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Biodiversity conservation and ecological function restoration in freshwater ecosystems

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Published in
Frontiers in Ecology and Evolution
Frontiers in Environmental Science

ISSN 1664-8714
ISBN 978-2-83251-717-8
DOI 10.3389/978-2-83251-717-8

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March 2023

Biodiversity conservation and ecological function restoration in freshwater ecosystems

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Citation
Zhang, M., Martinez-Capel, F., Qu, X., Wu, N., eds. (2023). *Biodiversity conservation and ecological function restoration in freshwater ecosystems*.
Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-717-8

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frontiers | Frontiers in Ecology and Evolution

TYPE Editorial
PUBLISHED 06 February 2023
DOI 10.3389/fevo.2023.1137985

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to
Conservation and Restoration Ecology,
a section of the journal
Frontiers in Ecology and Evolution

RECEIVED 05 January 2023
ACCEPTED 20 January 2023
PUBLISHED 06 February 2023

CITATION
Wu N, Zhang M, Qu X and Martinez-Capel F (2023) Editorial: Biodiversity conservation and ecological function restoration in freshwater ecosystems. *Front. Ecol. Evol.* 11:1137985.
doi: 10.3389/fevo.2023.1137985

Editorial: Biodiversity conservation and ecological function restoration in freshwater ecosystems

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KEYWORDS

anthropogenic factors, biodiversity, ecological function, freshwater, traits

Editorial on the Research Topic
Biodiversity conservation and ecological function restoration in freshwater ecosystems

The significance and problems of freshwater ecosystems

As an essential resource for human life, freshwater has no substitutes. Freshwater ecosystems are among the most diverse and dynamic ecosystems on the planet, covering <1% of the planet's surface, yet supporting ~9.5% of animal species and ~ one third of vertebrate species (Dudgeon et al., 2006; Balian et al., 2008; Wu et al., 2023). They also provide a wide range of ecosystem services to humans, such as flood regulation, food supply and cultural significance. These ecosystem services are intrinsically linked to the functional diversity of existing organisms and can therefore connect human societies to their habitat. Freshwater ecosystems are among the most degraded ecosystems on earth due to land use intensification, point and non-point source pollution, river modifications and overexploitation (Vörösmarty et al., 2010; Couto and Olden, 2018; Reid et al., 2019). Multiple stressors such as those described above will continue to have a profound impact on biodiversity and ecological system functions of freshwater ecosystems (Hering et al., 2015; Guo et al., 2020; Juvigny-Khenafou et al., 2020, 2021; Zhou et al., 2020), and the changes related with climate and global change are meaning additional threats for the freshwater communities (e.g., Martínez-Capel et al., 2017; Muñoz-Mas et al., 2018). In order to maintain biodiversity and key ecosystem processes, the conservation and restoration of freshwater ecosystems must be a top priority; thus global coordinated efforts are fundamental to advance in freshwater biodiversity science and conservation (Darwall et al., 2018).

Our goal is to address recent advances in the biodiversity-ecological functioning relationship, to illuminate the biodiversity maintaining mechanisms, and to proclaim the main environmental factors (flow discharge, river connectivity, etc.) that influence the biodiversity and ecosystem functions of freshwater ecosystems at different spatial scales. Through these scientific advances, we aim to improve our understanding of theories related to ecosystem function and provide support for biodiversity conservation and ecosystem restoration in freshwater ecosystems globally. This collection of 15 papers is our modest contribution to achieving this goal.

Impacts and solutions

In this research theme we have collected many latest pieces of research on freshwater ecosystems functions and freshwater biodiversity (including fish, micro-eukaryotic, phytoplankton, benthic algae, wetland plant and macroinvertebrates).

Uncovering the underlying drivers of biodiversity patterns has been a major Research Topic in ecology and biogeography for a long time (Myers et al., 2000; Chase, 2003; Leibold et al., 2004). Here we present three studies on the community assembly processes and their drivers. Ren at al. used 18S rRNA gene sequencing to assess the biogeography of micro-eukaryotic communities (MECs) and their driving factors in sediments of thermokarst lakes across the Qinghai-Tibet Plateau and concluded MECs in this region were jointly controlled by spatial and climatic factors as well as sediment properties. Wang, Li et al. showed through a temporal food web study of urban and woodland rivers that land use and seasonal changes in environmental conditions influence biological communities and their trophic interactions in riverine ecosystems. Xiang, Wang et al. studied the spatio-temporal dynamics of fish assemblages in the karst tributaries of the upper Yangtze River and showed that fish assemblages differed significantly between river reaches, whereas did not vary in a significant manner during 4 months. Fish communities in that region should therefore be protected by conserving intermediate habitats, particularly the many pools and riffles. The development of effective monitoring and management strategies to halt biodiversity decline has become an important topic in freshwater research (Myers et al., 2000; Stendera et al., 2012; Hermoso et al., 2016), and several of the above studies provide support for a comprehensive understanding of the processes that shape freshwater communities.

Recently, community ecologists have realized the need to start with information not only at the taxonomic level, but also from a functional perspective to understand the environmental interactions and the mechanisms shaping the community assembly in animals and plant communities (McGill et al., 2006; Tabacchi et al., 2019; Liu et al., 2021). Ao et al. harmonized and searched representative databases of macroinvertebrate traits from several continents and implemented this method in the Three Parallel Rivers Region, China, filling a research gap in those regions, including China, where macroinvertebrate trait studies were lagging. This approach has greatly contributed to the uniformity of global trait studies and to the accuracy and comparability of trait studies in different regions (Vieira et al., 2006; Sarremejane et al., 2020).

Furthermore, the relationship between biodiversity and ecosystem functioning is a central topic in ecological research. However, most relevant research publications mainly focus on grassland and forest ecosystems (Ptacnik et al., 2008; Filstrup et al., 2014). Zhang et al. highlighted the importance of functional diversity in maintaining the relationship between biodiversity and stability of phytoplankton community in the Xiangxi Bay of the Three Gorges Reservoir; this work contributes to the mechanistic understanding of the biodiversity-stability relationships in aquatic ecosystems.

In addition, several studies have shown that human infrastructure construction is a major driver of the losses in biodiversity and ecological function. For example, the construction of dams, reservoirs and hydropower stations threatens biodiversity, the ecosystem processes and services supported by rivers (Anderson et al., 2015; Grill et al., 2019); furthermore, the effects of flow regulation by dams can be intensified in an additive or synergistic way with climate and global change (Martínez-Capel et al., 2017; e.g., Bruno et al., 2019). Here we present a few articles on the impact of human engineering on organisms. Li Y. et al. elucidated the ecological response of planktonic eukaryotes by identifying their diversity and ecological distribution in trans-basin diversion channels. Liu et al. quantified the genetic diversity and population structure patterns of *Saurogobio dabryi* after habitat fragmentation caused by dams, which provided a reference for resource protection and management of this species in the upper Yangtze River. By studying the response of phytoplankton functional diversity to physicochemical conditions in subtropical cascade reservoirs, Shen et al. found inconsistent patterns concerning the cascading reservoir continuum concept (CRCC) and contributed to the further development of the theory of the CRCC. Wang, Wu et al., in the Xiangxi River, described how small run-of-river dams affect different facets of β-diversity and community assembly process of benthic diatoms, and suggested that such kind of studies could be extended to other aquatic organisms (such as macroinvertebrates, phytoplankton, fish).

Li D. et al. revealed the complex quantitative relationships of planktonic food chains in wild aquatic ecosystems from the perspective of species interactions. Ecological threshold detection of cladocera-cyanobacterial abundances provides a quantitative basis for early warning and a key to the mechanisms of cyanobacterial blooms, contributing to more effective control and prevention methods. Zhou et al. and Wagutu et al. elucidated the relationship between crayfish and hydrilla and their environment from a genetic ecology perspective, providing valuable information for management strategies of such species. The former study will contribute to control strategies for invasive species, thereby reducing the damage to ecosystem function caused by invasive species (Simberloff et al., 2013).

Finally, we also collected three novel and valuable papers. Higher water temperatures could accelerate the decomposition of leaf-litter. Xiang, Li et al. pointed out that this may lead to the depletion of food for detritivores during future summers. Golpour et al. showed the Shannon diversity index of water eDNA targeting fish (wf-eDNA) method was significantly higher than traditional sampling methods. Thus wf-eDNA seems to be a reliable and complementary approach for biomonitoring and ecosystem management of freshwater ichthyofauna. Finally, the study by Feng et al. provided guidance on the deployment of artificial spawning grounds at both temporal and spatial scales, and also supported the enhancement of lake fish breeding and fisheries in Chinese lakes.

Implications and conclusions

In summary, freshwater ecosystems provide us with numerous ecosystem services and are the ecosystems on which human beings depend for their survival. In the 21st century, human activities and climate change will continue to have a profound impact on freshwater ecology. We have collected numerous recent research advances on the conservation of biodiversity and restoration of ecological functions in freshwater ecosystems in this Research Topic. We believe this collection can provide additional useful information for the realization of the topic. From a broader perspective, we have a responsibility and an obligation to work together to protect freshwater ecosystems.

Author contributions

NW wrote the first draft. All authors contributed to the article and approved the submitted version.

Acknowledgments

We are very grateful to all our colleagues who submitted, reviewed, and edited manuscripts for this Research Topic. Guohao Liu drafted this editorial.

Conflict of interest

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

Publisher's note

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ORIGINAL RESEARCH
published: 29 April 2022
doi: 10.3389/fevo.2022.895328

Small Run-of-River Dams Affect Taxonomic and Functional β-Diversity, Community Assembly Process of Benthic Diatoms

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Being increasingly constructed worldwide, dams are a main driver of flow regime change and biodiversity decline. Although small run-of-river dams have exceeded the number of large dams, their impacts on taxonomic and functional β-diversity as well as community assembly process of aquatic organisms have been largely neglected. Ninety sites within twenty three small run-of-river dams in the Xiangxi River were selected, and the hydrological and physicochemical variables for each site were measured. We analyzed the traits and β-diversity of benthic diatoms, and explored the key driving mechanism of benthic diatom community assembly. Our results indicated that the construction of small run-of-river dams could affect the β-diversity of benthic diatoms and the mechanism of community assembly. Specifically, we found that small run-of-river dams could change the relative contribution of nestedness components to the trait-based β-diversity of benthic diatoms, but generally the taxonomy-based β-diversity was relatively higher than the trait-based β-diversity. Furthermore, the community assembly process of benthic diatoms was also affected. In areas affected directly by small run-of-river dams, dispersal assembly was the key mechanism for community assembly. Compared to unregulated habitats, the dispersal assembly process between the impacted and the unregulated habitats has been enhanced. We advocate that this study can be expanded to other organisms (such as macroinvertebrates, phytoplankton, fish) in future to fully understand impacts of small run-of-river dams on biodiversity from a multi-trophic level aspect. Based on our results, we suggest that maintaining genetic and ecological connectivity based on an effective impact assessment in dry seasons is a potential solution to mitigate the impacts of such dams, as key to adaptive management and sustainability.

Keywords: β-diversity decomposition, DNCI, nestedness, small hydropower, turnover, Xiangxi River

OPEN ACCESS

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Specialty section:
This article was submitted to Conservation and Restoration Ecology,
a section of the journal Frontiers in Ecology and Evolution

Received: *13 March 2022*
Accepted: *11 April 2022*
Published: *29 April 2022*

Citation:
Wang Y, Wu N, Tang T, Zhou S and Cai Q (2022) Small Run-of-River Dams Affect Taxonomic and Functional β-Diversity, Community Assembly Process of Benthic Diatoms. Front. Ecol. Evol. 10:895328. doi: 10.3389/fevo.2022.895328

INTRODUCTION

Due to its economic benefits, hydropower, as a clean and renewable energy source, often becomes the first choice in most countries (Huang and Yan, 2009). The hydropower plants can be divided into large hydropower plants (LHPs) and small hydropower plants (SHPs). The construction of LHPs brings huge social and ecological costs, such as negative impacts on water volume and quality, biodiversity, fisheries and other ecosystem services (Ansar et al., 2014; Kahn et al., 2014; Deemer et al., 2016). In contrast, SHP, as an important part of the future energy strategy, has become the consensus of all countries in the world due to its relatively small impact on the environment (Paish, 2002; Tang et al., 2012). Studies have shown that there are 82,891 SHPs in operation or under construction around the world. If all potential power generation capacity is to be developed, the number of SHPs will be twice as many as it is now (Couto and Olden, 2018). The construction of hydropower plants will affect the ecosystem in various ways, such as changing the continuity of rivers, changing water quality, increasing habitat fragmentation and degradation, and loss of biodiversity (Mallik and Richardson, 2009; Vaikasas, 2010). The research on the impact of LHPs on freshwater biological communities is relatively mature, but the research on the environmental impact of SHPs is still rare (Jia et al., 2009; Atilgan and Azapagic, 2016; de Faria et al., 2017; Aung et al., 2020). With the increasing number of SHPs, more and more scholars are aware of their impact on river ecosystems (Baskaya et al., 2011; Lange et al., 2018; Sarauskiene et al., 2021).

