increasing hydraulic and substrate heterogeneity. Habitat restoration projects monitored in these years received positive or no feedback toward biodiversity or ecosystem function. Some studies indicated that with the enhancement of habitat homogeneity moved aquatic communities in the direction of pristine status (Lin et al. 2019), while others found little change of biodiversity as a result of habitat restoration (Alexander & Allan 2007; Louhi et al. 2011), and, therefore, the underlying mechanisms are still unclear. Hence, it is important to understand the underlying mechanisms of habitat restoration, and most important, to test how heterogeneity habitat promotes changes in ecosystem structure and function. It is also important to know if a stream with a heterogeneous habitat is more resistant to change or resilient to future environmental disturbance, such as intermittent flows induced by changing climate or rapidly increasing human activities (Datry et al. 2017).

With both climate change and anthropogenic disturbance, it is predicted that there might be an increase in variability in water flow intermittent with extended droughts, with an increase rate of 5% by 2050 in Albarine River in France (Cipriani et al. 2014), for instance. Flow intermittent are occurred in stream ecosystems worldwide (Datry et al. 2017), which severely impacted the integrity and stability of fluvial ecosystems (Sutherland et al. 2008; Sabater et al. 2016). Intermittent flows disrupt hydrological connectivity (Larned et al. 2010; Datry et al. 2016), impact the durations and volumes of flow (Datry et al. 2017), shift habitat patches within channels (Datry et al. 2014), and hyporheic flow followed by (Febria et al. 2012), leading to perpetual fluctuation in ecological processes and resident communities (Dudgeon et al. 2006). Streams and rivers in many regions shift from perennial to intermittent flow regimes (i.e. Mediterranean streams) and this had large impacts on aquatic organisms and ecosystem functions (Sutherland et al. 2008; Döll & Schmied 2012). Prolonged drought threatens the integrity and activity of sediment biofilms (Timoner et al. 2012), bacterial biofilm community composition (Amalfitano et al. 2008; Febria et al. 2012), and reduces macroinvertebrate richness (Stubbington & Datry 2013). The intensity and duration of drying influences both the structural and functional resistance and resilience of biofilms (Stubbington & Datry 2013; Gionchetta et al. 2018). However, whether internal habitat heterogeneity can support biofilms (bioindicator of river health) in drying conditions remains poorly understood.

Resilience mechanisms indicate that resource competition/facilitation, recruitment and habitat heterogeneity provide ecological resilience to biological communities (Looy et al. 2019). Habitat-specific physicochemical characteristics may dictate the composition of microbial communities, easy dispersal characteristic of microbial communities throughout stream networks (Hempel et al. 2010) may aid the resilience of biofilm toward drought perturbation. Doreeto et al. (2018) indicated that one of the most important factors to guarantee resilience of benthic invertebrate communities is the presence of in-stream refuges, a set of microhabitats including pools (Chester & Robson 2011; Verdonschot et al. 2015), the hyporheic zone (Brunke & Gonser 1997; Wood et al. 2010), wet sediments, seeps, lateral aquatic habitats, and the organic debris (Robson et al. 2011). Recovery of invertebrate communities after droughts occurs not only by upward movements from subsurface (hyporheic) refuges (Vander et al. 2016) but also by downstream migration, notably by drift, and also hatching or reactivation of drought resistant stages, upstream movements, and aerial recolonization either by adults or through oviposition (Lake 2000). However, empirical evidence for the habitat resilience of biofilm microbial community remains poorly documented (Johnstone et al. 2016).

Hence, in this study, I compared biofilm-forming microbial communities in streams with three habitat types (low-level, medium-level and high-level habitat treatments), along with three flow conditions including a short drying time, a relative long time drying, and a rewetting. I investigated the composition of their microbial communities using 16S rRNA genes targeted Illumina MiSeq and linked this to habitat types. I aimed to investigate:

(i) How is the benthic bacterial community composition and their diversity affected by habitat heterogeneity?

(ii) What pattern do benthic bacterial community composition show under different flow regimes?

(iii) Can benthic bacterial communities persist in different flow regimes?

(iv) How resilient are the benthic bacterial community in streams with habitat heterogeneity?

I hypothesized that (i) heterogeneous habitat would provide variable hydrologic conditions, a mosaic of habitat patches that confer habitat heterogeneity and promote specialization of organisms (Townsend & Hildrew 1994). Thus, streams with a more heterogeneous habitat would support a more diverse benthic bacterial community composition. In turn, diverse living organisms promote better nutrient and energy cycling within stream ecosystems, making it healthier and more sustainable development; (ii) stream with a more heterogeneous habitat would provide numbers of strategies and refuges for living organisms encountering drying conditions, and hence would have a greater resilience than less heterogeneous ones. However, different species may act differently because of their life histories and their sensitivities to drying condition.

5.3 Materials and methods

5.3.1 Mesocosm system set-up

This study was performed in spring (7th April to 6th June 2019) on the right bank of the Yinxi stream, a near-pristine 2nd-order montane stream oriented from the Huangshan Jiulongfeng Nature Reserve Park (Huangshan city, Anhui province, China, 30°07'07''N, 118°01'24''E). The experiment was performed in an Ex-Stream System (Piggott et al. 2015) comprising 48-unit circular mesocosm systems (Figure 5.1). The stream mesocosm systems were supplied continuously with water pumped from Yinxi Stream thus enabling natural immigration and emigration of periphyton, microbes and invertebrates by drift and/or egg deposition.

5.3.2 Experimental design

A BACI (Before-After-Control-Impact) (Smith, 2002) experimental design were adopted (Figure 5.1). There were three substrate treatments representing three levels of habitat heterogeneity types: (1) Sediment, (2) Sediment+ Gravel and, (3) Sediment+ Gravel+ Big stones (henceforth denoted Low, Medium, and High level, respectively), in interaction with two levels of water flow treatment (1) Normal flow (continuous flow throughout the experiment), (2) Drying (3 days drying followed by 15 days drying) denoted Control, Drying (3dDrying and 15dDrying), respectively. Thus, there were six treatment combinations, each with eight replicates, all replicates of each of the six treatment combinations were assigned randomly to the mesocosms in a randomized block design with four blocks and two replicates of each treatment combination per block.

The mesocosms (Figure 5.1b) had an external diameter of 25 cm, an inner ring diameter of 6 cm and a volume of 3.5 litres, and they were sourced from Microwave Ring Moulds, Interworld, Auckland, New Zealand. The low-level habitat treatment (Low) resembled the stream bed of degraded urban rivers, which comprise 700ml fine sediment/silt; The medium-level habitat treatment (Medium) had substratum comprise of 300ml fine sediment, 300ml pebble (2-4mm), and 100ml gravel (4-30mm), to resemble the urban riverbed after habitat restoration; The High habitat treatment (high) comprised 300 mL fine sediment (<2 mm), 900 g gravel (2-30 mm), 4 stones (>30 mm) and 3 larger flat stones, this composition resembled that of the stream bed of head water streams next to the experiment (based on visual estimates, QY. Lin, personal observations). These substrata were collected from the downstream of Huangshan National Reserve Park and air-dried for 72 h before addition to the mesocosms. Twelve autoclaved ceramic tiles (dimensions 3.0 × 3.0 × 1 cm), were added horizontally on to the bed surface of each mesocosm to form biofilm collectors. Three leaf litter bags (8 mm mesh size, 2.500+0.005 g leaves) containing dried camphor (*Cinnamomum camphora* Linnaeus) leaves were added horizontally before the experiment to provide an additional food source and habitat for stream biota including macroinvertebrates. Apart from the drying period when part of the drought treatment was being implemented, water velocity through each mesocosm was maintained at 2 L/min, 12 cm/s, which was comparable to the medium velocity of the stream (Brooks et al. 2002), and calibrated daily.

The experiment ran for 61 days (Figure 5.2), with a 23-day precolonisation period followed by a 3-day drying manipulation period (26-day colonization in Control), a 15-day drying manipulation period (38-day colonization in Control), and a 23-day rewetting period (61-day colonization in Control). After 23 days of natural colonization of the sampling units, the water supply of the drying treatment was shut off for 15 days, sampling at 3d drying and 15d drying respectively, followed by 23 days of rewetting. The 3-day dewatering period here applied is in accordance with other studies (Ledger et al. 2011; Ledger et al. 2012; Lancaster & Ledger 2015), and it was effective to drain away the surface water from the mesocosms, and prevent catastrophic alterations of the water characteristics inside the substrate, guaranteeing their role as refuge. The 15-day dewetting is in accordance with studies conducted by Mckew et al. (2011).

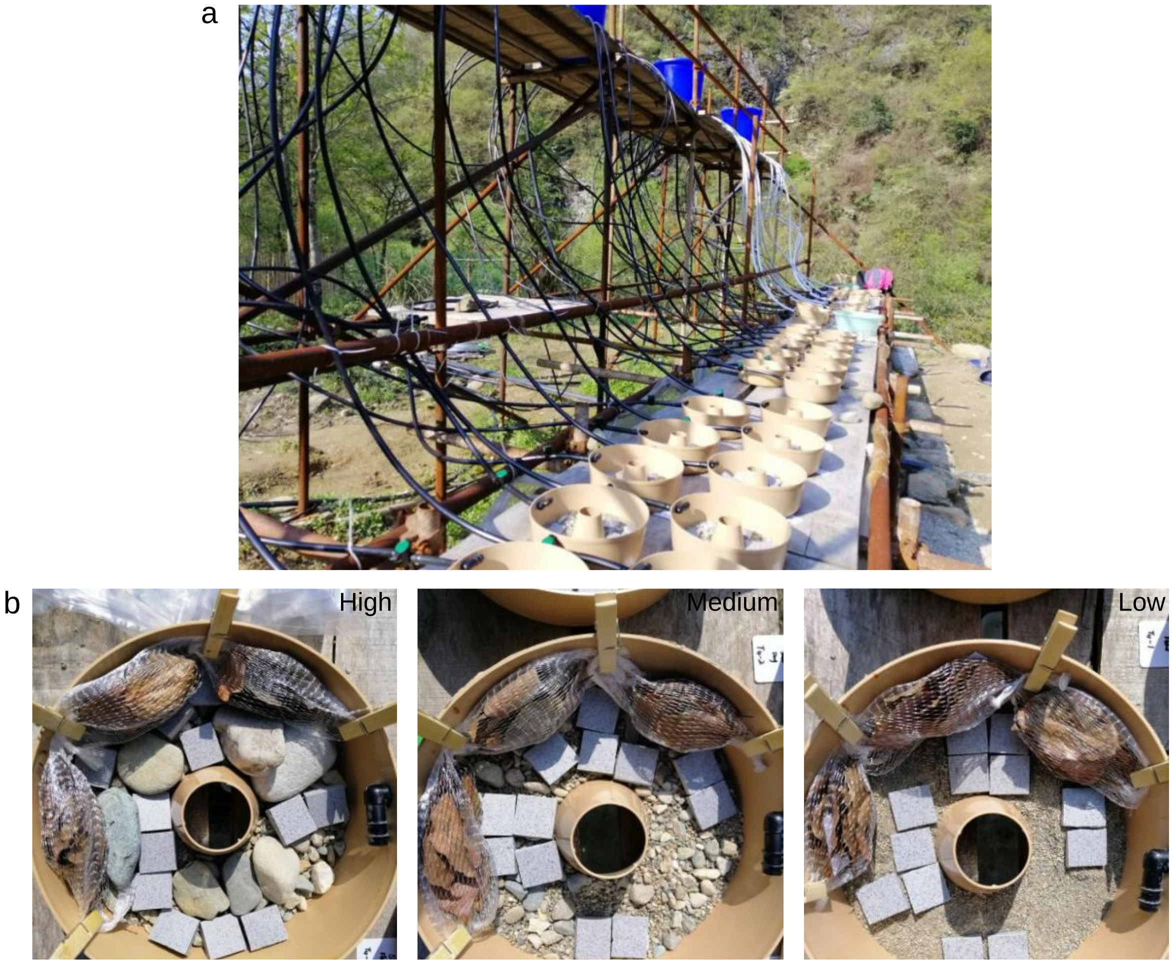

Figure 5.1 Ex-Stream system set up in Huangshan national reserve park (a) and experiment design (b) for the mesocosm experiment; Substrate treatments setup for streams of three habitats (High, Medium, Low), and separated into two drying treatment groups (3d and 14d) and one with continuous flow (control). There were eight replicates of each.