β-diversity not only includes the relationship between α-diversity and γ-diversity, but also can show the changes in species composition on a temporal and spatial scale. Recent advances in ecology believe that differences in species composition between communities originate from two different processes: species turnover and nestedness (Baselga, 2010). Among them, species turnover means species replacement between different communities, while a nestedness pattern presents if the communities with lower species richness is a subset of the communities with higher species richness (James et al., 2012; Staniczenko et al., 2013). β-diversity decomposition is to distinguish the effects of these two processes on total β-diversity, and to explore how these two processes together affect species distribution patterns in different temporal and spatial dimensions (Baselga, 2010; Podani and Schmera, 2011). Decomposition facilitates a deeper understanding of the distribution pattern of biodiversity and its maintenance mechanism (Baselga and Leprieur, 2015; Wu et al., 2022).

The formation of β-diversity is considered to be the result of combined effects of the two ecological processes of niche and dispersal (Soininen et al., 2007). The niche process believes that the range of the distribution area of a species is determined by the adaptation characteristics of the environment, while the dispersal process believes that the spreading ability of the species determines the distribution of the species (Green and Ostling, 2003). Probing the importance of niche and dispersal process in community assembly is conducive to strengthen the understanding of community assembly and protect biodiversity. Gaining more information on β-diversity allows for the correct identification of key mechanisms of community assembly for efficient selection of suitable protected areas (Sarkar, 2006). However, there are still controversies about how to apply appropriate statistical analysis methods to study β-diversity (Legendre et al., 2005; Tuomisto and Ruokolainen, 2006, 2008). The null model and variation partitioning methods are commonly used methods to quantify the process of community assembly, but they also have limitations. For example, the null model method relies on detailed data on community composition, environmental factors, and geographic location. Variation partitioning method cannot correctly represent the environmental and spatial components of community variation, making it difficult to explain the underlying process (Smith and Lundholm, 2010; Clappe et al., 2018). To overcome the data and methodological limitations of previous methods, a dispersal-niche continuum index (DNCI) has been proposed based on PER-SIMPER, which can effectively identify community assembly mechanisms (Vilmi et al., 2021). DNCI measures the relative strength of the community assembly process in a simple and easy way, which can be compared across data sets. Vilmi et al. (2021) developed DNCI based on stream diatoms, macroinvertebrates, fish, bacteria, and macroinvertebrates data and has been implemented in fleas and small mammals community assembly (Gibert et al., 2021). The construction of small run-of-river dams has caused the fragmentation of rivers and is likely to change the community construction mechanism. Currently, relevant research is still blank.

Benthic algae in rivers have the advantages of short life cycle, easy access, sensitive to physical and chemical changes in water bodies. As a consequence, they are increasingly used as indicators for water quality assessment (Lepisto et al., 2004; Wu et al., 2017). However, the current research on the impact of small run-of-river dam construction is mainly focusing on taxonomy-based α-diversity. For example, Wu et al. (2010) found that the construction of small run-of-river dams had affected the richness, diversity and traits of benthic diatoms. To our best knowledge, there are no studies on the impact of biological β-diversity yet.

In this study, we examined the potential impacts of small run-of-river dams from a Chinese mountain catchment (i.e., the Xiangxi River with 5 different habitat groups: H01–H05) on taxonomy- and trait-based β-diversity of benthic diatom communities and their components (i.e., turnover and nestedness) (**Figure 1**). We had three main research questions: (*i*) can small run-of-river dams change the relative contributions of turnover and nestedness components to total taxonomy- and trait-based β-diversity of benthic diatom communities? (*ii*) what is the key mechanism of community assembly (dispersal assembly, niche assembly or joint dispersal-niche assembly) in the area impacted by small run-of-river dams? (*iii*) do small run-of-river dams affect the community assembly process of benthic diatoms? Since species sharing similar niches are grouped into same traits, taxonomic species replacements do not always result in traits turnover. Furthermore, anthropogenic activities such as dam construction often cause segmentation and homogenization of stream habitats, and taxa in similar environments tend to share common traits (Goldenberg Vilar et al., 2014; Castro et al., 2018). Thus, we hypothesized that taxonomy-based β-diversity should be relatively higher, particularly a higher turnover, than trait-based β-diversity (HY1); the two facets of β-diversity at impacted habitats (e.g., H03 and H05) should be relatively lower than those at unregulated habitats (HY2). Since the main consequences from dam construction are losses of stream connectivity, we expected the dominant community assembly processes of both species and trait compositions will be affected. More specifically, the dispersal assembly process should be intensified between the impacted habitats (e.g., H03 and H05) and unregulated habitats (HY3).

MATERIALS AND METHODS

Study Area

Xiangxi River, originating from Shennongjia Mountain, lies in the west of Hubei Province. It is the largest river in Hubei Province and the closest tributary to the Three Gorges Dam. It has a total length of 469.7 km and an area of 3,099.4 km^2 (Fu et al., 2006). The characteristic of the river course is typical, and the basin is very rich in precipitation and water resources. In order to make full use of hydropower resources, many small run-of-river dams have been built on both sides of the Xiangxi River. Most of the water in the river channel is introduced into special penstocks by using the elevation difference to generate electricity, and only a small part of the river water is left to maintain the normal function of the river channel. In the Xiangxi River Basin, 23 small run-of-river dams and 5 sites on each of the river sections for investigation were selected (S1–S5) (**Figure 1**). Since the small run-of-river dams constructed by cascades caused site overlap, we selected a total of 90 sites for sampling in October 2005. 5 sites were selected before and after the small dams. S1 and S2 were located upstream of the water intake. S1 selected a point where the river course was not affected by the dam, which was located about 50–100 m upstream of the dam. S2 was located at upstream of the dam and was less affected. S3 and S4 were located between the water intake and the water outlet. S3 was a pool formed by overflow and erosion in the rainy season under the water intake. S4 was located at upstream of the water outlet, and the water flow could only be restored when the downstream of S3 collects water. S5 was a deep pool formed downstream of the water outlet. We divided the 90 sites into 5 habitat groups (H01–H05) according to the impact degree of small run-of-river dams. H01–H05 correspond to S1–S5, respectively.

Field Sampling and Processing

The sampling process was divided into two parts: sampling and identification. Algae samples were collected at the 90 sites. At each sample point, three to five representative stones with a diameter of ∼25 cm were selected, and the algae in the sampling area with a diameter of 2.7 cm was brushed, and then rinsed with 350 ml distilled water. Algae samples were then preserved with 4% formalin for subsequent identification. The identification of benthic algae included two steps, the identification of non-diatoms and the preparation of permanent diatom slides. First, a 400× magnification microscope was used to identify non-diatoms in the 0.1 ml counting chamber. Second, permanent diatom slides were prepared with nitric acid and sulfuric acid. We counted at least 300 diatom valves using a 1,000× magnification microscope under oil immersion conditions. We used available information to determine algae as the lowest classification level from Anonymous (1992). Hu et al. (1980), Zhang and Huang (1991), and Zhu (2000). Ind/m^2 represented the unit of density of benthic algae. As the most abundant group of benthic algae in the Xiangxi River was diatoms (about 80% of the total abundance) (Wu et al., 2010, 2012), we focused on diatoms in the following analyses.

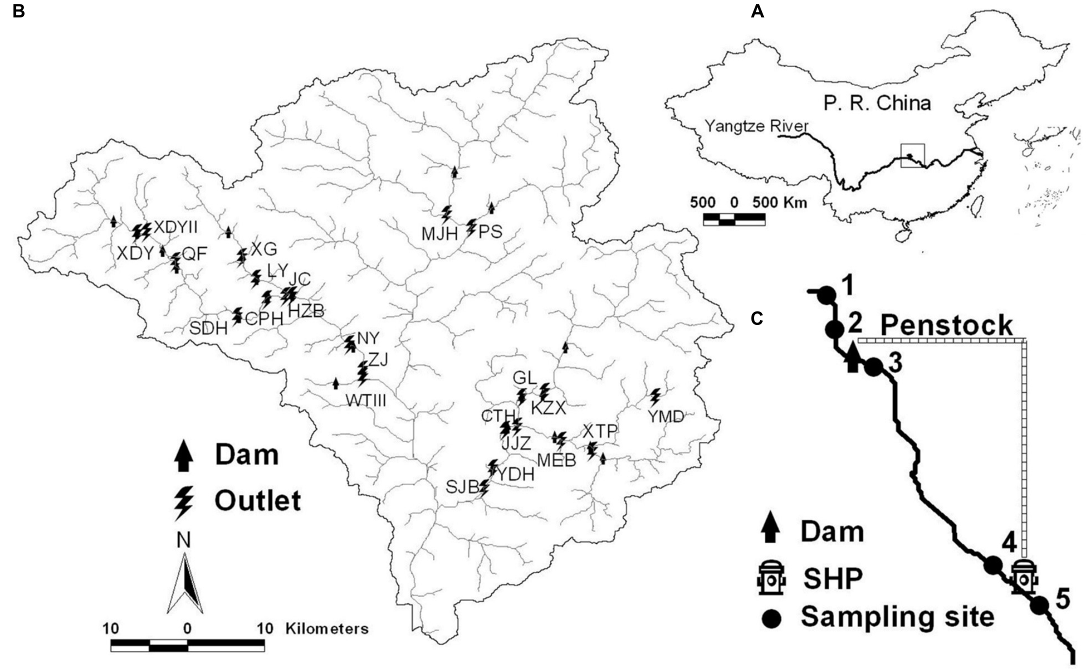

FIGURE 1 | Small run-of-river dams within the Xiangxi River watershed **(B)** in the People's Republic (P. R.) of China **(A)** and sketch map of sampling sites **(C)**. SHP, small hydropower station; letters in **(B)** are names of SHPs. The figure was modified from Wang et al. (2022).

Environmental Variables

Hydrological factors such as river width (Width), water depth (Depth), and velocity (Velocity) (using LJD-10 water current meter; Chongqing hydrological machines manufactory, Chongqing, China) were measured at each point. Meanwhiles, water temperature (WT), dissolved oxygen (DO), calcium concentration (Ca), pH, conductivity (COND), turbidity (TURB), total dissolved solids (TDS), salinity (Sal), Chloride (Cl), and oxidation reduction potential (orp) were measured *in situ* using a Horiba W-23XD (multiprobe sonde). ∼1 L of river water was collected in a pre-cleaned bottle to measure the two chemical variables of phosphate (PO_4) and total phosphorus (TP) in the laboratory (Chinese Environmental Protection Bureau, 1989).

Diatom Traits

The benthic diatoms collected at 90 sampling points were classified by trait. The trait information of all observed species comes from the literature (Passy, 2007; Wagenhoff et al., 2013; Wu et al., 2017; Witteveen et al., 2020). We divided it into three categories: cell size, guild, and life form, which included 15 different traits: cell size (large, macro, meso, micro, or nano), guild (high profile, low profile, motile, or planktonic guild), and life form (filamentous, unicellular, or colonial life form; high, medium, or low attachment) (**Table 1**). We have chosen as the study area the reaches of the river where small run-of-river dams are located to ensure that all communities at least partially share the same regional species pool.

TABLE 1 | Diatom traits, their categories, codes, and descriptions used in this study.

Traits	Categories	Codes	Descriptions
1. Cell size (Berthon et al., 2011; Rimet and Bouchez, 2012; Wu et al., 2017)	Nano (0–100 μm^3)	CellSize01	Due to the higher nutrient absorption rate and growth rate, smaller cells have greater anti-interference ability. Compared to larger cells, they have advantages under nutrient-limiting and high-disturbance conditions.
	Micro (100–300 μm^3)	CellSize02	
	Meso (300–600 μm^3)	CellSize03	
	Macro (600–1,500 μm^3)	CellSize04	
	Large ($\geq$1,500 μm^3)	CellSize05	
2. Guild (Passy, 2007; Rimet and Bouchez, 2012)	Low profile	LowPro	Differentiate ecological guilds based on their potential to tolerate nutritional limitations and physical disturbances.
	High profile	HigPro	
	Motile taxa	MotTax	
	Planktonic taxa	PlaTax	
3. Life form (Biggs et al., 1998; Wagenhoff et al., 2013; Lange et al., 2016)	Unicellular life form	LF_Uni	Distinguish life forms according to their resource gathering and disturbance tolerance.
	Filamentous life form	LF_Fil	
	Colonial life form	LF_Col	
	Low attachment	ATT_Low	According to the attachment of diatoms to the substrate to distinguish the intensity of their disturbance.
	Medium attachment	ATT_Med	
	High attachment	ATT_Hig	

Data Analysis

R (version 4.0.2) (R Core Team, 2020) was used to perform all analyses. First, we decomposed β-diversity into two aspects: taxonomy- and trait-based β-diversity. Using the *beta.pair* function in the R package *betapart*, based on presence-absence data (using Sørensen distance), the total taxonomy-based β-diversity was divided into two parts: turnover and nestedness (Baselga and Orme, 2012). The trait-based β-diversity matrix included three components: total β-diversity, turnover, and nestedness, which were generated using the above 15 characteristics (using Sørensen distance). First, the *gowdis* function in the R package *FD* was used to calculate the distance between species based on trait data. Second, the Gower distance was used for principal coordinate analysis (PCoA), and the *pco* function in the R package *labdsv* was used to generate the trait vector. Third, the function *functional.beta.pair* in the R package *betapart* was used to generate three trait-based β-diversity matrices, using the first two PCoA vectors and species data. By decomposing β-diversity, we can know the relative contribution of turnover and nestedness components to the total taxonomy- and trait-based β-diversity, and compare the differences among the 5 different habitats (H01–H05) (*question i*). Furthermore, we ran Mantel test (using the function *mantel* in R package *vegan*) (Oksanen et al., 2019) to test the relationship between taxonomy- and trait-based β-diversity components to examine whether both facets of β-diversity provide complementarity of ecological information.

To explore what is the key mechanism of community assembly processes in the study area impacted by small run-of-river dams (*question ii*) and how do they affect community assembly processes of benthic diatoms (*question iii*), we calculated and compared the pairwise DNCI values (function *DNCI_multigroup* in R package *DNCImper*) (Gibert, 2021) for both species and trait composition of benthic diatoms among 5 different habitats. The DNCI can effectively estimate whether dispersal or niche processes dominate community assembly without environmental or spatial data, and it allows the comparisons of processes among different datasets (Vilmi et al., 2021). At the same time, the DNCI method relies on the distribution of taxa, which can better clarify the importance of the niche and dispersal process in the community assembly, and strengthen the understanding of the community assembly (Chase et al., 2011; Vilmi et al., 2021). In addition, the interpretation of DNCI values is relatively simple:

if the DNCI is not significantly different from 0, we can assume that the dispersal and niche processes contribute equally to variations in community composition; if the DNCI is significantly lower than 0, dispersal processes are the primary determinants of community composition, whereas if the DNCI is significantly higher than 0, community composition is mainly driven by niche processes. In order to produce more robust results, we made pairs of groups even by random sampling of the largest groups (i.e., symmetrize = TRUE).

RESULTS

Environmental Variables

Hydrological factors such as velocity, width and depth were obviously affected by the construction of small run-of-river dams (**Supplementary Table 1**), especially habitats such as H03, H05 that were disconnected due to the small dams. The rest 12 environmental variables (i.e., WT, DO, TP, PO_4, pH, Sal, COND, orp, TURB, TDS, Cl, and Ca) had no significant differences among the 5 habitat groups (H01–H05). It could be seen that the construction of small run-of-river dams mainly affected hydrological factors, while no obvious impact was found on other physical and chemical variables.

Small Run-of-River Dams Affect Two Facets of β-Diversity and Its Components

The main patterns observed in the Xiangxi River Basin were turnover of taxonomy-based β-diversity and nestedness of trait-based β-diversity, and the contribution of turnover (0.489) was greater than nestedness (0.338) (**Figure 2**). As expected by HY1, taxonomy-based β-diversity (0.584) was relatively higher than trait-based β-diversity (0.243). The contribution of nestedness to trait-based β-diversity was significantly lower in impacted habitats (e.g., H03: 0.147, H05: 0.143) than unregulated habitats (e.g., H01: 0.214, H02: 0.253, H04: 0.232). From the perspective of environmental variables, the construction of small run-of-river dams had a great impact on the hydrological factors of H03 and H05 two types of habitats. The difference between habitats had a great influence on the trait of the benthic diatom community, and changed the relative contribution of nestedness components to the total trait-based β-diversity of the benthic diatom community, which answered our first question (*question i*). It could be seen from the **Figure 2** that the total trait-based β-diversity of impacted habitats (e.g., H03: 0.192, H05: 0.168) were lower than the unregulated habitats (e.g., H01: 0.257, H02: 0.296, H04: 0.269), partially supporting our HY2.

The Mantel test showed that the correlation between taxonomy- and trait-based β-diversity components were significant ($p < 0.001$), but correlation coefficient (i.e., Mantel r) was not high, indicating that the two provide different ecological information. Among them, the Mantel correlation coefficient between nestedness was the highest ($r = 0.474$), followed by total β-diversity ($r = 0.188$) and turnover ($r = 0.149$) (**Supplementary Figure 1**). In different habitat groups, the results of the Mantel test were different. Compared with other habitat groups, the Mantel test showed that the nestedness in the two impacted habitats H03 ($r = 0.413$) and H05 ($r = 0.558$) was higher, followed by turnover (H03: $r = 0.302$, H05: $r = 0.364$), the correlation was significant ($p < 0.001$) (**Supplementary Figures 2–6**). However, Mantel correlation coefficient between nestedness in H04 ($r = 0.837$) was the highest, which was significant ($p < 0.001$) (**Supplementary Figure 5**). There were also insignificant correlations in different habitat groups.

Small Run-of-River Dams Influence Community Assembly Process

The pairwise DNCI values of 5 different habitat groups of benthic diatom species and traits were all negative (**Figure 3**), demonstrating that dispersal was the main assembly process. This answered our second question (*question ii*), and we found that the key mechanism of small run-of-river dams affecting benthic diatom communities was dispersal assembly. Among the pairwise DNCI values based on species, the highest absolute value appeared in the comparison between H03 and H05 (DNCI = –11.01), indicating that the potential intensity of the dispersal-dominant assembly process between H03 and H05 was the largest, while the absolute value of DNCI between H02 and H04 was the lowest (DNCI = –6.27), indicating that the intensity of dispersal was the smallest (**Supplementary Table 2**). However, in the pairwise DNCI values based on trait, the absolute value of DNCI between H02 and H03 was the highest (DNCI = –16.90), followed by H03 and H05 (DNCI = –10.76), and the smallest absolute value between H01 and H04 (DNCI = –6.27) (**Supplementary Table 2**). The absolute value of the pairwise DNCI average value based on trait (DNCI = –8.74) was greater than the absolute value of the pairwise DNCI average value based on species (DNCI = –8.37), reflecting the greater intensity of the dominant process of dispersal of the trait (**Supplementary Table 2**). We found that whether it is based on species or trait, the absolute values of DNCI between other habitats and H03 and H05 are relatively large, which answered our third question (*question iii*). Small run-of-river dams affected the community assembly process of benthic diatoms. Due to the construction of small run-of-river dams, the dispersal assembly process between impacted habitats (e.g., H03 and H05) and unregulated habitats has been strengthened, confirming our third hypothesis (HY3).

DISCUSSION

Small Run-of-River Dams Changed the Contribution Rate of Turnover and Nestedness

As hypothesized by HY1, for the benthic diatom assemblages in the entire study area, taxonomy-based β-diversity was higher, particularly a higher turnover, than trait-based β-diversity. In the context of spatial and environmental gradients, factors such as competition, geographic barriers, and environmental filtering can all lead to species turnover (Angeler, 2013; Gutiérrez-Cánovas et al., 2013; Legendre, 2014). We speculated that the

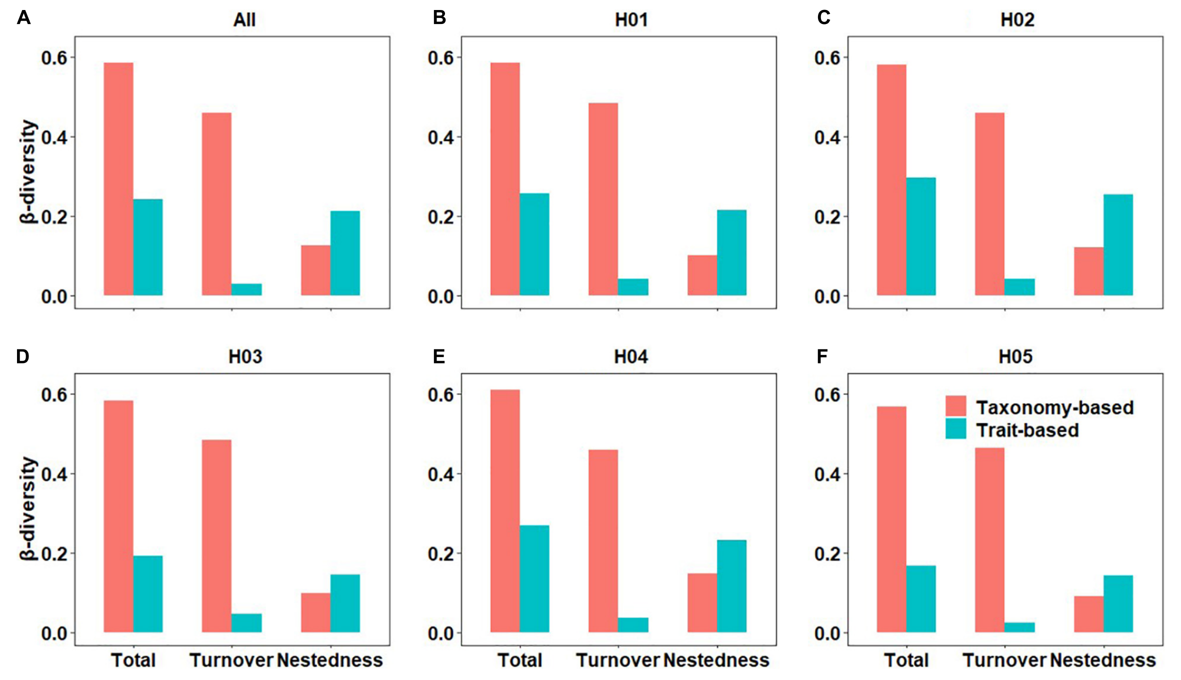

FIGURE 2 | Statistical descriptions of two facets (taxonomy- and trait-based) of the β-diversity components (i.e., total, turnover, and nestedness) for benthic diatoms at all sites **(A)** and different habitat groups (**B–F**, H01–H05).

reason why the species turnover of the Xiangxi River Basin was much higher than that of nestedness may be as follows. First of all, the construction of small run-of-river dams led to the fragmentation of habitats, and there were obvious differences in hydrological variables in the watershed. Geographical isolation might limit the dispersal of benthic diatoms that were originally continuously dispersal, leading to the formation of allopatric speciation (Leprieur et al., 2011). Through species filtering on environmental gradients, different species appeared in their specific habitats suitable for survival, which led to rapid species turnover among communities. Second, huge human disturbances such as the construction of intensive small run-of-river dams have provided abundant hydropower resources, but they have caused huge changes to the river habitat and overall environmental conditions of the Xiangxi River Basin, and may also affect the current benthic diatom communities. Priority effect might also play a role here. In a specific habitat, species that colonize first would reject species that arrive later, resulting in a higher rate of species turnover between communities (Fukami, 2015). The main contribution of turnover to taxonomy-based total β-diversity was also found in previous studies (Rocha et al., 2019; Branco et al., 2020; Wu et al., 2021).

The trait-based β-diversity in the impacted habitats (such as H03, H05) was relatively lower than that of unregulated habitats, but the taxonomy-based β-diversity was not much different, which partially supported our HY2. The construction of small run-of-river dams changed the relative contribution of nestedness component to the trait-based β-diversity of benthic diatom communities, but had little effect on the taxonomy-based β-diversity. Due to the construction of small run-of-river dams, the two habitat groups H03 and H05 have been greatly affected. The hydrological factors of H03 and H05, such as velocity, width and depth, were very different from other habitat groups. Similarly, H05 was a deep pool formed by the outlet of a small run-of-river dams. Habitat homogeneity and dispersal limitations due to huge human disturbance might cause the traits of benthic diatoms to converge, resulting in lower trait-based β-diversity (Logez et al., 2010; Svenning et al., 2011). For example, benthic diatoms in high-velocity habitats mostly exhibit high

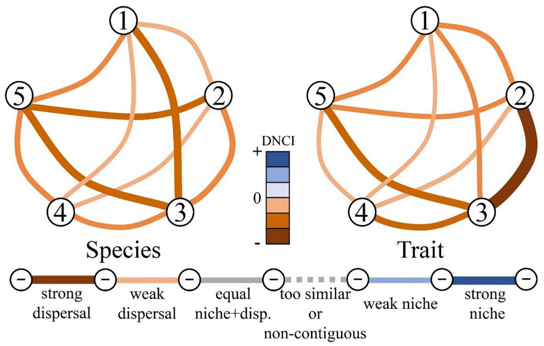

FIGURE 3 | Dispersal-niche continuum index (DNCI) value calculated based on species and trait. The overall and pairwise analysis are pictured as a colored gradient bar and network plots, respectively. The width and color of the links between nodes correspond to assembly process intensity. The darker and wider the link, the stronger the assembly process. The figure on the left is based on species, whereas figure on the right is based on trait. ①–⑤ corresponds to H01–H05.

attachment (Wang et al., 2022). In some highly heterogeneous habitats, benthic diatoms also showed the characteristics of high heterogeneity. Perhaps we could also use selective extinction to explain why nestedness had little effect on trait-based β-diversity in these two habitats. Because different benthic diatoms had different sensitivity to environmental changes, species with higher sensitivity were likely to disappear in habitats with higher environmental pressure due to selective extinction, but species with higher tolerance to environmental changes could survive in the habitat (Gutiérrez-Cánovas et al., 2013; Si et al., 2017). Nestedness was dominant in trait-based β-diversity patterns, which was in the line with previous studies (Rocha et al., 2019; Wu et al., 2021).