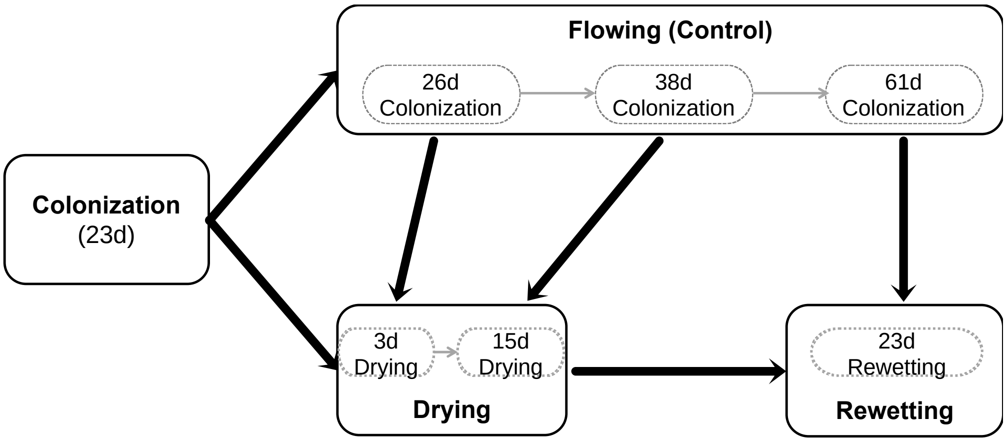

Figure 5.2 Conceptual module of the Ex-Stream experiment. According to the experiment design, 23-day colonization of bacterial community in flowing condition was followed by 38-day continuant flowing and 15-day drying plus 23-day rewetting respectively, aiming to determine the impact of drying and the resilience of bacteria to drying conditions.

5.3.3 Biofilm sampling procedure

To sample the biofilms on the ceramic, the ceramic tiles were placed in each mesocosm randomly. After 23 days exposure, 3 days drying, 15 days drying, and 23 days rewetting, twelve tiles were collected (three tiles for each session), biofilm samples were collected by scraping accumulated materials into 50ml tubes covered with aluminum foil for transporting in a cool box to the laboratory. Each 50ml tube containing biofilm samples scrapped from three tiles of each mesocosm were then filtered on 0.22 μm pore size polycarbonate membrane filters (Millipore, USA) using a vacuum pump, and these filters were stored in sterile Petri dishes at -20 °C until DNA extraction.

5.3.4 DNA extraction and bacterial community composition analysis

Genomic DNA of the biofilms was extracted using an MO BIO extraction Kit (MO BIO PowerBiofilm® DNA Isolation Kit, USA), thereafter DNA quality was assessed using a NanoDrop spectrophotometer to measure the absorbance ratio at 260nm and 280nm. All DNA samples obtained were preserved at -80 °C before sending to Sangon Biotech (Shanghai) Co., Ltd. for Illumina Miseq sequencing.

Sequences were treated and analysed via QIIME 1.8.0. Following removal of the primer, all low-quality reads that containing ambiguous characters, a sequence length less than 200 bp, and a tail quality score < 20 were discarded. Chimeras were assessed using the UCHIME software (Edgar et al. 2011) and discarded. The remaining high-quality reads were clustered into OTUs (Operational Taxonomic Units) via USEARCH with a 97% similarity (Edgar, 2010). All OTUs were then assigned to taxonomic category against the Silva database using the Ribosomal Database Project (RDP) classifier at a confidence threshold of 0.8.

5.3.5 Statistical analysis

Based on the results of the operational taxonomic units (OTUs) analysis, α-diversity indices (Shannon-Weiner index; bacterial richness) were calculated in QIIME1.8.0 (Wang et al. 2018). Differences in bacterial α-diversity in various experiment phases in three stream types were analyzed using ANOVA (one-way analysis of variance), followed by the Tukey–Kramer post hoc test for comparison of means. Non-metric Multi-dimensional Scaling (NMDS) was then used to examine β-diversity based on Euclidean dissimilarities using the 'vegan' package (Oksanen et al. 2018; R Core Team 2017). Analysis of similarities (ANOSIM) was performed based on Bray-Curtis to access the similarity of bacterial community among three stream types in difference colonization phases using the R package vegan. Welch's t-test (White et al. 2009) was performed in STAMP to detect the different-abundant taxonomic groups at phylum level between different treatments. Standardised effect sizes (Partial eta-square) range from 0 to1 are presented to interpret the percentage of variance explained (Garson 2015). Effect sizes can be classified as follows: <0.01 very small, ≥0.01 small, ≥0.06 medium, and ≥0.14 large (Richardson 2011).

Finally, shifts in the functional structures of bacterial communities under drying and rewetting conditions were assessed in the METAGENassist web server (Arndt et al. 2012). Metabolic function was tested among twenty functional categories to evaluate biochemical processes of bacterial communities. A MANOVA analysis was performed on the abundant functions (>1% of the total reads). Response variables were log-transformed where necessary to improve normality and homogeneity of variances. Differences were considered significant at $p < 0.05$.

5.4 Results

5.4.1 Biofilm bacterial community in three microhabitats

The dataset had a total of 5,529,461 raw reads for the 192 samples. After filtering, denoising, and chimera removal, 5,146,771 reads were assigned to 78,100 OTUs. In permanent streams (Control), diversity metrics of bacterial community (Figure 5.3) showed different pattern with habitat changes in different time periods. Habitat heterogeneity did not affect bacterial richness and Shannon diversity after three weeks of colonization (Figure 5.3). After three days, a slight decrease of bacterial richness and diversity in low and medium-level habitat was observed, a reverse pattern were detected in high-level habitat, which induced a greater bacterial richness and diversity in low-level habitat than medium ones (p = 0.0346, p = 0.0216, respectively, Figure 5.3). Both metrics presented a continuant decline in three kinds of streams after then (Figure 5.4, Table 5.2), at day 38, both Low and Medium habitat streams had significant greater bacterial richness and diversity than High habitat ones ($p < 0.05$, Figure 5.3, Table 5.1). Furthermore, after about another three weeks colonization, bacterial richness and diversity were significantly increased ($p < 0.05$ for all three stream types, Figure 5.4, Table 5.2). By day 61, bacterial richness reached a similar level in streams of different habitats ($p > 0.05$, Figure 3g). Streams of high-level habitat got much diverse bacteria than low-level streams (p = 0.031, Figure 5.3b, Table 5.1).

Table 5.1 Variance of biofilm bacterial α- diversity (bacterial richness and Shannon diversity) in different habitats under flowing and drying condition. Significant difference observed at the p = 0.05 level, $p < 0.05$ were marked in bold.

Treatment	Comparison	Flow Condition		Drying Condition	
		Richness *p*	**Shannon *p***	**Richness *p***	**Shannon *p***
23d Colonization	Low-Medium	0.593	0.4191	0.593	0.4191
	Low-High	0.1379	0.1051	0.1379	0.1051
	Medium-High	0.2358	0.39	0.2358	0.39
	Low-Medium-High	0.2832	0.2543	0.2832	0.2543
26d/ 3dDrying	Low-Medium	**0.0346**	**0.0216**	0.0811	0.0612
	Low-High	0.2361	0.2132	0.5062	0.6785
	Medium-High	0.2272	0.2505	0.4368	0.1564
	Low-Medium-High	0.0729	0.0607	0.3001	0.1267
38d/ 15dDrying	Low-Medium	0.6903	0.5656	0.7314	0.9209
	Low-High	**0.0094**	**0.0025**	0.1753	0.5348
	Medium-High	**0.0217**	**0.0122**	0.1066	0.5043
	Low-Medium-High	**0.0139**	**0.0028**	0.1885	0.7292
61d/ Rewetting	Low-Medium	0.6237	0.2302	0.8057	0.8755
	Low-High	0.2895	**0.031**	0.5439	0.2294
	Medium-High	0.3466	0.3798	0.4069	0.1535
	Low-Medium-High	0.4865	0.1008	0.6577	0.2824

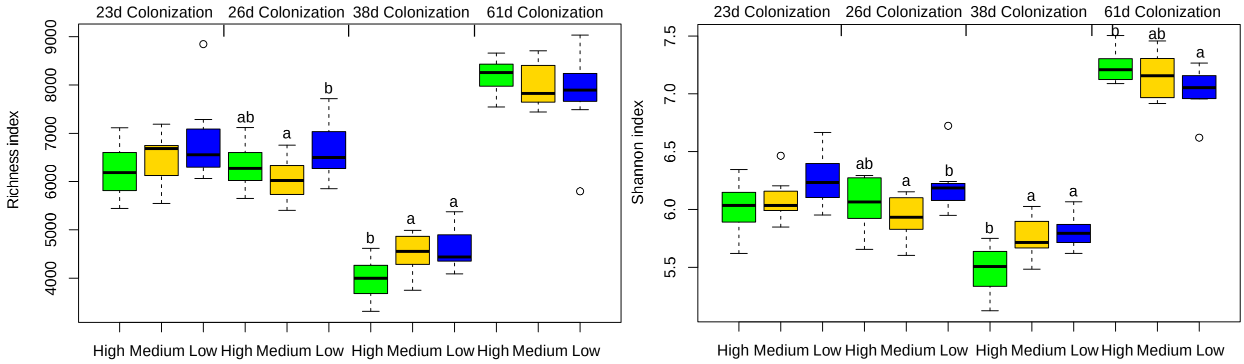

Figure 5.3 Boxplot representing the variance of diversity metrics bacterial Richness (a) & Shannon diversity Index (b) of bacterial community in streams of high/medium/low-level habitat streams in 23-day, 26-day, 38-day and 61-day colonization. Black line: medium value; box: quartile interval; whiskers: minimum and maximum value. Different lowercase letters indicate the significant difference observed at the p = 0.05 level.

Table 5.2 Variance of biofilm bacterial α- diversity (bacterial richness and Shannon diversity) in different experiment phases under flowing and drying condition. Significant difference observed at the p = 0.05 level, p < 0.05 were marked in bold.