Mantel test showed that the correlation coefficient between taxonomy- and trait-based β-diversity components was not high, indicating that they provided different ecological information. We compared the contribution of turnover and nestedness to β-diversity, and found that the taxonomy-based total β-diversity was much higher than that of total trait-based β-diversity. The reason was that the turnover of trait-based β-diversity was lower. Due to the limited dispersal caused by the construction of small run-of-river dams, the functional convergence between benthic diatoms might lead to lower trait-based β-diversity (Logez et al., 2010; Svenning et al., 2011). In trait-based β-diversity, the contribution of nestedness was greater than the turnover. This indicated that the benthic diatom community in the Xiangxi River Basin has experienced a high level of trait convergence. For the nestedness contribution of trait-based β-diversity, impacted habitats (e.g., H03 and H05) were lower than unregulated habitats. This showed that the construction of small run-of-river dams had a great impact on the trait of the benthic diatom community, and reduced the relative contribution of nestedness components to its total β-diversity.

The large amount of overlap between benthic diatom assemblages in the functional space could explain the low level of functional conversion. Among the 5 habitat groups, the water flow of H04 can only be restored after H03 water is collected. Human disturbance led to high homogeneity of the habitat, and the nestedness of environmental conditions led to the high nestedness of trait (Rocha et al., 2019). H03 and H05 were also seriously affected by the construction of small run-of-river dams. Compared with turnover, nestedness also played a greater role in community composition.

Assembly Process

Community assembly is affected by both the dispersal process and the niche process, but the relative importance of the two processes differs depending on the scale, geographic area, and species group (Qian and Ricklefs, 2007; Buckley and Jetz, 2008). The dispersal process considers that the dispersal of a single species tends to be concentrated, and the ability of dispersal determines the distribution of species (Ney-Nifle and Mangel, 1999; Green and Ostling, 2003). The significantly negative DNCI result did not indicate that the niche-based process did not play a role in the community assembly process, but represented that the niche process was weak, and its role in species assembly was not as important as the dispersal-based process. They always appear in natural combinations (Araújo and Rozenfeld, 2014). By comparing multiple DNCI values, the differences in the assembly process between different groups can be more accurately identified. In our research, all 5 different habitats had the advantage of dispersal-based assembly process. The species and trait of benthic diatoms mainly depended on historical processes (dispersal). This answered our second question (*question ii*). In areas impacted by small run-of-river dams, dispersal assembly was a key mechanism for community assembly.

Studies have found that the structure of diatom communities can be explained by environmental and spatial variables in river, such as altitude, substrate type, and water quality effects (Schmera et al., 2018; Wang et al., 2020). Hydrological, physical and chemical factors might also affect the process of community assembly (Isabwe et al., 2018). Wu et al. (2021) found that spatial and local environmental factors exceeded the geographic climate gradient when constructing the β-diversity of benthic diatoms based on taxonomy and trait. The increase in flood residence time leads to changes in the chemical and physical properties of the water body, which affects community assembly and abundance (Corline et al., 2021). The construction of small run-of-river dams has led to changes in the hydrological and environmental factors between habitat groups, and also has a certain impact on hydrological connectivity. Studies have found that hydrological connectivity was a key factor affecting the assembly of phytoplankton communities (Lopez-Delgado et al., 2020; Meng et al., 2020). Hydrological connectivity affected the structure and assemblage of phytoplankton by affecting the spatial variability of CDOM, and might also interact with species dispersal functions, thereby affecting community assembly (Mayora et al., 2016). The penstock connecting H02 and H05 transports river water directly from H02 to H05, causing the connected environment to be cut off, but not completely. The different intensities of the dispersal capacity among these habitats might be caused by changes in some environmental factors between the habitat groups due to the construction of small run-of-river dams, such as velocity, width, and depth. The extremely small negative value of DNCI indicated severe habitat homogeneity, resulting in no significant difference in species richness and trait richness of benthic diatom communities. Whether based on species or trait, the absolute value of DNCI between other habitat groups and H03 and H05 was relatively large, this showed that especially in the habitat separated by the construction of small run-of-river dams, the signs of dispersion driving benthic diatom communities were particularly obvious. The construction of small run-of-river dams seriously affected the environmental factors between habitats, resulting in insufficient ecological differences between habitats. It answered our third question (*question iii*). Small run-of-river dams affected the community assembly process of benthic diatoms, especially the impacted habitats (e.g., H03 and H05). This also supported HY3 that the construction of small run-of-river dams have affected the community assembly process of benthic diatoms, and strengthened the dispersal assembly process between the impacted habitat and the unregulated habitat.

Management Implications

β-diversity decomposition is to explore how the two processes of species turnover and nestedness together affect species distribution patterns in different spatial and temporal dimensions, and helps to understand the distribution patterns and driving mechanisms of various biological groups (Baselga, 2010; Podani and Schmera, 2011). But at present, the research on the β-diversity of benthic diatoms is mostly based on taxonomy, whereas little attention is paid to the trait-based β-diversity (Rocha et al., 2019; Branco et al., 2020; Wu et al., 2021). In the spatial dimension, the species turnover and nestedness components of β-diversity can reflect different conservation strategies. Exploring how the two processes of species turnover and nestedness together affect species distribution patterns is particularly critical in the research of biogeography and conservation biology (Williams, 1996; Baselga and Leprieur, 2015; Socolar et al., 2016). In our research, the turnover process dominated the taxonomy-based total β-diversity, which means that all research sites contribute similarly to β-diversity, which can help decision makers decide on protected areas and conduct restoration progress assessments (Lopez-Delgado et al., 2020). Therefore, in river management, in addition to considering severely homogenized habitats, it should also include highly heterogeneous habitats without priority (Baselga, 2010; Gutiérrez-Cánovas et al., 2013).

In order to better protect biodiversity and maintain the healthy development of ecosystems, areas with high trait diversity should be protected first, and then the trait diversity of impacted habitats should be restored. Due to the differences in β-diversity among taxa, a conservation plan based on one taxon may not be able to protect the diversity of other taxa well. In the future, this study can be expanded to other organisms (such as macroinvertebrates, phytoplankton, fish) to study species distribution patterns and interactions, so as to fully understand the impact of small run-of-river dams on biodiversity from a multi-trophic level aspect, and provide suggestions on the planning and layout of the protected area.

From the research results, species with similar biological taxonomy would replace each other, and dispersal assembly was the key mechanism of community assembly in the study area. Changes in the composition of benthic diatom communities may be better explained by spatial distance and environmental variables. Due to the construction of small run-of-river dams, river connectivity has been interrupted, and there were large differences in hydrological variable between habitat environments. The distribution of species with strong dispersal ability may be more restricted by the niche process. The stronger the dispersal ability, the more likely it can occupy a suitable habitat. Ecological flow refers to the minimum flow required to maintain or restore the basic structure and function of the river, lake, and reservoir ecosystem in order to ensure the ecological service function of the river (Poff and Matthews, 2013). Our research results showed that the ecological flow should not only consider water quality and the structure and function of the river ecosystem, but also include the protection of biodiversity. We hereby suggest that maintaining genetic and ecological connectivity based on an effective impact assessment in dry seasons.

CONCLUSION

Our research proved that the construction of small run-of-river dams affected the β-diversity of benthic diatoms and the community assembly mechanism, thus verifying our hypothesis. We concluded that small run-of-river dams changed the relative contribution of nestedness components to the trait-based total β-diversity of benthic diatoms, and the taxonomy-based β-diversity was relatively higher than the trait-based β-diversity. Another important result was the discovery that small run-of-river dams affect the community assembly process of benthic diatoms. In areas impacted by small run-of-river dams, dispersal assembly was a key mechanism for community assembly. Compared to unregulated habitats, the dispersal assembly process between the impacted habitat and the unregulated habitats has been enhanced. Therefore, our research suggests that maintaining river connectivity and approving ecological flows, and maintaining genetic and ecological connectivity based on an effective impact assessment in dry seasons to reduce the impact of such dams, as key to adaptive management and sustainability.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

NW and QC conceived the ideas. YW and NW performed the data analyses and led the writing. NW, TT, and SZ performed the sampling collection and processing. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the National Key R&D Program of China (No. 2017YFC0506406), the Strategic Priority Research Program of the Chinese Academy of Sciences (No. XDA23080101), and the National Natural Science Foundation of China (No. 30330140).

ACKNOWLEDGMENTS

We thank Xiaocheng Fu, Fengqing Li, and Wanxiang Jiang for their field supports. Jianjun Wang and Kun Guo helped with data analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.895328/full#supplementary-material

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

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ORIGINAL RESEARCH
published: 06 May 2022
doi: 10.3389/fevo.2022.901107

Biogeography of Micro-Eukaryotic Communities in Sediment of Thermokarst Lakes Are Jointly Controlled by Spatial, Climatic, and Physicochemical Factors Across the Qinghai-Tibet Plateau

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Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *21 March 2022*
Accepted: *29 March 2022*
Published: *06 May 2022*

Citation:
Ren Z, Zhang Y, Li X and Zhang C (2022) Biogeography of Micro-Eukaryotic Communities in Sediment of Thermokarst Lakes Are Jointly Controlled by Spatial, Climatic, and Physicochemical Factors Across the Qinghai-Tibet Plateau. Front. Ecol. Evol. 10:901107. doi: 10.3389/fevo.2022.901107

Thermokarst lakes are formed following ice-rich permafrost thaw and widely distribute in the cold regions with high latitude and elevation. However, the micro-eukaryotic communities (MECs) in thermokarst lakes are not well studied. Employing 18S rRNA gene sequencing, we assessed the biogeography of MECs and their driving factors in sediments of thermokarst lakes across the Qinghai-Tibet Plateau (QTP). Results showed that Diatom, Gastrotricha, Nematozoa, Ciliophora, and Cercozoa were dominant lineages in relative abundance and OTU richness. MECs varied substantially across the lakes in terms of diversity and composition. Structural equation modeling and mantel test showed that both OTU richness and community structure of MECs had close relationships with spatial factors, climatic factors, and sediment properties, particularly with latitude, mean annual precipitation, pH, as well as nutrient concentrations and stoichiometric ratios. Moreover, different groups of microbial eukaryotes (taxonomic groups and co-occurrence modules) responded differentially to the measured environmental variables. The results suggested that the biogeography of sediment MECs of thermokarst lakes on the QTP were jointly controlled by spatial and climatic factors as well as sediment properties. This study provides the first view of the composition, diversity, and underlying drivers of MECs dynamic in surface sediments of thermokarst lakes across the QTP.

Keywords: thermokarst lakes, sediment, microbial eukaryotes, latitude, climate, nutrient

INTRODUCTION

Formed as a result of ice-rich permafrost thaw, thermokarst lakes and ponds are the most important aquatic ecosystems in the Arctic and sub-Arctic regions (Kokelj and Jorgenson, 2013; Farquharson et al., 2016; In'T Zandt et al., 2020a; Veremeeva et al., 2021) and pervasively distributed on the Qinghai-Tibet Plateau (QTP; Zou et al., 2017)

In cold regions with high elevation and altitude, thermokarst lakes have been recognized as biogeochemical hotspots, particularly due to their important roles in organic carbon (OC) stock and greenhouse gases emission (Walter et al., 2006; Du Toit, 2018; In'T Zandt et al., 2020a; Jongejans et al., 2021). In circumpolar thermokarst lakes, the storage of OC is estimated to 102 Pg C (Olefeldt et al., 2016) and the methane (CH_4) emission is estimated to be 4.1 ± 2.2 Tg CH_4 per year (Wik et al., 2016). Over the past decades, global permafrost temperature increased by 0.3°C per decade (Biskaborn et al., 2019), leading to tremendous permafrost thaw and consequential changes of thermokarst lakes in size and abundance (Karlsson et al., 2012; Luo et al., 2015; Pastick et al., 2019), as well as serious influences on regional and global biogeochemistry (Shirokova et al., 2013; In'T Zandt et al., 2020a; Le Moigne et al., 2020). Given their crucial roles in biogeochemical processes, microbial diversity and community have been attracting increasing research interests, while most of the studies focused on bacteria and archaea, particularly on those mediating carbon dioxide (CO_2) and CH_4 metabolism (He et al., 2012; Matheus Carnevali et al., 2018; Vigneron et al., 2019; In'T Zandt et al., 2020b). However, the micro-eukaryotic communities in thermokarst lakes are not well known.

In lake ecosystems, microeukaryotes are a versatile group, encompassing an enormous diversity and playing fundamental roles in ecosystem structure and function (Schaechter, 2012; Keck et al., 2020). Microeukaryotes (algae, fungi, protozoa, metazoan, etc.) act as primary producers, consumers, decomposers, parasites, and saprotrophs, structuring aquatic food webs and driving biogeochemical cycles (Schaechter, 2012; Rodríguez-Martínez et al., 2020). Hence, microeukaryotes are crucial in maintaining ecosystem functions and stability. Moreover, microeukaryotes have sensitive responses to local and global environmental changes by shifting their abundance and diversity, and thus being recognized as important ecological indicators of lacustrine ecosystems (Payne, 2013; Capo et al., 2016; Keck et al., 2020; Pearman et al., 2020). Surface sediments also host a huge number and diversity of microorganisms (Lozupone and Knight, 2007; Roeske et al., 2012). Sediment microeukaryotes drive important biogeochemical processes. For example, Bacillariophyta (diatoms) contribute to primary production, and osmotrophic-saprotrophic protists are important for detrital decomposition (Rodríguez-Martínez et al., 2020). Many previous studies focused on the paleolimnology to reveal the long-term dynamics of lacustrine microeukaryotes and their reflected environmental changes by studying sediment cores (Smol, 1992; Capo et al., 2016; Keck et al., 2020). In thermokarst lakes, sediments are originated from thawing permafrost and continuously supplemented by collapse of surrounding permafrost (West and Plug, 2008). These organic-rich sediments represent an important stock of C (Strauss et al., 2013; Anthony et al., 2014) and are an active area of C metabolism (Heslop et al., 2015; Winkel et al., 2019). Aside from the studies associated with C cycling, we lack of knowledge about how micro-eukaryotic communities change across a large spatial scale and how environmental variables drive these communities.

In this study, we investigated the micro-eukaryotic communities (MECs) in sediments of thermokarst lakes across the QTP using 18S rRNA gene sequencing. Our aim was to document the diversity and structural properties of the MECs, and to address the question: how do MECs in thermokarst lake sediments respond to spatial and climatic factors as well as sediment physicochemical properties across the QTP?

MATERIALS AND METHODS

Study Area, Field Sampling, and Chemical Analysis

This work was conducted on the QTP in July 2021. In total, 44 thermokarst lakes (TL01–TL44) were investigated spanning an extensive area with longitude (LON) from 90.6 E to 98.6 E, latitude (LAT) from 30.2 N to 35.0 N, and elevation from 3569 to 4959 m above the sea level (**Figure 1**). The average elevation of the study sites is 4426 m. The map of permafrost distribution (**Figure 1**) on the QTP (Zou et al., 2017) was downloaded from the National Tibetan Plateau Data Center. The spatial dataset of climate on the Tibetan Plateau (Zhou, 2018) was also downloaded from the National Tibetan Plateau Data Center[1] and used to extract the mean annual temperature (MAT) and mean annual precipitation (MAP) of our study regions. For sediment sampling of each lake, the top 15 cm of the sediment was collected from 3 points and mixed together for the following analyses. The microbial samples were collected in a 45 mL sterile centrifuge tube and frozen in liquid nitrogen immediately in the field. In the laboratory, the sediment samples were air-dried to determine sediment organic carbon (SOC), total nitrogen (TN), and total phosphorus (TP). SOC was measured by the potassium dichromate oxidation spectrophotometric method (Chinese standard method HJ615-2011) (Wang et al., 2020; Luo et al., 2021). TN was measured using the modified Kjeldahl Method (Chinese standard method HJ717-2014) (Qian et al., 2019; Wang et al., 2020). TP was measured using the ascorbic acid colorimetric method after microwave extraction with nitric acid (Dancer et al., 1998).

DNA Extraction, Polymerase Chain Reaction, and Sequencing

DNA was extracted using the Magen Hipure Soil DNA Kit (Magen, Guangzhou, China) following the manufacture instruction. DNA extracts were quantified using a Qubit 3.0 Fluorometer (Life Technologies, Guangzhou, China). A total of 20 ng DNA was as the template for the polymerase chain reaction (PCR) to generate amplicons. The 18S rDNA hypervariable regions of V9 were amplified using forward primer 1380F (CCCTGCCHTTTGTACACAC) and reverse primer 1510R (CCTTCYGCAGGTTCACCTAC). The 25 μl PCR mixture was prepared with 2.5 μl of TransStart buffer, 2 μl of dNTPs, 1 μl of each primer, 0.5 μl of TransStart Taq DNA polymerase, and 20 ng template DNA. The PCR reaction was conducted on

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[1] https://data.tpdc.ac.cn/en/

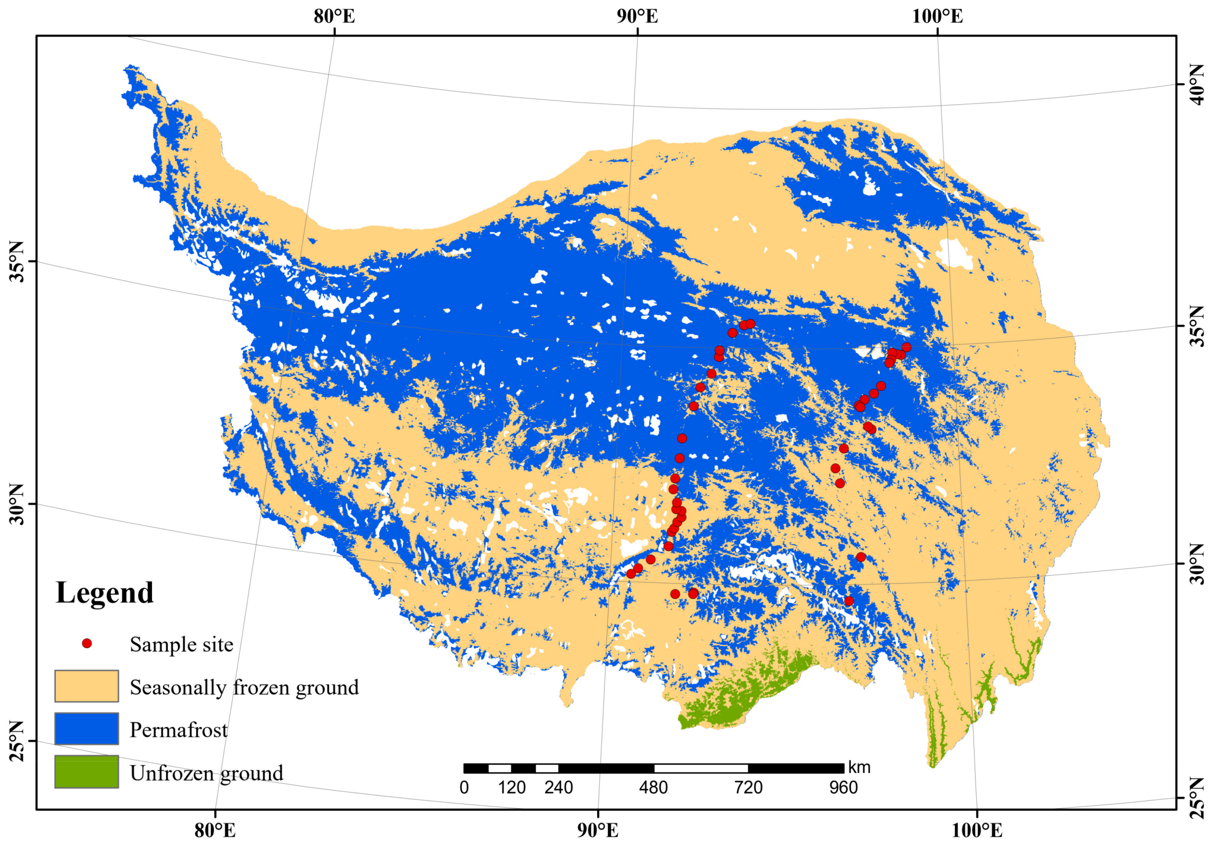

FIGURE 1 | Map of the 44 study thermokarst lakes across the Qinghai-Tibet Plateau. The distribution of the permafrost was cited from Zou et al. (2017).

a thermal cycler (ABI GeneAmp$^{®}$ 9700, Guangzhou, China). DNA libraries were verified on 1.5% agarose gels (Biowest Agarose, Guangzhou, China) and quantified using a Qubit 3.0 Fluorometer (Life Technologies, Guangzhou, China). DNA libraries were multiplexed and loaded on an Illumina NovaSeq PE250 platform according to manufacture instructions (Illumina, San Diego, CA, United States). Raw sequence data were analyzed using QIIME 1.9.1 (Caporaso et al., 2010). Sequences were quality filtered and clustered to generate operational taxonomic units (OTUs) at a threshold of 97% similarity against the SILVA 138 database (Quast et al., 2013) using QIIME. Raw sequence data can be accessed at the China National Center for Bioinformation (PRJCA005279).

Statistical Analyses

Spearman correlations were used to assess the relationships between OTU richness of the overall micro-eukaryotic communities (MECs) and major phylum (phylum with relative abundance >1%) versus environmental variables, as well as between the relative abundance of the major phylum versus environmental variables. The first structural equation modeling (SEM) analysis was conducted to depicting the relationships between environmental factors (spatial factors, climatic factors, pH, nutrient, and nutrient ratios) and community structure and alpha diversity of MECs. The second SEM was conducted to depict the relationships between environmental factors and the major modules (contain more than 10% of the nodes) of the co-occurrence network of the MECs. In SEMs, the environmental factors, MECs, and major modules were reduced in dimensions by non-metric multidimensional scaling (NMDS) using the R package vegan 2.5-7 (Oksanen et al., 2007) and the first axis of NMDS was used. SEMs were conducted using the R package lavaan 0.6-10 (Rosseel, 2012). Phylogenetic tree of microbial eukaryotes was constructed in the R package ggtree 3.2.1 (Yu et al., 2017) using OTUs which presented at least 10 lakes and had an average relative abundance >0.01%. A heatmap was built in the phylogenetic tree to show the relationships (Spearman correlation) between these OTUs and environmental factors. Mantel test was used to assess the relationships between environmental variables and the variations of MECs in terms of overall MECs, major phylum, and major network modules.

The co-occurrence network of the MECs was constructed based on the OTUs used in the phylogenetic tree using the R package igraph 1.2.11 (Csardi, 2013) and visualized using Gephi 0.9.2 (Bastian et al., 2009). The spearman correlation between all pairs of OTUs was calculated, and the *P*-values were adjusted using the FDR method for false discovery (Benjamini and Hochberg, 1995). Only strong and significant correlations ($R > 0.5$, or $R < -0.5$, $P < 0.05$) were used in constructing the network. Topological parameters were calculated, such as