Treatment	**Comparison**	**Flow Condition**		**Comparison**	**Drying Condition**	
		Richness *p*	**Shannon *p***		**Richness *p***	**Shannon *p***
Low	23d-26d	0.6042	0.6446	Colonization-3dDrying	0.2224	**0.0497**
	23d-38d	**0**	**0.0002**	Colonization-15dDrying	0.4339	0.2910
	23d-61d	0.0552	**0**	Colonization-Rewetting	**0**	**0**
	26d-38d	**0.0347**	**0**	3dDrying-15dDrying	0.0874	0.9165
	38d-61d	**0**	**0**	15dDrying-Rewetting	**0.0001**	**0.0031**
	23d-26d-38d-61d	**0**	**0**	Colonization-3dDrying-15dDrying-Rewetting	**0**	**0**
Medium	23d-26d	0.0930	0.1306	Colonization-3dDrying	0.3418	0.4396
	23d-38d	**0**	**0.0028**	Colonization-15dDrying	0.5325	0.3801
	23d-61d	**0**	**0**	Colonization-Rewetting	**0**	**0**
	26d-38d	**0**	0.0725	3dDrying-15dDrying	0.8533	0.2327
	38d-61d	**0**	**0**	15dDrying-Rewetting	**0.0001**	**0.0099**
	23d-26d-38d-61d	**0**	**0**	Colonization-3dDrying-15dDrying-Rewetting	**0**	**0**
High	23d-26d	0.6891	0.6951	Colonization-3dDrying	0.6352	0.1584
	23d-38d	**0**	**0.0002**	Colonization-15dDrying	**0.0327**	0.9296
	23d-61d	**0**	**0**	Colonization-Rewetting	**0**	**0**
	26d-38d	**0**	**0.0001**	3dDrying-15dDrying	**0.0397**	0.6633
	38d-61d	**0**	**0**	15dDrying-Rewetting	**0.0001**	**0.0079**
	23d-26d-38d-61d	**0**	**0**	Colonization-3dDrying-15dDrying-Rewetting	**0**	**0.0006**

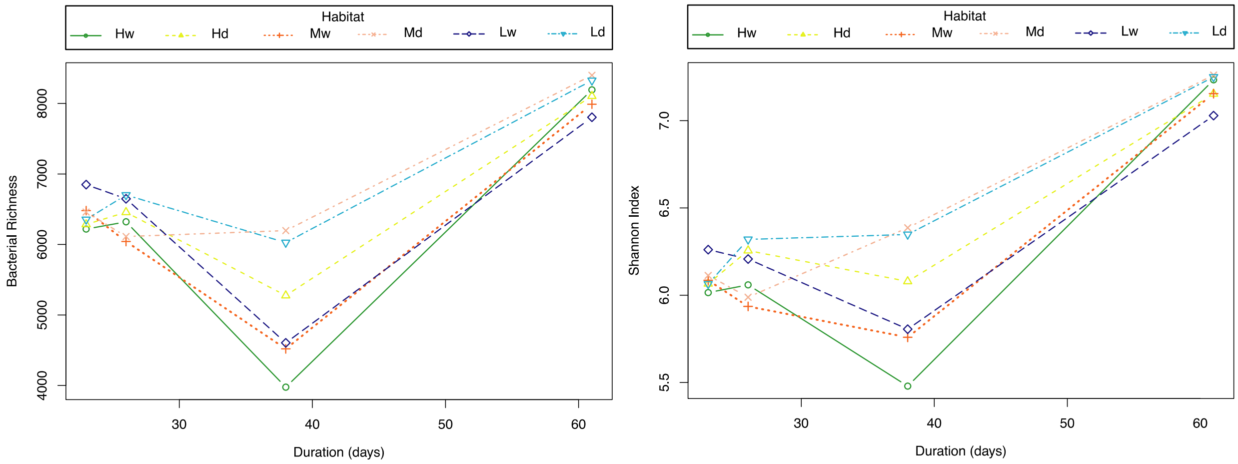

Figure 5.4 Shift of bacterial richness (a) and Shannon diversity (b) along temporal variation under continuous flowing (Hw/Mw/Lw) and drought perturbation (Hd/Md/Ld) in high/medium/low diverse habitat streams.

According to the analysis of similarities (ANOSIM), no difference of the bacterial community structures were detected among three stream types at four incubation periods (23-day: R = 0.03, p = 0.11; 26-day: R = 0.01, p = 0.36; 38-day: R = -0.01, p = 0.53; 61-day: R = 0.06, p = 0.13, respectively). However, the bacterial community composition in low-level habitat streams were distinct from those in high-level streams after 23-day and 61-day colonization (p = 0.04, p = 0.02, respectively), and especially for the bacterial community in the final colonization period. There was a clear shift in bacterial community composition from low-level to high-level habitat streams along the first axes, the bacteria in Medium habitat streams were scattered among them (Figure 5.5d).

Over time, the shift of bacterial community composition displayed a similar pattern in different habitats (High: R = 0.82, p = 0.001; Medium: R = 0.7, p = 0.001; Low: R = 0.67, p = 0.001; Figure 5.6a-c). In all three stream types, the bacterial community composition assemblage at 61-days was distinct from bacteria at 38-day (p < 0.001 in all three habitats; Table 5.3), the bacteria at 38-days also showed significant differences with those colonized at 26-days (p < 0.001 for all three habitats; Table 5.3). The bacteria colonizing at 23-days were distinct from those at 26-days in high-level streams (p = 0.001), whereas, no difference was observed between these two periods in medium and low-level habitat streams ($p = 0.06$ and $p = 0.08$, respectively).

Table 5.3 Similarities of biofilm bacterial community composition in different flowing phases under flowing and drying condition. Significant difference observed at the $p = 0.05$ level, $p < 0.05$ were marked in bold.

Treatment	Comparison	Flow Condition			Comparison	Drying Condition		
		ANOSIM Statistic R	***p* value**	**Permutations**		**ANOSIM Statistic R**	***p* value**	**Permutations**
Low	23d-26d	0.09	0.080	999	Colonization- 3dDrying	0.29	**0.004**	999
	23d-38d	0.47	**0.001**	999	Colonization-15dDrying	0.5	**0.001**	999
	23d-61d	0.95	**0.001**	999	Colonization-Rewetting	0.99	**0.001**	999
	26d-38d	0.47	**0.001**	999	3dDrying-15dDrying	0.46	**0.001**	999
	26d-61d	0.98	**0.002**	999	3dDrying-Rewetting	1	**0.002**	999
	38d-61d	0.99	**0.001**	999	15dDrying-Rewetting	0.53	**0.001**	999
	23d-26d-38d-61d	0.67	**0.001**	999	Colonization-3dDrying-15dDrying-Rewetting	0.61	**0.001**	999
Medium	23d-26d	0.14	0.060	999	Colonization-3dDrying	0.56	**0.001**	999
	23d-38d	0.78	**0.001**	999	Colonization-15dDrying	0.68	**0.001**	999
	23d-61d	0.99	**0.001**	999	Colonization-Rewetting	1	**0.001**	999
	26d-38d	0.5	**0.002**	999	3dDrying-15dDrying	0.68	**0.001**	999
	26d-61d	0.98	**0.001**	999	3dDrying-Rewetting	0.99	**0.001**	999
	38d-61d	0.95	**0.001**	999	15dDrying-Rewetting	0.65	**0.001**	999
	23d-26d-38d-61d	0.7	**0.001**	999	Colonization-3dDrying-15dDrying-Rewetting	0.75	**0.001**	999
High	23d-26d	0.35	**0.001**	999	Colonization-3dDrying	0.31	**0.001**	999
	23d-38d	0.92	**0.002**	999	Colonization-15dDrying	0.61	**0.001**	999
	23d-61d	1	**0.002**	999	Colonization-Rewetting	1	**0.003**	999
	26d-38d	0.69	**0.001**	999	3dDrying-15dDrying	0.49	**0.002**	999
	26d-61d	1	**0.002**	999	3dDrying-Rewetting	0.94	**0.001**	999
	38d-61d	1	**0.001**	999	15dDrying-Rewetting	0.61	**0.002**	999
	23d-26d-38d-61d	0.82	**0.001**	999	Colonization-3dDrying-15dDrying-Rewetting	0.64	**0.001**	999

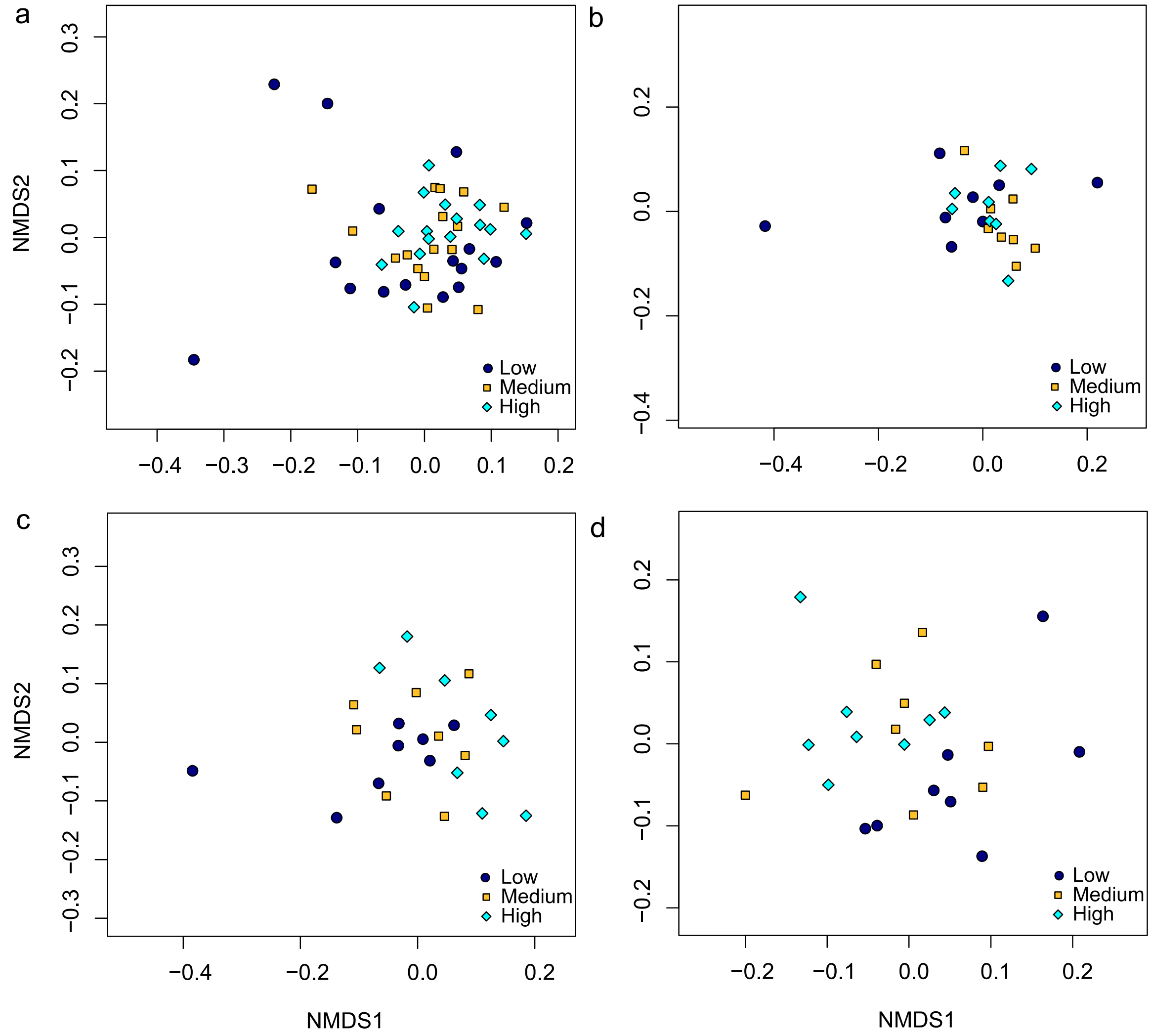

Figure 5.5 Non-metric multi-dimensional scaling (NMDS) ordination of biofilm bacterial communities in high/medium/low diverse habitat streams under flowing condition at four incubation periods: Day 23 (a), Day 26 (b), Day 38 (c) and Day 61 (d).