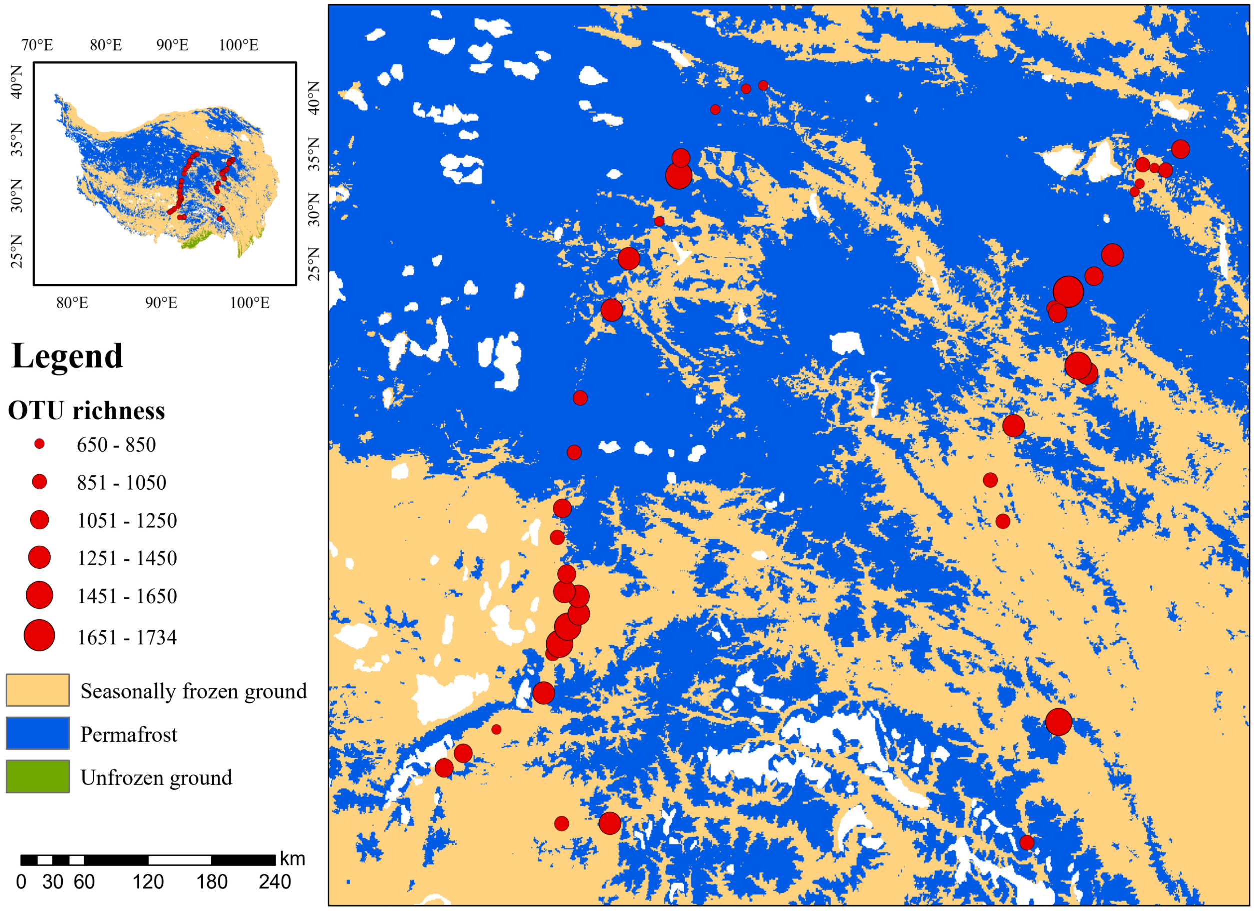

FIGURE 2 | Alpha diversity (OTU richness) of the microbial eukaryotes in sediments of thermokarst lakes across the Qinghai-Tibet Plateau.

clustering coefficient (a measure of local connections), average degree (average number of neighbors for all nodes), and modularity (the tendency to contain subclusters of nodes). Module structures were established and the major modules were identified as those containing more than 10% of the nodes. The topological role of each node (OUT) was identified according to the within-module connectivity (Zi) and among-module connectivity (Pi). Module hubs are the highly linked nodes within the modules ($Zi \geq 2.5$ and $Pi < 0.62$). Connectors are the linking nodes between different modules ($Pi \geq 0.62$ and $Zi < 2.5$). Network hubs are those with $Zi \geq 2.5$ and $Pi \geq 0.62$. Other nodes were determined as peripherals ($Zi < 2.5$ and $Pi < 0.62$). All the analyses were carried out in R 4.1.2 (R Core Team, 2017).

RESULTS

Alpha Diversity and Environmental Association

Across these studied thermokarst lakes, a total of 11,499 OTUs were identified from the quality filtered sequences. The OTU richness ranged from 650 to 1734 (**Figure 2**) with an average value of 1130 (**Figure 3A**), and was positively correlated ($p < 0.05$) with MAP, SOC, TN, C:N, C:P, and N:P, while negatively correlated ($p < 0.05$) with latitude and pH (**Figure 3B**). The SEM result further showed that nutrient stoichiometric ratios had significantly direct effects on alpha diversity of the micro-eukaryotic communities (**Figure 4**). However, pH and climatic factors had significantly indirect effects on alpha diversity through the influences on nutrient concentrations and stoichiometric ratios (**Figure 4**).

In terms of each major phylum, Ciliophora had the highest OTU richness of 161 on average, followed by Diatom (148 OTUs on average), Cercozoa (122 OTUs on average), and others (**Figure 3A**). The OTU richness of Gastrotricha, Nematozoa, Annelida, Ascomycota, and Apicomplexa was significantly correlated ($p < 0.05$) with spatial factors (longitude, latitude, and/or elevation) (**Figure 3B**). The OTU richness of Diatom, Gastrotricha, Annelida, Chlorophyta, and Euglenozoa were significantly correlated ($p < 0.05$) with a climatic factor (MAP). In addition, the OTU richness of the most phylum was significantly correlated ($p < 0.05$) with sediment properties, particularly with sediment pH, SOC, TN, C:P, and N:P (**Figure 3B**).

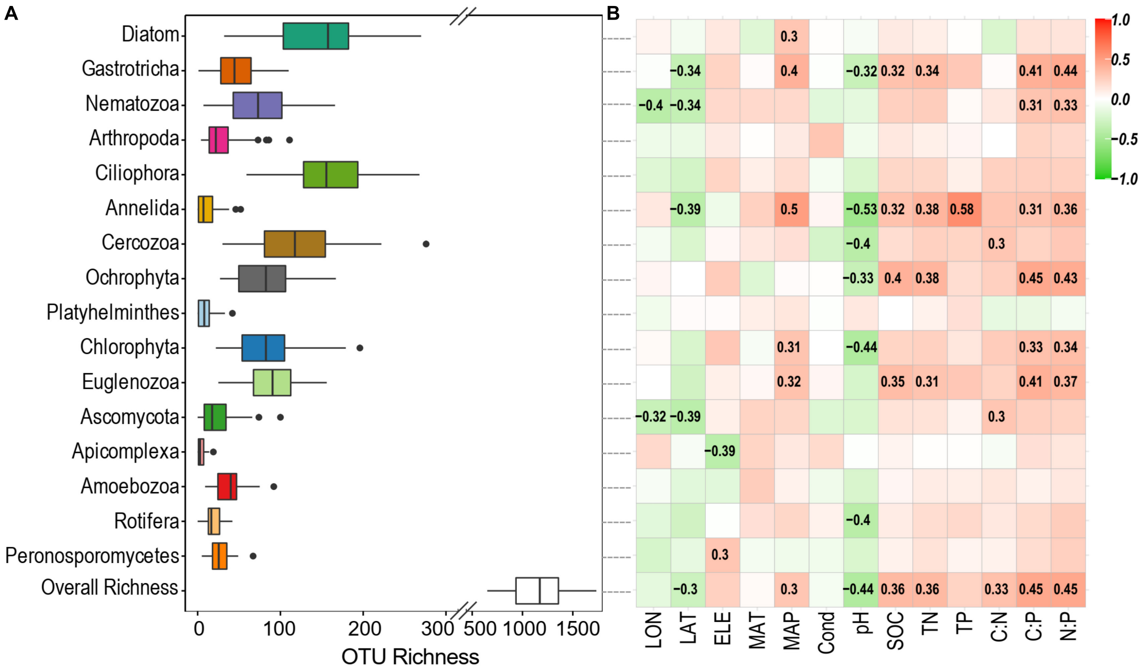

FIGURE 3 | (A) OTU richness of the overall micro-eukaryotic communities and major phylum. **(B)** Spearman correlations show the relationships between alpha diversity and environmental factors. The color represents the correlation coefficient, which is shown in number when the result is significant ($p < 0.05$).

Community Composition and Variation

The overall micro-eukaryotic communities were dominated by Diatom (16.9%), followed by Gastrotricha (11.3%), Nematozoa (11.0%), Arthropoda (9.2%), Ciliophora (8.8%), Annelida (8.2%), and Cercozoa (5.5%) (**Figure 5A**). The taxonomic composition was largely different between any pair of lakes, supported by the high community dissimilarities (Bray-Curtis distance), which ranged from 0.534 to 0.986. However, the relative abundance of the major phylum was rarely associated with environmental factors (**Figure 5B**). On the contrary, in terms of individual OTUs (OTUs with the average relative abundance >0.01% and appeared in at least 10 lakes), most of them had significant relationships with one or more environmental variables, particularly with latitude, MAP, pH, SOC, TN, C:P, and N:P (**Figure 6**).

Mantel tests showed that the variation of micro-eukaryotic communities across the lakes was significantly associated with all the measured spatial factors (except longitude), climatic factors, and sediment properties (**Table 1**). The variation of the subcommunities of Diatom, Gastrotricha, Arthropoda, Ciliophora, Cercozoa, Ochrophyta, Chlorophyta, Euglenozoa, and Amoebozoa was also strongly associated with most of the environmental variables (**Table 1**). The fitted SEM model explained 53.5% of the variation in micro-eukaryotic communities, further suggesting that climatic factors and pH had positive and negative direct effects on MECs, respectively (**Figure 4**).

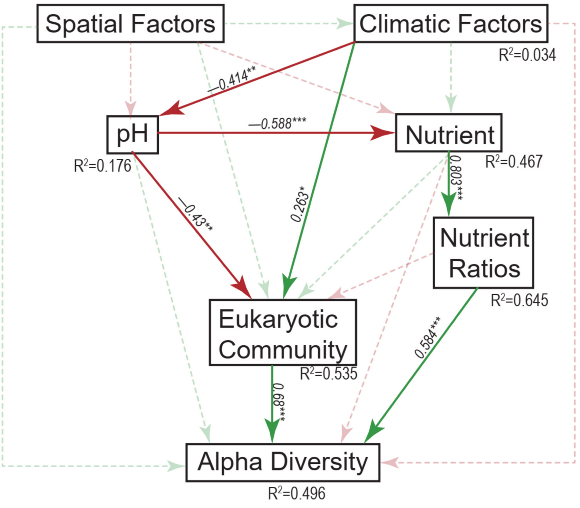

FIGURE 4 | Structural equation modeling analysis depicting the relationships between spatial factors (including latitude, longitude, and elevation), climatic factors (including mean annual temperature and mean annual precipitation), pH, nutrients (including sediment organic carbon, total nitrogen, and total phosphorus), nutrient ratios (including C:N, C:P, and N:P ratios), micro-eukaryotic community, and alpha diversity. Solid and dashed arrows represent the significant and non-significant relationships, respectively. Red and green arrows represent negative and positive relationships, respectively. The significant path coefficients were shown adjacent to the path with *, **, and *** denote the significant level of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Co-occurrence Network

The resulting eukaryotic co-occurrence network consisted of 690 nodes and 2,909 edges (**Figure 7**). Only 4.8% of the edges were negative. Some topological properties were calculated to describe the complex co-occurrence relationships between OTUs. The

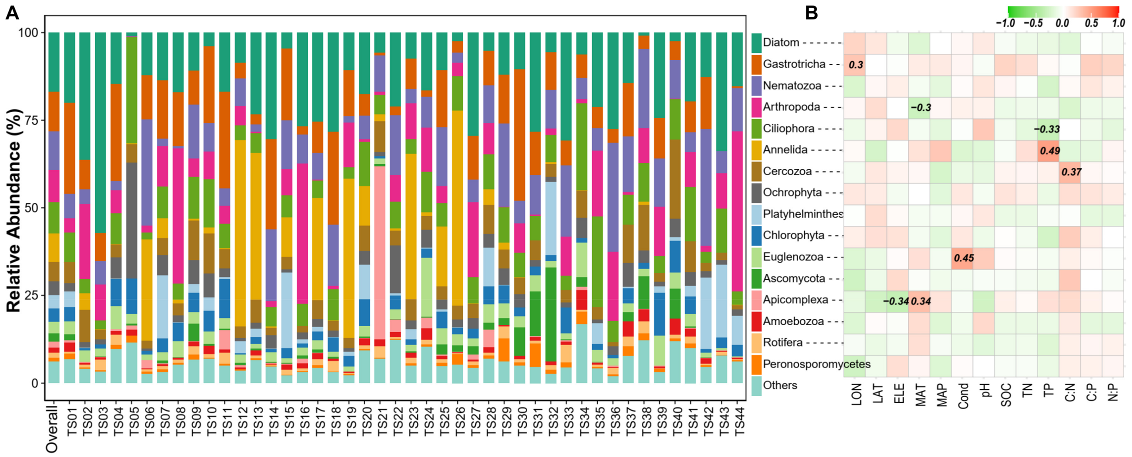

FIGURE 5 | (A) Community composition of sediment microbial eukaryotes at the phylum level. Only phyla with a relative abundance >1% are shown. "Overall" represents the whole dataset including all samples. **(B)** Spearman correlations show the relationships between the relative abundance of the major phylum and environmental factors. The color represents the correlation coefficient, which is shown in number when the result is significant ($p < 0.05$).