5.4.2 Taxonomic difference of biofilm bacteria under drying and rewetting condition

Under flow intermittent, low-level and high-level habitat streams had similar development pattern in bacterial diversity metrics, in contrast to these in medium-level streams. After three-day drying, a slight increase in bacterial richness in low and high-level streams and a marked increase in bacterial diversity in low-level streams (p = 0.0497) was detected, whereas, no difference of bacterial α-diversity was detected for Medium ones ($p > 0.05$). Low-level habitat streams had the greatest bacterial richness and diversity, following by high-level and medium-level ones. Thereafter, when compared biofilms with three-day drying ones, bacterial richness experienced a sharp decline in high-level habitat streams ($p = 0.0397$) and a weak decrease in low-level streams ($p = 0.0874$) (Figure 5.3a), bacterial diversity didn't change much in all three stream types ($p > 0.05$, Figure 5.3b). After 15 days continuant drying, medium-level streams had the greatest bacterial richness and diversity, high-level habitat streams had the lowest bacterial richness and diversity among three stream types, though the differences were not significant as it is ($p > 0.05$, Table 5.1). After 23 days rewetting, three stream groups displayed similar developing pattern in bacterial α-diversity (Figure 5.3). When compared with drought ones, bacterial richness in those streams were significantly increased (Low/Medium/High: $p = 0.0001$), bacterial diversity showed similar pattern (Low: $p = 0.0031$; Medium: $p = 0.0099$; High: $p = 0.0079$, respectively). By the end of rewetting, bacterial richness and diversity were greatest in medium-level streams, lowest in high-level streams, though the differences were not distinct among stream types (Richness: $p = 0.6577$; Shannon: $p = 0.2824$).

When compared drying bacteria with those colonized in permanent streams (Control) at same period, however, bacterial richness and diversity in all three stream types displayed an increasing trend under drying perturbation. Though no significant difference were observed after three-day drying for bacterial richness (High: $F_{1,14} = 0.073$; Medium: $F_{1,14} = 0.013$; Low: $F_{1,14} = 0.066$) and bacterial diversity (High: $F_{1,14} = 1.84$; Medium: $F_{1,14} = 0.093$; Low: $F_{1,14} = 0.818$), after 15 days drying, the bacterial richness in three stream types were all significantly greater than those in Control streams (High: $F_{1,14} = 7.987$, $p = 0.0093$; Medium: $F_{1,14} = 20.18$, $p = 0.0006$; Medium: $F_{1,14} = 15.2$, $p = 0.0017$, respectively), bacterial diversity in medium/low-level habitat streams were greater than control circumstance ($F_{1,14} = 3.554$, $p = 0.0527$; $F_{1,14} = 3.977$, $p = 0.0495$, respectively). No difference in bacterial diversity was found in high-level streams between control and drought condition ($F_{1,14} = 2.136$, $p = 0.1091$). Rewetting induced no difference in bacterial richness for each stream type ($F_{1,14} = 0.133$, $F_{1,14} = 2.499$, $F_{1,14} = 1.708$, for high/medium/low-level streams respectively) and no difference in bacterial diversity in high and medium-level streams (H: $F_{1,14} = 1.156$; M: $F_{1,14}$

= 1.636, respectively), when compared with those Control ones. Whereas, the bacterial diversity was remarkably greater than bacteria experienced continuant flowing in low-level habitat streams ($F_{1,14}$ = 6.349, p = 0.0237).

5.4.3 Shift of biofilm bacterial community composition under drying and rewetting condition

For bacterial community composition undergoing drying condition, according to analysis of similarities (ANOSIM), the ordination revealed an obvious clustering of samples collected at the same period in each stream type (Figure 5.6). Significant differences in bacterial community composition were shown at two drying periods in all three stream types (Figure 5.6, Table 5.4). The bacterial community composition underwent three-day drying in each stream types were distinct from those assemblage after 23-day colonization (Low: R = 0.29, p = 0.04; Medium: R = 0.56, p = 0.01; High: R = 0.31, p = 0.01, respectively), the bacteria maintained after fifteen-day drying were also differed from those sampled after three-day drying (Low: R = 0.46, p = 0.001; Medium: R = 0.68, p = 0.001; High: R = 0.49, p = 0.002, respectively; Table 5.4) along the second axis in all three stream types. Rewetting bacteria clustered in each stream group were distinctly separated from bacteria after fifteen-day drought perturbation along the first axis (Low: R = 0.53, p = 0.001; Medium: R = 0.65, p = 0.001; High: R = 0.61, p = 0.002, respectively) and bacteria in initial 23-day colonization (Low: R = 0.99, p = 0.001; Medium: R = 1, p = 0.001; High: R = 1, p = 0.003, respectively).

Table 5.4 Similarities of biofilm bacterial community composition in different habitats under flowing and drying condition. Significant difference observed at the $p = 0.05$ level, $p < 0.05$ were marked in bold.

Treatment	Comparison	Flow Condition			Drying Condition		
		ANOSIM Statistic R	*p* value	Permutations	ANOSIM Statistic R	*p* value	Permutations
23d Colonization	Low-Medium	0.01	0.32	999	0.01	0.32	999
	Low-High	0.07	**0.04**	999	0.07	**0.04**	999
	Medium-High	0.02	0.24	999	0.02	0.24	999
	Low-Medium-High	0.03	0.11	999	0.03	0.11	999
26d/ 3dDrying	Low-Medium	0.07	0.14	999	0.04	0.26	999
	Low-High	-0.01	0.53	999	0.04	0.24	999
	Medium-High	-0.04	0.67	999	0	0.46	999
	Low-Medium-High	0.01	0.36	999	0.03	0.26	999
38d/ 15dDrying	Low-Medium	-0.08	0.88	999	-0.06	0.85	999
	Low-High	0.06	0.18	999	0.02	0.32	999
	Medium-High	-0.01	0.46	999	0.1	0.06	999
	Low-Medium-High	-0.01	0.53	999	0.02	0.27	999
61d/ Rewetting	Low-Medium	-0.02	0.49	999	-0.02	0.54	999
	Low-High	0.19	**0.02**	999	0.04	0.26	999
	Medium-High	0.04	0.24	999	0.02	0.36	999
	Low-Medium-High	0.06	0.13	999	0.02	0.29	999

Table 5.5 Similarities of biofilm bacterial community composition between flowing, drying and rewetting condition in different habitats. Significant difference observed at the $p = 0.05$ level, $p < 0.05$ were marked in bold.

Treatment	Comparison	ANOSIM Statistic R	*p* value	Permutations
High	3dDrying - 26d	0.39	**0.001**	999
	15dDrying - 38d	0.62	**0.001**	999
	Rewetting - 61d	0.67	**0.001**	999
Medium	3dDrying - 26d	0.62	**0.001**	999
	15dDrying - 38d	0.73	**0.001**	999
	Rewetting - 61d	0.78	**0.001**	999
Low	3dDrying - 26d	0.36	**0.001**	999
	15dDrying - 38d	0.59	**0.001**	999
	Rewetting - 61d	0.85	**0.001**	999

When compared the drying bacteria with Control ones, drying bacterial community composition were significantly different from bacteria colonized in continuant flow (Figure 5.7a-f, Table 5.5), either after three-day drying (Low: R = 0.36, $p = 0.001$; Medium: R = 0.62, $p = 0.001$; High: R = 0.39, $p = 0.001$, respectively) or fifteen-day drying (Low: R = 0.59, $p = 0.001$; Medium: R = 0.73, $p = 0.001$; High: R = 0.62, $p = 0.001$, respectively). After

23-day rewetting, bacterial community composition were also distinct from bacteria in Control ones (Low: R = 0.85, p = 0.001; Medium: R = 0.78, p = 0.001; High: R = 0.67, p = 0.001, respectively), bacteria clustered in both rewetting and Control condition were obviously separate along the first axis (Figure 5.7g-i).

According to taxonomic classification, fourteen abundant bacterial taxa were observed in all mesocosm streams (Table 5.6). The univariate results displayed the contribution of each treatment on those abundant taxa in contrast with Control bacteria. Three-day drying significantly affected 21.4% of bacterial taxa (positive: Gammatimonadetes, Armatimonadetes; negative: Candidatus_Saccharibacteria) in streams with high-level habitat, 42.9% (positive: Proteobacteria, Gemmatimonadetes, Armatimonadetes; negative: Planctomycetes, Verrucomicrobia, Candidatus Saccharibacteria) in streams with medium-level habitat, and only 7.1% (Armatimonadetes, positives) in low-level habitat streams. Under fifteen-day drying, 42.9% of taxa (positive: Bacteroidetes, Gemmatimonadetes, Armatimonadetes; negative: Proteobacteria, Candidatus Saccharibacteria, Parcubacteria) were affected in high-level streams, 42.9% (positive: Gemmatimonadetes, Armatimonadetes, Chloroflexi; negative: Proteobacteria, Candidatus Saccharibacteria, Parcubacteria) in medium ones, and 28.6% (positive: Gemmatimonadetes, Chloroflexi; negative: Candidatus Saccharibacteria, Parcubacteria) in low-level ones. Rewetting impacted 50.0% of taxa (positive: Proteobacteria, Gemmatimonadetes, Firmicutes, Deinococcus-Thermus; negative: Verrucomicrobia, Candidate division WPS-1, Parcubacteria) in streams with high-level habitat, 50% (positive: Proteobacteria, Gemmatimonadetes, Firmicutes, Chloroflexi, Parcubacteria, Deinococcus-Thermus; negative: Planctomycetes) in Medium ones, and 35.7% (positive: Gemmatimonadetes, Chloroflexi, Deinococcus-Thermus; negative: Candidatus Saccharibacteria, Parcubacteria) in low-level streams.

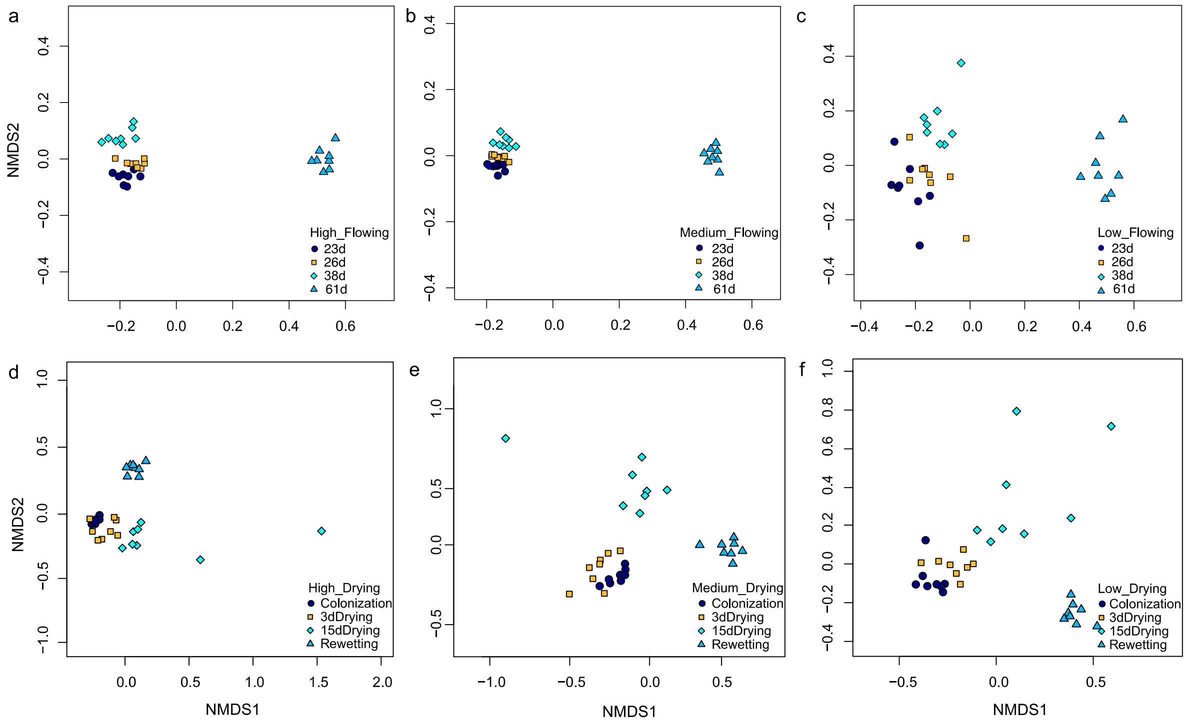

Figure 5.6 Non-metric multi-dimensional scaling (NMDS) ordination of biofilm bacterial communities under flowing (a-c) and drying condition (d-f) at four experiment phases (23-day Colonization, 26-day Colonization/ 3-day Drying, 38-day Colonization/ 15-day drying, 61-day Colonization/ Rewetting) in high, medium, and low-level habitat streams.

Table 5.6 Results (p-values and partial-η_2 effect sizes) of the MANOVA (multivariate and univariate results) on the 14 abundant bacterial phyla under three days drying, 15 days drying and rewetting condition in streams of different habitat.

Bacteria taxa	High			Medium			Low		
	3dDrying	15dDrying	Rewetting	3dDrying	15dDrying	Rewetting	3dDrying	15dDrying	Rewetting
Proteobacteria	0.248	**0.004**	**0.012**	**0.010**	**0.006**	**0.031**	0.896	0.202	0.649
	-(0.002)	-(0.319)	(0.133)	(0.121)	-(0.362)	(0.106)	-(0.008)	-(0.245)	-(0.002)
Planctomycetes	0.183	0.217	0.074	**0.008**	0.359	**0.01**	0.962	0.253	0.4
	-(0.013)	-(0.374)	-(0.191)	-(0.302)	(0.100)	-(0.274)	(0.000)	-(0.247)	-(0.344)
Bacteroidetes	0.876	**0.04**	0.603	0.788	0.558	0.54	0.943	0.286	0.237
	-(0.038)	(0.108)	-(0.003)	-(0.009)	(0.001)	(0.013)	-(0.001)	-(0.001)	(0.016)
Verrucomicrobia	0.182	0.242	**0.009**	**0.008**	0.414	0.087	0.551	0.335	0.532
	-(0.150)	(0.000)	-(0.340)	-(0.342)	(0.024)	-(0.126)	(0.021)	-(0.175)	-(0.039)
Actinobacteria	0.964	0.384	0.679	0.226	0.319	0.67	0.252	0.054	0.36
	-(0.022)	-(0.182)	-(0.001)	-(0.091)	(0.009)	-(0.002)	-(0.157)	(0.028)	-(0.082)
Gemmatimonadetes	**0.014**	**0.005**	**<0.001**	**<0.001**	**0.011**	**0.002**	0.099	**0.013**	**<0.001**
	(0.526)	(0.399)	(0.428)	(0.696)	(0.512)	(0.627)	(0.339)	(0.423)	(0.577)
Acidobacteria	0.589	0.315	0.282	0.283	0.06	0.345	0.342	0.06	0.1
	-(0.065)	(0.002)	-(0.041)	-(0.092)	(0.161)	(0.047)	-(0.107)	(0.055)	(0.060)
Armatimonadetes	**0.003**	**0.008**	0.197	**<0.001**	**0.029**	0.772	**0.003**	0.127	0.3
	(0. 488)	(0.209)	-(0.107)	(0.767)	(0.444)	(0.030)	(0.489)	(0.040)	(0.054)
Candidatus Saccharibacteria	**0.034**	<**0.001**	0.091	**0.025**	**<0.001**	0.425	0.302	**<0.001**	**0.04**
	-(0.242)	-(0.861)	-(0.117)	-(0.364)	-(0.611)	-(0.012)	-(0.077)	-(0.759)	-(0.334)
Firmicutes	0.212	0.127	**0.006**	0.097	0.056	**0.023**	0.113	0.074	0.485
	(0.032)	(0.561)	(0.677)	(0.145)	(0.615)	(0.507)	(0.140)	(0.717)	(0.082)
Chloroflexi	0.908	0.052	0.107	0.589	**0.004**	**0.002**	0.876	**0.004**	**0.007**
	-(0.039)	(0.111)	(0.153)	(0.031)	(0.432)	(0.389)	(0.000)	(0.358)	(0.116)
Candidate division WPS-1	0.25	0.715	**0.016**	0.252	0.221	0.764	0.282	0.106	0.626
	-(0.118)	-(0.003)	-(0.446)	(0.072)	(0.035)	(0.024)	(0.016)	(0.064)	-(0.015)
Parcubacteria	0.567	**0.02**	**<0.001**	0.662	**<0.001**	**0.026**	0.282	**0.032**	**0.003**
	(0.014)	-(0.461)	-(0.675)	-(0.019)	-(0.711)	(0.392)	(0.039)	-(0.484)	-(0.621)
Deinococcus-Thermus	0.374	0.197	**0.022**	0.626	0.277	**<0.001**	0.096	0.453	**0.001**
	-(0.077)	(0.059)	(0.413)	-(0.003)	(0.066)	(0.817)	-(0.229)	-(0.009)	(0.608)

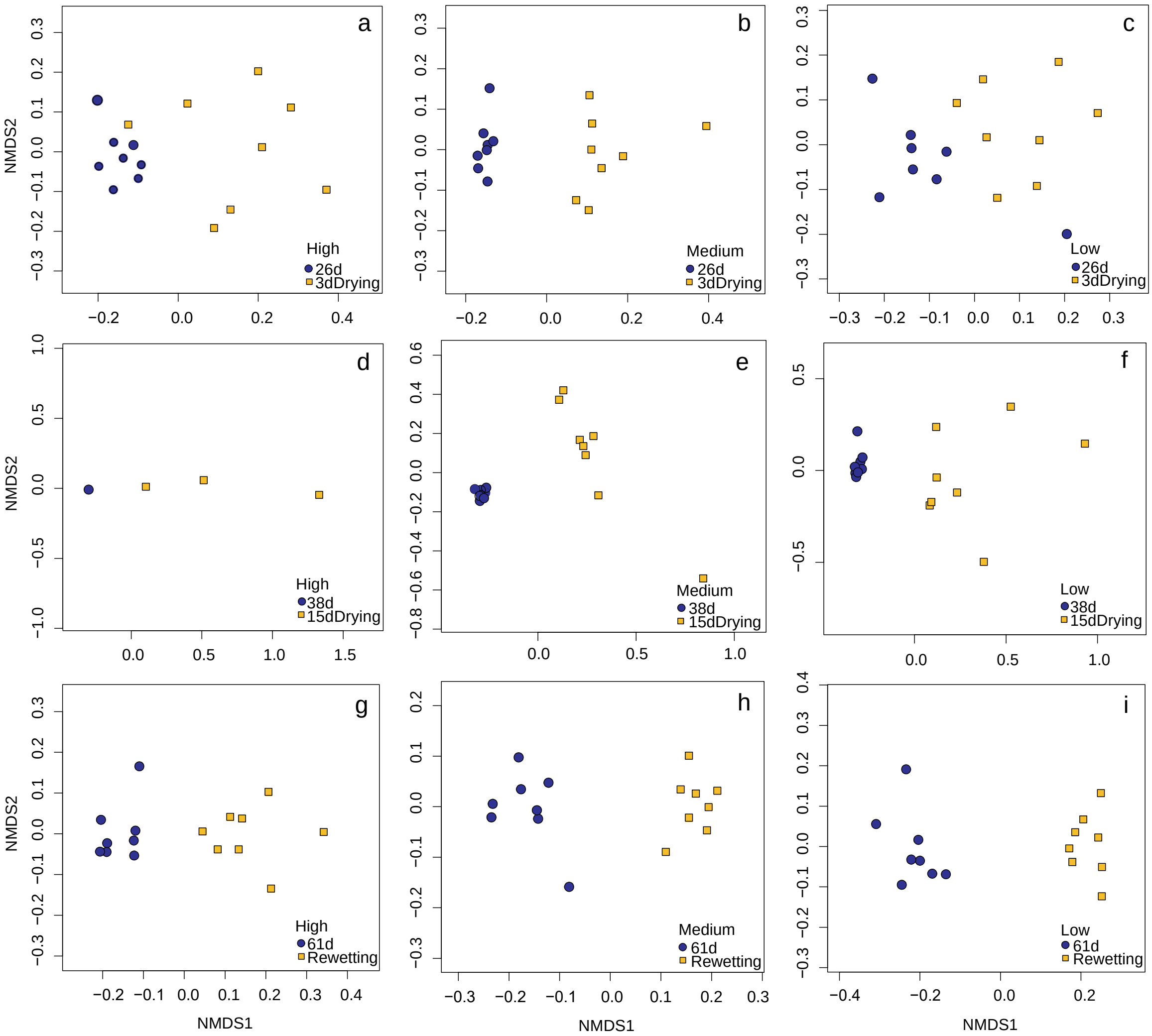

Figure 5.7 Non-metric multi-dimensional scaling (NMDS) ordination of biofilm bacterial communities between flowing (a-c), drying(d-f), and rewetting condition (g-i) in high, medium, and low-level habitat streams.

5.4.4 Functional structure changes of biofilm bacteria under drying and rewetting condition

The Metagenassist generate a total of 29 predicted bacterial metabolic functions, with a prevalence of functions associated with sulfate reducer (22.49%, mean across the whole dataset), dehalogenation (16.88%), ammonia oxidation (15.94%), nitrite reducer (12.93%), xylan degradation (8.88%), and sulfide oxidizer (6.98%) across the experimental streams.

Eleven abundant metabolism functions were distributed in all mesocosm streams (Table 5.7), constituting 97.72% of all functions identified. Among these abundant functions, the multivariate results of the MANOVA showed that three-day drying slightly increased hehalogenation function in high-level habitat streams, and enhanced the dehalogenation, decline the nitrite reducer function in Medium ones, no difference were detected in these functions in low-level streams under three-day drying. Under fifteen-day drying, sulfate reducer and nitrogen fixation were all increased in three types of streams, though the increase intensity of sulfate reducer was not that significant in Medium ones. Additionally, a significant decline in chitin degradation and atrazine metabolism were observed in streams with medium and low-level habitats, atrazine metabolism was also declined in high-level habitat streams. After flow resumption, a dramatic increase in chitin degradation, aromatic-hydrocarbons degradation, atrazine metabolism, and a slight increase in sulfide oxidizer were detected in streams with high-level habitat, no change was observed in other types of streams, except for a slight increase in chitin degradation in Medium ones.