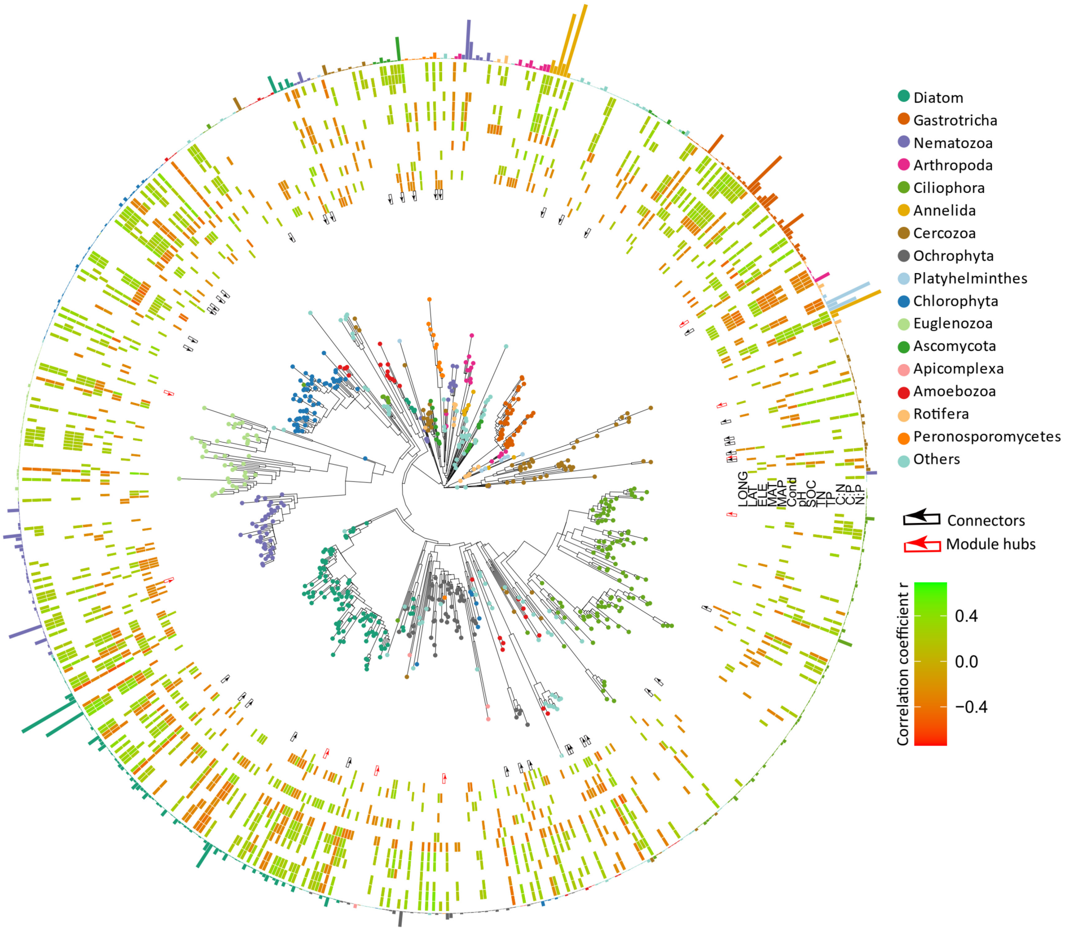

FIGURE 6 | Phylogenetic tree of microbial eukaryotes was constructed by OTUs with relative abundance >0.01%. Tree tips are colored by major phylum. The red and black arrows in the inner ring represent module hubs and connectors of the co-occurrence network. The heatmap indicates the relationships between OTUs and environmental variables (the color represents Spearman's correlation coefficient and only significant results are shown). The bars in the outer ring represent the relative abundance of the OTUs and are colored by phylum.

TABLE 1 | Mantel tests between environmental factors and micro-eukaryotic communities (overall, dominant phyla, and network modules).

	LONG	LAT	ELE	MAT	MAP	Cond.	pH	SOC	TN	TP	C:N	C:P	N:P
Overall community	0.006	__0.242**__	__0.165**__	__0.074*__	__0.285**__	__0.095**__	__0.202**__	__0.075*__	__0.106**__	__0.178**__	__0.095**__	__0.066*__	__0.095**__
Diatom	0.004	__0.207**__	0.038	−0.067	__0.317**__	__0.086**__	__0.233**__	0.029	0.058	__0.097**__	__0.194**__	0.059	__0.101**__
Gastrotricha	−0.008	__0.160**__	__0.114**__	__0.115**__	__0.160**__	__0.091**__	__0.180**__	__0.202**__	__0.209**__	__0.174**__	0.061	__0.178**__	__0.173**__
Nematozoa	0.038	__0.131**__	__0.099**__	0.018	__0.217**__	__0.129**__	0.047	0.05	0.027	−0.005	−0.049	0.027	0.025
Arthropoda	0.035	__0.077*__	__0.096**__	__0.128**__	__0.160**__	−0.011	__0.134**__	__0.074*__	__0.107**__	__0.179**__	__0.114**__	0.048	__0.068*__
Ciliophora	−0.006	__0.094**__	__0.165**__	−0.047	__0.165**__	__0.132**__	__0.197**__	__0.138**__	__0.082*__	__0.153**__	__0.144**__	__0.092**__	0.046
Annelida	−0.036	0.055	0.056	−0.026	__0.119**__	−0.027	−0.046	−0.008	−0.021	__0.159**__	0.041	−0.004	−0.029
Cercozoa	−0.013	__0.216**__	__0.154**__	__0.117**__	__0.227**__	__0.168**__	__0.150**__	0.05	__0.088**__	__0.083*__	__0.092**__	0.018	0.06
Ochrophyta	0.058	__0.189**__	__0.189**__	0.039	__0.205**__	__0.216**__	__0.221**__	__0.127**__	__0.123**__	0.042	0.024	__0.097**__	__0.101**__
Platyhelminthes	0.036	__0.086*__	−0.087*	0.057	−0.043	0.049	0.015	__0.086*__	__0.117**__	0.063	−0.064	__0.112**__	__0.139**__
Chlorophyta	−0.005	__0.131**__	__0.117**__	0.053	__0.173**__	__0.107**__	__0.181**__	−0.035	−0.03	__0.131**__	__0.077*__	−0.037	−0.028
Euglenozoa	−0.026	__0.196**__	__0.159**__	__0.072*__	__0.159**__	__0.201**__	__0.190**__	0.049	__0.068*__	__0.136**__	__0.115**__	0.039	0.056
Ascomycota	__0.068*__	__0.151**__	__0.068*__	0.002	__0.117**__	0.041	0.039	−0.04	−0.017	−0.081	0.053	−0.049	−0.018
Apicomplexa	−0.011	__0.125**__	0.025	__0.096**__	__0.118**__	__0.105**__	__0.105**__	0.014	0.065	0	0.059	0.056	__0.100**__
Amoebozoa	−0.019	__0.128**__	0.013	0.034	__0.092**__	__0.095**__	__0.216**__	__0.089**__	0.062	__0.185**__	0.03	__0.097**__	__0.075*__
Rotifera	−0.073	0.026	−0.105**	−0.037	−0.017	−0.014	__0.122**__	0.031	0.006	__0.076*__	0.038	0.021	0.001
Peronosporomycetes	0.013	__0.123**__	0.042	−0.034	__0.130**__	−0.082	__0.082*__	−0.121	−0.084	__0.072*__	0.052	−0.095	−0.053
ModA	−0.012	__0.132**__	__0.127**__	0.05	__0.089**__	__0.216**__	__0.117**__	__0.092**__	0.051	__0.103**__	−0.042	__0.066*__	0.032
ModB	−0.025	__0.202**__	0.009	0.058	__0.301**__	−0.016	__0.139**__	−0.053	0.043	__0.087**__	__0.185**__	−0.008	0.06
ModC	−0.051	__0.103**__	−0.025	−0.051	__0.129**__	0.046	__0.191**__	__0.079*__	0.015	__0.103**__	0.038	0.045	0.005
ModD	0.014	__0.169**__	−0.035	0.019	__0.227**__	0.063	__0.108**__	0.059	0.062	0.041	__0.087**__	0.036	0.037

The numbers represent Pearson's correlation coefficients. P-values are adjusted for false discovery using FDR adjustment.
** and ** represent $P < 0.05$ and $P < 0.01$, respectively.*
Significant correlations ($P < 0.05$) are shown in bold.

average path length (the average shortest path lengths between all pairs of nodes) was 4.15 edges with a diameter (the maximum length) of 11 edges. The clustering coefficient was 0.33, the average degree was 8.43, and the modularity was 0.57 (>0.4 suggests modular structure of the network). The results suggested clustered topology and modular structure of the eukaryotic co-occurrence network. In this network, OTUs were clustered into four major modules containing more than 10% of the nodes (**Figure 7**). These modules consisted of OTUs from different taxonomic groups and responded differently to environmental variables (**Figure 7**). Module-A was dominated by Ciliophora, Cercozoa, Ochrophyta, and Euglenozoa in terms of OTU richness. Module-B was dominated by Diatom, Chlorophyta, and Ciliophora. Module-C was dominated by Ciliophora, Chlorophyta, and Cercozoa. Module-D was dominated by Ciliophora, Ascomycota, Ochrophyta, and Gastrotricha. Mantel tests showed that all the modules were significantly associated with latitude, MAP, and pH (**Table 1**). In addition, these modules were also associated differently with elevation, conductivity, SOC, TP, C:N, and C:P (**Table 1**). The SEM result further suggested that module-A was directly affected by nutrient stoichiometric ratios, module-B was directly affected by spatial factors and climatic factors, and module-C was directly affected by nutrient concentration (**Figure 8**). In the network, keystone taxa were identified according to the within-module and among-module connectivity (Zi and Pi, respectively) of the nodes. There were nine module hubs ($Pi < 0.62$ and $Zi \geq 2.5$) and 38 connectors ($Pi \geq 0.62$ and $Zi < 2.5$) identified in this network (**Figure 7**). Latitude, MAP, pH, SOC, TN, C:P, and N:P had strong relationships with some of the keystone taxa (**Figure 6**).

DISCUSSION

In our study, diverse microbial eukaryotes (a total of 11,499 OTUs) were identified from the sediments of the studied thermokarst lakes with an average OTU richness of 1,130 per lake (**Figure 3**). In general, Diatom, Gastrotricha, Nematozoa, Arthropoda, Ciliophora, Annelida, and Cercozoa had a high relative abundance of the sequences (>5% of relative abundance on average). In fact, the relative proportion of microbial eukaryotes in a sample inferred from 18S rRNA gene sequencing can be either greater or lower than their actual abundance, because eukaryotic genomes can encode for multiple copies of this gene (Gong et al., 2013). Changes observed in gene reads proportion have to be considered with caution. However, our data also showed that these microbial eukaryotes had a high diversity. For example, Ciliophora, Diatom, and Cercozoa had more than 100 OTUs on average, and Euglenozoa, Chlorophyta, Ochrophyta, and Nematozoa had more than 50 OTUs (**Figure 3**). These microbial eukaryotes are also observed in many other lacustrine environments with high abundance and diverse (Kammerlander et al., 2015; Filker et al., 2016; Capo et al., 2017; Debroas et al., 2017). MECs are crucial components of aquatic environments, contributing to ecosystem structure and function, such as biodiversity, biogeochemical cycling, and climate regulation (Schaechter, 2012; Simon et al., 2015;