Table 5.7 Results (*p*-values and partial-η_2 effect sizes) of the MANOVA (multivariate and univariate results) on the 11 abundant metabolism functions under three days drying, 15 days drying and rewetting condition in streams of different habitats.

Metabolism Function	High			Medium			Low		
	3dDrying	15dDrying	Rewetting	3dDrying	15dDrying	Rewetting	3dDrying	15dDrying	Rewetting
Sulfate reducer	0.47	**0.032**	0.688	0.727	**0.099**	0.371	0.783	**0.011**	0.341
	(0.038)	(0.288)	-(0.012)	-(0.009)	(0.183)	-(0.057)	-(0.006)	(0.377)	-(0.065)
Ammonia oxidizer	0.249	0.113	0.303	0.942	0.206	0.255	0.737	0.259	0.609
	(0.094)	(0.170)	(0.076)	-(0.000)	(0.112)	(0.092)	(0.008)	(0.090)	(0.019)
Dehalogenation	**0.074**	0.618	0.772	**0.042**	0.544	0.384	0.307	0.815	0.346
	(0.211)	(0.018)	-(0.006)	(0.263)	(0.027)	-(0.054)	(0.074)	(0.004)	-(0.064)
Nitrite reducer	0.654	0.435	0.185	**0.038**	0.589	0.444	0.669	0.872	0.69
	-(0.015)	(0.044)	(0.122)	-(0.272)	(0.021)	(0.042)	-(0.013)	(0.002)	(0.012)
Sulfide oxidizer	0.976	0.344	**0.064**	0.609	0.314	0.629	0.214	0.675	0.742
	-(0.000)	-(0.064)	(0.225)	-(0.019)	-(0.072)	(0.017)	-(0.108)	-(0.013)	-(0.008)
Xylan degrader	0.104	0.123	0.237	**0.03**	0.75	0.183	0.891	0.175	0.482
	(0.177)	(0.161)	-(0.098)	(0.293)	(0.007)	-(0.123)	(0.001)	(0.127)	-(0.036)
Nitrogen fixation	0.503	**0.012**	0.614	0.338	**0.026**	0.925	0.285	**0.054**	0.421
	(0.033)	(0.376)	(0.018)	-(0.066)	(0.308)	-(0.001)	-(0.081)	(0.241)	-(0.047)
Degrades_aromatic_hydrocarbons	0.893	0.218	**0.027**	0.337	0.319	0.295	0.156	0.536	0.184
	(0.001)	-(0.106)	(0.302)	-(0.066)	-(0.071)	(0.078)	-(0.139)	(0.028)	(0.122)
Chitin degradation	0.63	0.117	**0.001**	0.875	**0.001**	**0.079**	0.813	**0.001**	0.269
	(0.017)	-(0.166)	(0.560)	-(0.002)	-(0.546)	(0.204)	-(0.004)	-(0.543)	(0.087)
Sulfur oxidizer	0.117	0.586	0.576	0.174	0.134	0.186	0.133	0.72	0.314
	(0.166)	(0.022)	(0.023)	(0.128)	(0.153)	(0.121)	(0.153)	(0.009)	(0.072)
Atrazine metabolism	0.296	**0.045**	**0.033**	0.33	**0.002**	0.969	0.837	**0.005**	0.182
	(0.078)	-(0.258)	(0.286)	-(0.068)	-(0.514)	-(0.000)	-(0.003)	-(0.444)	-(0.123)

5.5 Discussion

5.5.1 Impact of habitat on biofilm bacteria

Our results indicated that under permanent condition, three stream habitats didn't lead to the structural difference in bacterial communities in different experiment periods, except for the dissimilarity between low-level and high-level habitat in 23-day and 61-day colonization. Whereas, a significant difference in benthic bacterial community compositions was observed along experimental phases in each stream type. Suggesting that experiment time had greater impact on benthic bacterial communities than habitat types, habitat impact benthic bacteria on the condition that the living habitat is heterogeneous to enable the assemblage of communities of diverse living spaces. This finding is in agree with our research hypothesis that different habitat would promote specialization of organisms, and in line with previous research that different compartments present contrasting environments for the dominance of various microbial heterotrophic taxa (Gao et al. 2005; Zeglin 2015).

Bacterial α-diversity didn't change with habitat changes in the first 23 days colonization. Nevertheless, the bacterial richness and diversity revealed to be greater developed in low-level habitat than medium ones in day-26 and greater in low-level and medium-level habitats than high-level ones in day-38. In day-61, constant bacterial richness was obtained among habitat types, whereas, high-level habitat possessed greater bacterial diversity than low-level ones. This developing strategy suggested that low-level habitat exhibited greater bacterial richness and diversity in the early colonization period, whereas, high-level habitat develop greater diverse of bacteria in a stable experiment system. These results partially support our research hypothesis that more heterogeneity habitat would support more diverse benthic bacteria. As mixed substrate would provide a greater range of divided habitat spaces for the colonization of diverse taxa preferring in different living spaces (Giller & Malmqvist 1998).

5.5.2 Impact of drying on bacterial community

Flow intermittence caused by climate change or anthropogenic activities were reported to disrupting hydrological connectivity (Boulton et al. 2017), creating heterogeneous mosaics of stream habitat (Datry et al. 2014), which lead to perpetual fluctuations in the ecology and community structure and ecosystem process of the lotic ecosystem (Dudgeon et al. 2006). In this study, three-day drying and fifteen-day drying were arranged after 23-day colonization, the result showed that compared with bacteria assemblage in continuant flow, bacterial α-diversity didn’t change after three-day drying in streams of each habitat type. Fifteen-day drying significantly increased the bacterial richness in three stream types, and increased the bacterial diversity in streams of low-level and medium-level habitats, high-level habitat developed comparable diverse of bacteria in either flowing condition or drying condition. Suggesting that short time drying remained comparable bacterial α-diversity in diverse habitat streams, whereas, relative long time drying created more finely mosaic habitat in low and medium-level habitat streams, which supported greater numbers and more diverse of taxa with preferences for each particular habitat (Datry et al. 2017). Although hydrological variability was highly related to the variation in bacterial community composition (Portillo et al. 2012), the remained surface waters, sequence of disconnected pools and connected hyporheic flow after short time drying (3-day drying; Boulton 2003; Bhamjee et al. 2016) provide refuges for the surviving of bacterial communities, which might attribute to the maintenance of bacteria in diverse habitat. On the other hand, a relative long time drying (15-day drying) created terrestrial habitat along dry riverbeds (Boulton et al. 2017), impairing ecological process such as oxygenation of the hyporheic zone (Datry & Larned 2008; Boulton et al. 2010) in streams of each type. Bacterial richness and diversity may decline during the drying phase (Rees et al. 2006; Timoner et al. 2014a). However, substrate that comprise greater surface area in low-level and medium-level habitat might possess greater water-holding capacity, protecting microbial cells from drying events by forming refugia (Romaní et al. 2013) under the surface area. Moreover, the finely mosaic habitat widely created in low-level and medium-level habitat streams might form hypoxic or anoxic environment (Lillebø et al. 2007; von Schiller et al. 2011), increasing the bacterial diversity through stimulating the develop of anaerobic communities along the depletion of oxygen (Briée et al. 2007) and substantial changes in nutrients and DOM (von Schiller et al. 2017).

When looking into details of community change, it appeared that the bacterial community compositions were significantly shifted under drying condition (either three-day drying or fifteen-day drying) in all three stream types. Supporting the judgement that dry substrates, moist substrates and substrates in flowing waters sustain contrasting microbial community composition and activity (Zeglin et al. 2011; Fazi et al. 2013; Pohlon et al. 2013). Three-day drying increased the dehalogenation function in medium-level and high-level habitat, and increased the xylan degrader, decrease the nitrite reducer in medium-level ones. With the extend of flow intermittent, fifteen-day drying in three stream types all lead to the similar functional changes, which increased the sulfate reducer and nitrogen fixation, and declined the chitin degradation and atrazine metabolism function. In line with previous study that flow intermittent induced anoxic and accumulation of OM might inhibit microbial metabolism process (Medeiros et al. 2009), including the metabolism of atrazine, and select for anaerobic sulfate-reducing bacteria responsible for OM mineralization (Briée et al. 2007), additionally, flow intermittent streams were dominated by N-fixation microbes that highly suited for changing redox conditions (Febria et al. 2015; Koach et al. 2015). Moreover, several bacterial groups in these habitats formed cyst walls or endospore that mainly composted of chitin during drying phases (Romaní et al. 2017), constituting the major resilience strategy under drying, which may explain the reduction in chitin degradation function in this extreme condition.

5.5.3 Resilience of biofilm bacteria to drying perturbation

Flow resumption reappears the rewetting condition driven by rainfall or water management, which stimulate the reversal of terrestrial habitat to lotic environment (Boulton et al. 2017), adjust microbial community composition followed by (Lyautey et al. 2005; Zoppini et al. 2010). In our study, except for the increasing bacterial diversity in streams with low-level streams, all three stream types underlying rewetting condition had comparable bacterial richness and diversity with that in control streams. Implying that biofilm bacteria have great resilience to hydrological stress, the bacterial α-diversity in those rewetted habitats were recovered to control status. In accordance with the assumption that resident communities are more tolerant of “harsh” conditions and are more resilient or resistant to perturbation induced by flow intermittent (Boulton et al. 2000). The speed active of some autochthonic “seed-bank” microbial communities that resist during drying period (Placella et al. 2012; Zeglin et al. 2011) and recolonization of microbes dispersed from upstream environment (Leibold et al. 2004; Rosado et al. 2015) remediate the drying effect on microbes. The persist of microbial mat formed during drying period and related microbes may explain the greater diverse bacteria in streams of low-level habitat.

Though the α-diversity didn’t change under rewetting condition, the bacterial community composition in three stream types were all distinct from control ones. In accordance with statement that flow resumption would lead to considerable changes in microbes (Romaní et al. 2017). Low-level habitat had comparable community function with bacteria in control streams. The formation of microbial mat that formed by cyanobacteria and heterotrophic bacteria that related to sulfur metabolism during prolonged drying condition (Dupraz & Visscher 2005; Stanish et al. 2013) might explained the speed recolonization of similar functional bacteria in streams with low-level habitat. Streams of medium-level and high-level habitat possessed greater chitin degradation ability, associated with increasing relative abundance of Proteobacteria, Firmicutes, Parcubacteria. This community shift pattern agrees with the statement in Febria et al. (2015) that bacterial communities from the permanent stream showed high centrality among Proteobacteria phylum. Firmicutes has a gram-positive cell-wall type and includes many endospore-forming genera, which may help succeed surviving during desiccation periods (Klappenbach et al. 2000).

Moreover, as found in arid-zone stream, rehydration led to marked increase in functional diversity (Timoner et al. 2014b), the bacterial functional diversity in medium-level and high-level habitat streams were also increased after rewetting. Bacteria in these two stream types possessed greater chitin degradation function than control ones. Implying that upon the restore of hydrological and physico-chemical environment, rewetting enabled the active and fast assemblage of bacteria and induced the rapid excystment through chitin degradation (Verni & Rosati 2011).

In spite of the community difference between drying and control condition, similar bacterial community composition was detected in streams of analogous habitats in either flowing or drying condition, experimental period contributed more to the variance in benthic bacterial communities than instream habitat. These results might be attributed to the aerial colonization of bacterial communities across these manipulated streams, as the bacterial sporulation may ease the contamination the bacterial communities in mesocosms with short distance through air dispersal, and it's especially the case for the sporulation of bacteria when encountering drying condition (Romaní et al. 2017). To avoid the cross-contamination, covering each mesocosm with transparent and fine-mesh size netting to form a closed artificial stream might help control the spore dispersal while minimizing the risks of contamination (Flum et al. 1993) for future research.

5.6 Conclusions

An Ex-Stream experiment was performed to investigate the resilience of aquatic community structure to drying perturbation in streams of different habitats, using benthic biofilm bacteria as a bioindicator. The experiment demonstrated that different habitats and hydrological phases promoted specialization of microbial communities. More heterogeneity habitat supported more diverse benthic bacteria in permanent streams. Short time drying didn't change the α-diversity in diverse habitat streams, whereas, relative long time drying supported greater abundance and more diverse of taxa with preferences for finely mosaic habitat in streams of low-level and medium-level habitat. Upon the form of hypoxic or anoxic environment, and accumulation of OM, flow intermittent induced greater sulfate degradation, nitrogen fixation, reduced atrazine metabolism function in all kinds of streams and reduced chitin degradation in low-level and medium-level habitat streams for the resilience of microbial communities. Rewetting remediated the drying effect on microbes through microbial activation and recolonization, developed comparable bacterial richness and diversity with that in permanent streams, except for an increased in bacterial diversity in low-level habitat streams, and adversely change the functional pattern through increasing chitin degradation in medium and high-level habitat streams, as well as enhancing atrazine metabolism and aromatic_hydrocarbons degradation functions in high-level ones. Implying that biofilm bacteria have great resilience to hydrological stress, especially in streams with more heterogeneous habitat. Habitat restoration applied for the management of degraded streams may possess greater resilience capacity toward future environment changes.

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Chapter 6 General conclusion

6.1 General introduction

Upon the wide implementation of ecological restoration to mitigate anthropogenic disruption on freshwater ecosystems, monitoring and evaluation of restoration programmes is critical in increasing our knowledge of restoration progress and associated mechanisms. Hydromorphological, water chemical properties and a few bioindicators were assessed in the monitoring of the restoration progress, however, the responses of benthic community composition to ecological restoration approaches are varied and unclear, little knowledge was obtained on the effect of ecological restoration on the ecosystem function, such as leaf litter decomposition. Moreover, the resilience of freshwater ecosystems to future climate and anthropogenic disturbance following river ecological restoration has rarely been considered, particularly for restoration projects implemented in China. Aiming at investigating the restoration progress of aquatic community and ecosystem functioning following stream ecological restoration in south China, and link restoration mechanisms to restoration process, variables and techniques, a field research comprise two seasons field sampling and an mesocosm experiment were performed. Using biofilm bacteria and macroinvertebrate as bioindicators, and leaf litter breakdown and ecosystem resilience as indicators of ecosystem health, this research focused on following objectives:

(i) Determine how habitat restoration affected benthic biofilm bacterial community composition;

(ii) Test the response of benthic macroinvertebrate communities to ecological restoration in urban rivers;

(iii) Investigate the impact of stream ecological restoration on leaf litter decomposition;

(iv) Explore the contributing factors (i.e. abiotic or biotic) for the shifts in community composition and leaf litter decomposition;

(v) Test the resilience of restored streams to flow intermittent caused by anthropogenic disturbance and climate changes.

This thesis is divided into four main chapters based one the outlined research objectives. The results and conclusions of each chapter are summarized as followings.

6.2 Chapter 2 - Effect of river ecological restoration on biofilm microbial community composition

For the shortage of knowledge on the structure and function of microbial communities in riverine systems following habitat restoration, 16S rRNA genes targeted high-throughput Illumina Miseq sequencing was used to characterise the difference in biofilm bacterial communities in forest rivers (reference sites), urban degraded rivers and urban rivers undergoing habitat restoration from the same watershed, with the aim to determine the shift pattern of biofilm bacterial community and linked environmental variables in rivers following habitat restoration. The results obtained from this chapter provide evidence that ecological restoration positively changed the bacterial community composition in the degraded rivers. Ecological restoration led to a drop of bacterial diversity, but a greater abundance of taxa that degrade organic pollutants, attributing to the variance in habitat diversity, and subsequent changes in dissolved oxygen and total organic carbon in the restored rivers. These results support the statement that through enhancing habitat heterogeneity, ecological restoration can in turn alter the water chemistry and the physico-chemical related microbial community composition. Although microbial community composition has not been recovered to "pristine" status, ecological restoration efficiently promoted the development of microbial community composition toward natural status and microbial related ecosystem processes. Therefore, this study supports the ecological theory that enhancing habitat heterogeneity via ecological restoration could be applied for sustainable freshwater restoration and management.

6.3 Chapter 3 - The effect of habitat restoration on macroinvertebrate communities in urban rivers

The use of macroinvertebrates as bioindicators for restoration have been studied in Europe and North America, however, the response of macroinvertebrates to habitat restoration differs among river studied, there have been few assessments of restoration in Asia and, in particular China. In this chapter, the macroinvertebrate community composition was compared in three types of rivers within the same watershed, forest rivers (reference sites), urban degraded rivers and urban rivers undergoing habitat restoration. The aim was to determine how macroinvertebrate community composition and taxonomic diversity differed in restored sites relative to degraded and reference sites, the environmental factors shaping macroinvertebrate communities across the three river types. The results obtained from this chapter indicate a greater Shannon diversity, a greater total richness and total abundance of macroinvertebrate, and greater richness and abundance of intolerant EPT taxa in rivers undergoing ecological restoration, supporting our hypothesis that benthic macroinvertebrate community structure could be positively shifted under stream ecological restoration. The variance in macroinvertebrate communities was closely correlated with the increased substrate diversity, flow velocity, and reduced total nitrogen, total organic carbon. Habitat characteristics contributed to most (22%) of the variation of the macroinvertebrate community, followed by water chemistry (5%) and spatial factors (4%). Accordingly, ecological restoration recovered the aquatic biodiversity to some extend mainly based on the enhanced habitat heterogeneity, therefore, habitat restoration is a positive manner to restore the ecosystem health for freshwater conservation and management. This study enhances our knowledge of the restoration progress by understanding the recovery process of macroinvertebrate community after habitat restoration and its important controlling variables, it could be used as important evidence and guidance for future endeavors on stream ecological restoration.

6.4 Chapter 4 - Evaluating ecosystem function following river restoration: the role of hydromorphology, bacteria, and macroinvertebrates

For the rare assessment and evaluation of ecosystem function in post-restoration monitoring projects, leaf litter decomposition has been used as an indicator of ecosystem integrity to assess the ecosystem function of restored rivers in China. By comparing the leaf breakdown rates in urban rivers undergoing habitat restoration with that in degraded urban rivers and rivers in forested areas (i.e., reference conditions), and linking the leaf decomposition to abiotic and biotic factors, the impact of habitat restoration on leaf litter decomposition could be measured, and the contributing factors that cause the variance in leaf litter breakdown rates assessed. The results obtained from this study demonstrated faster leaf breakdown rates (120.40% in summer, 28.06% in winter) in the rivers undergoing ecological restoration on contrast to the degraded rivers. All evaluated abiotic and biotic factors contribute appreciably to the variance in leaf litter decomposition. Macroinvertebrates (mainly shredders) contribute to the most of the variance, 52% in summer and 33% in winter, followed by habitat features (e.g. substrate diversity, water velocity; 17% in summer, 29% in winter), water chemical elements (e.g. nutrient and organic pollutants; 11% in summer, 1% in winter) and biofilm bacteria (0% in summer, 15% in winter). Ecological restoration improved degraded streams through enrich habitat composition, increase channel connectivity, restore water quality and aquatic communities (e.g., microbe, macroinvertebrate), all of which combine to improve nutrient and energy cycling process measured using leaf decomposition rates. The overall findings of this study enhance our understanding of the restoration progress of ecosystem function in degraded urban rivers and its important controlling variables. This knowledge guide us that habitat feature and aquatic organisms should be taken into consideration in future ecological restoration strategies to restore the ecosystem integrity and related ecosystem process. Moreover, for the comprehensive evaluation of the stream ecosystem function, leaf-associated fungal community and microbial production should also be tested in future determinations.

6.5 Chapter 5 - Resilience of stream biofilm bacterial communities to drying perturbation in stream ecosystems: The effect of habitat heterogeneity

To understand the resilience of freshwater ecosystems, especially the restored rivers to future climate and human disturbance, an Ex-Stream experiment was conducted in Anhui Jiulongfeng Nature Reserve to investigate the resilience of aquatic community structure to different flows (intermittent/ drying perturbations) in streams with different habitats, using benthic biofilm bacteria as bioindicators. With the aim of assessing the shift pattern of benthic bacterial community composition under flow intermittence, and the resilience of benthic bacterial community to drying condition in streams of different habitats. The results obtained from this study demonstrated a shift of bacterial community compositions either after drying events or flow resumption. Relative longtime drying induced increased bacterial richness and diversity and diminished chitin degradation in streams with low-level and medium-level habitat, and improved sulfate degradation, nitrogen fixation, reduced atrazine metabolism function in streams with three different habitat types. Controversially, flow resumption remediated the drying effects, which developed a comparable bacterial richness and diversity with permanent ones in all stream types, except for an increased bacterial diversity in low-level habitat streams. Rewetting increased the microbial metabolism activities such as chitin degradation, atrazine metabolism and aromatic hydrocarbons degradation in high-level habitat streams, and chitin degradation to some extend in medium ones, accounting for the variance in microbial community composition. Implying that biofilm bacteria hold great resilience capacity towards hydrological stress, particularly in freshwater with more heterogeneous habitat. Medium-level habitat streams, representing rivers undergoing ecological restoration may possess greater resilience capacity toward future environment changes than degraded rivers. This study enriches our knowledge of the restoration mechanisms by understanding the resilience capacity of biofilm bacteria assemblage in streams with three different habitat types, it confirmed the critical roles of habitat heterogeneity in the recovery of ecosystem structure and function, and provide powerful evidence on the restoration process of stream management.

6.6 Overall conclusions

In conclusion, this thesis demonstrated that ecological restoration arms freshwater with greater habitat environment, which promote the efficient restoration of the aquatic community, ecosystem process, and ecosystem resilience for freshwater sustainable development. This study strengthens our knowledge of the restoration progress of ecosystem structure and functioning, informs us the restoration mechanisms and critical factors (e.g. habitat heterogeneity) that kick in the restoration processes, which could be used as an important evidence and guidance for future stream ecological restoration programmes.

6.7 Limitations of the study

In this thesis, quantitative approaches were taken to investigate the response of biodiversity and ecosystem function to river ecological restoration, which forms a relative integrative platform to assess the river health. Whilst there are still some deficiency of experimental design and technical problems that need to be addressed. Statistically, only one urban system with three replicates of restored rivers were studied in the two-season field monitoring, which inhibit the formation of integrated view of restoration progress in China, for the short monitoring period and limited number of sampling sites. Further, for the shortage of data for pre-restoration monitoring and limited research time, the field monitoring experiment conducted in this research programme only compared the benthic community composition and ecosystem function in the post-restoration rivers to reference forest rivers and urban degraded rivers, though seems reasonable, but cannot fully represent the actual situation of the restoration progress, before-after experiment design might interpret the restoration progress more accurately.

Technically, litter decomposition can be controlled by the biodiversity, biomass, and activities of bacteria, evaluating α-diversity alone in this study may obscure the contribution of bacteria in leaf mass loss. Aquatic fungi, mainly hyphomycetes, are more efficient than bacteria in leaf breakdown. Unfortunately, fungi were not taken into consideration and this limits the comprehensive interpretation of river restoration progress. Further, the traditional methodology used for macroinvertebrate sampling, sorting and identification might not override the full scale of community compositions in the studied aquatic ecosystems, more advanced molecular techniques such as testing of environmental DNA could better compromise the short backs brought by the traditional approach.

6.8 Future directions

The overall findings of this study providing useful evidence that habitat restoration can be used as an effective measure of freshwater management via recovering ecosystem structure and function. For future water conservation and management, it is highly recommended to take habitat features, physico-chemical properties and aquatic organisms into consideration in ecological restoration strategies to restore the ecosystem integrity and related ecosystem process. Prolonged monitoring and evaluation of restoration programmes should be included as a key component of restoration strategy to assure the sustainable development of the restored systems, either for pre-restoration or post-restoration.

Moreover, the restoration response may be varied both spatially and temporally, hence, longtime and multi-city monitoring and study of the river restoration programmes around China would help form an integrated view of restoration progress and efficiency of different restoration approaches, which provides water managers and policy makers an integrated guidance for future planning of ecological restoration and management strategies.

Technically, efficient monitoring and assessment of aquatic communities requires reliable methods. Molecular techniques could be used for the simultaneous mapping of taxonomic and functional diversity in stream communities. Apart from assessing the α-diversity, community composition, the activities and metabolic processes of aquatic organisms (i.e. bacteria, fungi, and macroinvertebrates) should be better acknowledged in bioassessment for comprehensive interpretation of the river restoration progress.

Appendices, supplementary information

Chapter 2

Table S2.1 Detailed location data and habitat information for the nine study sites within the Anji City Region, PRC tested in winter 2017; Habitat information include canopy cover, habitat types, substrate composition and substrate Shannon index (H’). F = forest streams; R = restored streams; D = degraded streams.

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

Chapter 3

Table S3. 1 Detailed location and habitat information for the nine study sites within the Anji City Region, PRC tested in summer 2018; Habitat information include canopy cover, habitat types, substrate composition and substrate Shannon index (H'). F = forest streams; R = restores streams; D = degraded steams.

Site code	River name	Location (Longitude & Latitude)	Canopy cover (%)	Habitat types present			Substrate composition (%)				Substrate Shannon Index(H')
				Island	Pool	Riffle	Boulder	Cobble	Pebble	Granule	
F-1	Longwang Mountain	30°25'3.93"N 119°24'30.52"E	78	✓	✓	✓	21.2	74.3	3.6	0.9	0.71
F-2	Yangjiao Mountain	30°26'59.18"N 119°27'55.03"E	95	✓	✓	✓	23.1	70.1	5.7	1.1	0.80
F-3	Zhebei Valley	30°25'24.05"N 119°30'33.60"E	92	✓	✓	✓	12.7	46.9	34.8	5.6	1.15
R-1	Shima Port	30°37'52.98"N 119°41'57.03"E	3	✓	✓	✓	0	11.9	39.2	48.9	0.97
R-2	Depu Gang	30°36'22.34"N 119°41'39.80"E	5	✓	✓	✓	0	13.2	57.1	29.7	0.95
R-3	Wuxiangba	30°38'43.04"N 119°36'32.29"E	6	✓	✓	✓	0	69.4	27.9	2.7	0.71
D-1	Tongxin	30°38'13.96"N 119°41'28.86"E	28	-	✓	-	0	0	0	100	0
D-2	Wanmu	30°38'7.99"N 119°39'2.36"E	9.5	✓	✓	-	0	0	0	100	0
D-3	Chiyi	30°38'28.69"N 119°36'12.85"E	75	-	✓	-	0	0	0	100	0

Table S3.2 Summary of (M)ANOVA results for different types of rivers within Anji City Region, PRC. Significant *p*-values (<0.05) are printed in bold.

Invertebrate Community Matric	Forest vs. Degraded		Forest vs. Restored		Restored vs. Degraded	
	p	difference	*p*	difference	*p*	difference
Total abundance	**0.001**	3.162	0.692	-0.379	**<0.001**	3.541
Total richness	**<0.001**	1.870	0.426	0.133	**<0.001**	1.737
EPT abundance	**<0.001**	5.018	0.596	-0.458	**<0.001**	5.477
EPT richness	**<0.001**	2.208	0.330	0.321	**<0.001**	1.887
Shannon-Wiener index	**<0.001**	0.744	0.387	0.115	**<0.001**	0.629
Pielou's evenness	0.589	0.084	0.819	0.050	0.911	0.034
Intolerant taxa richness	**<0.001**	2.057	0.068	0.394	**<0.001**	1.663
Chironomidae	0.991	-0.022	0.897	0.077	0.838	-0.098
Leptophlebiidae	**<0.001**	0.201	**<0.001**	0.176	0.639	0.025
Perlidae	**<0.001**	0.071	**<0.001**	0.071	1.000	0.000
Coenagriidae	**<0.001**	0.014	**<0.001**	0.014	0.885	0.000
Leptoceridae	**0.015**	0.057	**0.018**	0.054	0.982	0.002
Caenidae	0.739	0.066	0.136	-0.196	0.052	0.262
Baetidae	0.685	0.030	0.260	-0.063	0.088	0.093
Corbiculidae	1.000	0.000	**<0.001**	-0.025	**<0.001**	0.025
Gossiphonidae	1.000	0.000	0.053	-0.008	0.053	0.008
Heptageniidae	0.636	0.010	0.219	-0.020	0.066	0.030
Tubificidae	0.109	-3.552	1.000	1.064	0.109	-3.552
Viviparidae	0.197	-0.108	0.980	-0.010	0.251	-0.098

Table S3.3 Spearman correlation coefficients between environmental variables (i.e. habitat characteristics, physico-chemical variables) and macroinvertebrate alpha diversity for different types of rivers within Anji City Region, PRC.

	Total Abundance	Total Richness	EPT abundance	EPT richness	Intolerant richness	Shannon diversity
pH	0.23	0.41	0.08	0.44	0.44	0.50
Turbidity	-0.13	-0.13	-0.05	-0.17	-0.13	-0.10
DO	0.57	0.65	0.55	0.66	0.64	0.62
NH_4-N	-0.63	-0.64	-0.59	-0.61	-0.60	-0.59
NO_3-N	-0.22	-0.35	-0.12	-0.40	-0.35	-0.35
TN	-0.68	-0.79	-0.62	-0.79	-0.77	-0.80
TP	-0.57	-0.72	-0.62	-0.76	-0.77	-0.65
TOC	-0.73	-0.90	-0.72	-0.90	-0.89	-0.85
Chl-*a*	-0.06	0.04	0.04	0.04	0.07	-0.03
COD	-0.44	-0.72	-0.40	-0.79	-0.73	-0.74
Water velocity	0.50	0.30	0.39	0.16	0.08	0.40
Substrate diversity	0.84	0.97	0.85	0.95	0.95	0.90
Canopy cover	-0.04	0.35	-0.09	0.47	0.49	0.39

Chapter 4

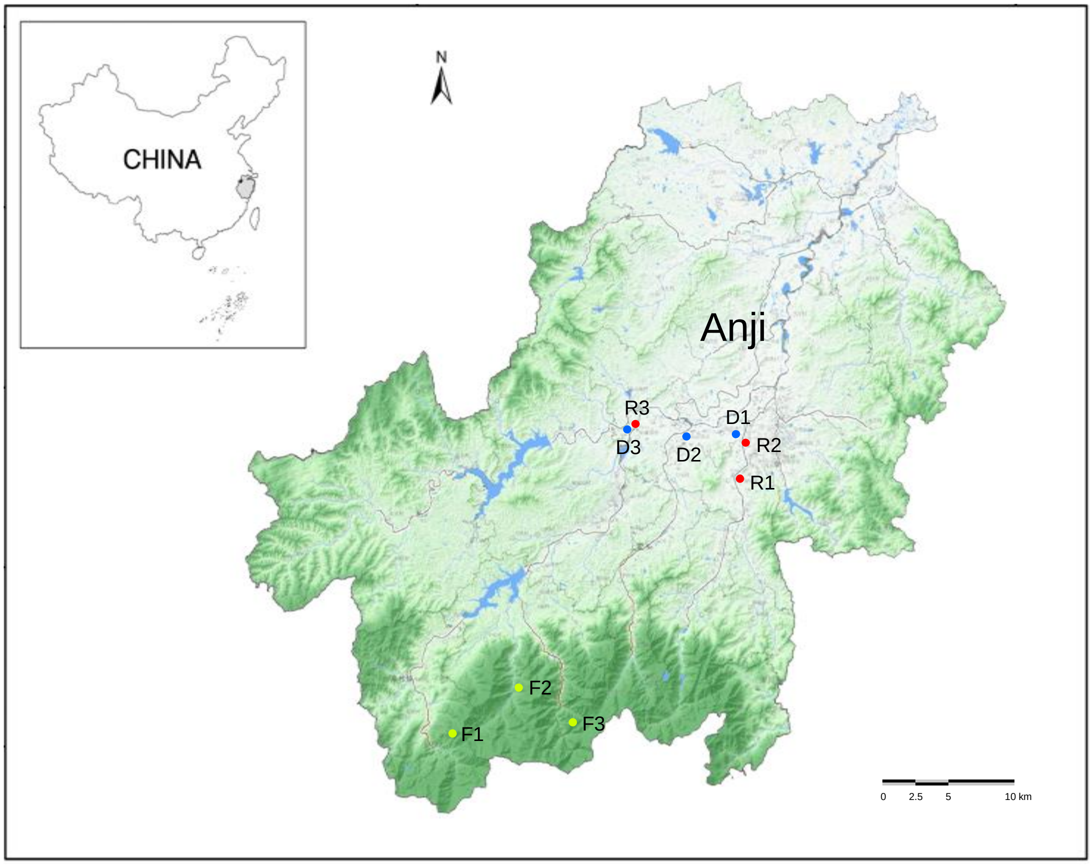

Figure S4.1 Study area and locations of sampling sites within the Anji City Region, People's Republic of China (PRC), including three degraded urban rivers (D), three rivers under habitat restoration (R), and three forested rivers (F).

Table S4.1 Nomenclature and Abbreviation List

N	Nitrogen
P	Phosphorus
CO_2	Carbon dioxide
DO	Dissolved oxygen
NH_4-N	Ammonium nitrogen
NO_3-N	Nitrate-nitrogen
TN	Total nitrogen
TP	Total phosphorus
TOC	Total organic carbon
COD	Chemical oxygen demand
FPOM	Fine particular organic carbon
FFGs	Functional feeding groups
C-F	Collector-filterer
C-G	Collector-gatherer
Scr	Scraper
Shr	Shredder
Prd	Predator
PRC	People republic of China
PCNM	Principal Coordinates of Neighborhood Matrices
OTUs	Operational Taxonomic Units
RDP	Ribosomal Database Project
Camphor	Cinnamomun camphora
Habitat	Habitat variable
ENV	Physico-chemical variables
Spatial	Spatial factors
Macroinvertebrate	Macroinvertebrate matrics
Bacteria	Bacterial alpha diversity
Alpha diversity	α-diversity