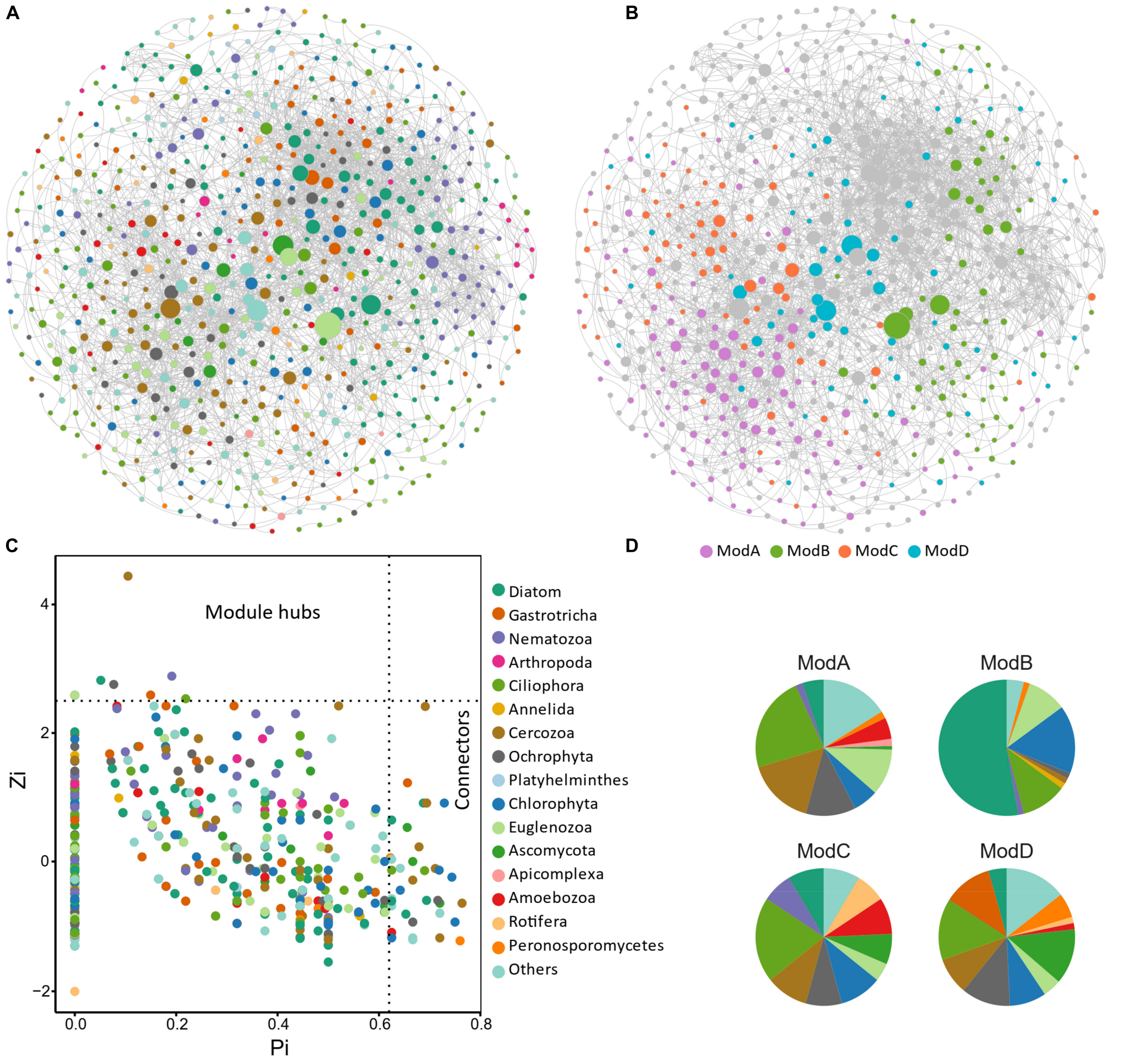

FIGURE 7 | Co-occurrence network and module structure of micro-eukaryotic communities in sediments of the thermokarst lakes. Nodes represent OTUs and are colored by **(A)** taxonomic groups and **(B)** major modules (modules contain more than 10% of the nodes). Edges represent strong and significant Spearman's correlations ($R > 0.5$, or $R < -0.5$, $P < 0.05$). *P*-values were adjusted using the FDR method for false discovery. **(C)** Topological roles of the nodes in the network. Zi represents within-module connectivity. Pi represents among-module connectivity. Each dot represents an OTU colored by phylum. **(D)** The taxonomic composition of the major modules shown in number of OTUs.

Rodríguez-Martínez et al., 2020). However, MECs in aquatic ecosystems vary drastically along time and across space, leading to consequent variations in ecosystem structure and function (Allison and Martiny, 2008; Capo et al., 2016; Keck et al., 2020; Pearman et al., 2020).

Based on accumulating evidence proposed by previous studies, the dynamics of MECs are thought to be influenced by various abiotic and biotic factors (Simon et al., 2015; Wang et al., 2015). For example, the distribution patterns of MECs are proposed to be influenced by spatial factors (Olsen et al., 2013; Wang et al., 2016), local precipitation and irradiation (Williamson et al., 2010; Jones et al., 2013), pH (Liu et al., 2014; Shen et al., 2014; Gong et al., 2015), temperature (Salonen et al., 2018; Liu et al., 2021), and nutrients (Salonen et al., 2018; Liu et al., 2021). Understanding how environmental variables shape community structures of MECs in thermokarst lakes is of importance for comprehensive biogeography studies as well as for predictions of the lake ecosystems in future climate change. In our studied thermokarst lakes, MECs varied substantially across these lakes in terms of diversity, composition, and structure (**Figures 3**, **4**). Multiple analyses showed that both OTU richness and community structure of MECs had close relationships with spatial factors, climatic factors, and sediment properties, particularly with latitude, MAP, pH, as well

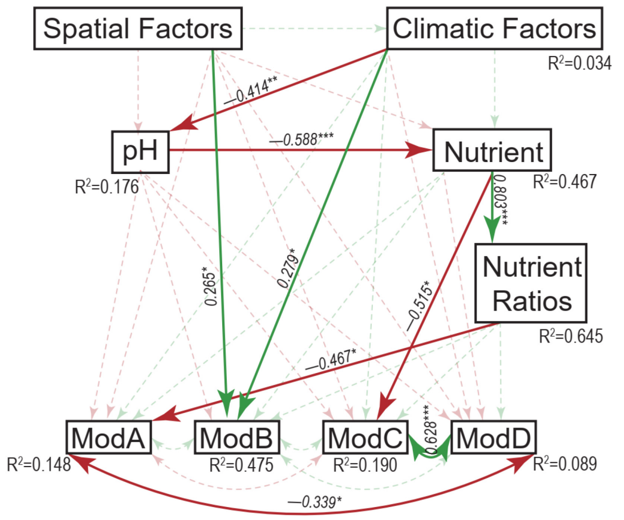

FIGURE 8 | Structural equation modeling analysis depicting the relationships between spatial factors (including latitude, longitude, and elevation), climatic factors (including mean annual temperature and mean annual precipitation), pH, nutrient (including sediment organic carbon, total nitrogen, and total phosphorus), nutrient ratios (including C:N, C:P, and N:P ratios), and the major modules (ModA, ModB, ModC, and ModD). Solid and dashed arrows represent the significant and non-significant relationships, respectively. Red and green arrows represent negative and positive relationships, respectively. The significant path coefficients were shown adjacent to the path with *, **, and *** denote the significant level of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

as nutrient concentrations and stoichiometric ratios. Moreover, different groups of microbial eukaryotes (taxonomic groups and co-occurrence modules) responded differentially to the measured environmental variables.

It has been well demonstrated that pH is an overriding important factor in controlling diversity and composition of both bacterial communities (Fierer and Jackson, 2006; Lauber et al., 2009; Griffiths et al., 2011) and MECs (Mulder et al., 2005; Tsyganov et al., 2013; Shen et al., 2014) in soil from local to global scales. In our study of lake sediments, pH had direct effects on MECs (**Figure 4**) and had negative relationships with the OTU richness of MECs as well as major phylum, including Gastrotricha, Annelida, Cercozoa, Ochrophyta, Chlorophyta, and Rotifera (**Figure 3**). The divergence of pH also had significant relationships with the variation of most of the major taxonomic groups and all the major modules (**Table 1**). These results suggest that pH is an important factor in shaping MECs in sediments of thermokarst lakes. In addition, climatic conditions, particularly warming, have been suggested to influence MECs in terrestrial (Bates et al., 2013) and aquatic (Domis et al., 2014; Rigosi et al., 2014; Capo et al., 2017) ecosystems. In our study, however, MAP had significant relationships with diversity and composition of MECs. Moreover, our study also supported the important role of nutrient concentration and stoichiometric ratios. Nutrients are undoubtedly other major drivers in ecosystem structure and function. Even moderate changes in nutrient concentration and relative balance (stoichiometric ratio) can lead to significant modifications of MECs (Wu et al., 2011; Domis et al., 2014; Rigosi et al., 2014; Capo et al., 2017).

Our data identified a large number of OTUs and a high relative abundance of sequences as Diatom, which predominantly function as phototrophic organisms. In addition, other phototrophic groups, Chlorophyta and Ochrophyta were also high in OTU richness (**Figure 3**) and sequence proportion (**Figure 5**). The shallow depth and low suspended sediment concentrations of the thermokarst lakes allow greater light penetration to lake sediments. Moreover, sediments had plenty of nutrients that were further supplemented from the terrestrial input. Thus, phototrophic organisms are presented in sediments with high abundance and diversity. In the studies of limnology and paleolimnology (Davidson et al., 2013; Soares et al., 2013; Capo et al., 2017), Chlorophyta and diatoms are sensitive to nutrient changes and have high abundance in eutrophic conditions. Ciliophora was commonly found to have high abundance in polar sediment and permafrost soil with high organic matter content (Mackelprang et al., 2011; Jansson and Tas, 2014; Geisen et al., 2015; Hindshaw et al., 2017). Ciliophora is predominantly heterotrophic, and often bacterivorous and algae grazers (Sherr and Sherr, 2002), exhibiting a very clear response to nutrient changes (Capo et al., 2017). High abundance of their preys following nutrient enrichment can lead to the predominance of Ciliophora taxa (Capo et al., 2017). Paleolimnological studies have observed a positive relationship between P concentration and relative abundance of Ciliates and suggested that Ciliophora could be ecological indictors of eutrophication levels in aquatic environments (Xu et al., 2014; Pawlowski et al., 2016). In our study, we found that the variation of the Ciliophora had significant relationships with nutrient concentrations and stoichiometric ratios (**Table 1**). In aquatic ecosystems, Cercozoans are also bacterivorous and algae grazers. Previous studies showed that temperature fluctuations have strong influence on richness and relative abundance of Cercozoa (Tveit et al., 2015; Capo et al., 2016). In our study, however, the richness and relative abundance of Cercozoa were significantly associated with pH and C:N (**Figures 3**, **5**), and the variation of Cercozoa had close relationships with most of the measured spatial, climatic, and physicochemical variables (**Table 1**).

In our case, the results showed strong relationships between environmental factors and MECs, as well as differential relationships between some microbial eukaryotic groups and measured environmental variables, which are consistent with many previous studies as discussed above. However, a number of gaps still exist in our understanding of the dynamic of MECs. For example, our measured environmental variables only explained half of the variation in MECs, suggesting potential influences of other abiotic variables, but most likely, abiotic variables (such as predation and mutualistic/parasitic interactions) (Zhao et al., 2019; Shi et al., 2020) on MECs. Moreover, in thermokarst lakes, the sediments are initially originated from permafrost soil and continuously supplemented by collapse of surrounding permafrost (Zhao et al., 2019; Shi et al., 2020). The sinking of plankton eukaryotes also contributes significantly to the sequences of MECs from sediment samples (Capo et al., 2017; Broman et al., 2019). Thus, the sediments have a considerable proportion of terrestrial and plankton microbial eukaryotes. The limited abiotic and biotic variables as well as the complex composition of the MECs in sediment samples may impede our comprehensive understanding of the biogeography of MECs in thermokarst lakes and require further studies.

CONCLUSION

Thermokarst lakes are widely distributed on the Qinghai-Tibet Plateau. In this study, we investigated the micro-eukaryotic communities in sediment of the thermokarst lakes across the QTP. We found that Diatom, Ciliophora, Nematozoa, and Cercozoa were dominant in terms of both OTU richness and relative abundance. OTU richness of the overall communities and major phylum were significantly correlated with multiple environmental variables. SEM and mantel test suggested that micro-eukaryotic communities in sediment of thermokarst lakes were jointly controlled by spatial, climatic, and physicochemical factors. Within these factors, latitude, MAP, pH, nutrient concentrations, and stoichiometric ratios were the most relevant variables. Different micro-eukaryotic groups, such as different phyla and network modules, responded differently to these variables. Due to the fact that sediment MECs in thermokarst lakes also contain the microbial eukaryotes from water column and surrounding terrestrial soil, a microbial source tracking will deepen further our understanding of the environmental responses of sediment MECs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: ngdc.cncb.ac.cn (Accession: PRJCA005279).

AUTHOR CONTRIBUTIONS

ZR designed the study, did the analyses, performed the field and laboratory work, and prepared the manuscript. YZ did the visualization. XL did the analyses and performed the laboratory work. CZ performed the laboratory work and prepared the manuscript. All authors read and reviewed the manuscript.

FUNDING

This study was supported by the start-up funding for the new introduced talents of the Beijing Normal University (28707-111032105).

ACKNOWLEDGMENTS

We are grateful to Yongming Deng and Xuan Jia for their assistance in the field work.

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

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ORIGINAL RESEARCH
published: 03 June 2022
doi: 10.3389/fevo.2022.890475

Genetic Diversity and Population Differentiation of Chinese Lizard Gudgeon (*Saurogobio dabryi*) in the Upper Yangtze River

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Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *06 March 2022*
Accepted: *10 May 2022*
Published: *03 June 2022*

Citation:
Liu H, Xiong F, Zhai D, Duan X, Chen D, Chen Y, Wang Y and Xia M (2022) Genetic Diversity and Population Differentiation of Chinese Lizard Gudgeon (Saurogobio dabryi) in the Upper Yangtze River. Front. Ecol. Evol. 10:890475. doi: 10.3389/fevo.2022.890475

Dam construction on the upper Yangtze River has dramatically altered riverine ecosystems and caused habitat fragmentation of fishes, which might influence the genetic structure of fish populations. In this study, we examined the possible genetic effects of dam construction on Chinese lizard gudgeon (*Saurogobio dabryi*) populations in the upper Yangtze River, China. Seven populations were sampled, and genetic structure was analyzed using single nucleotide polymorphism (SNP) markers through the specific locus amplified fragment sequencing (SLAF-seq) method. The numbers of SNPs were lower in the upstream populations than in the downstream populations. Genetic similarity was increased from downstream to upstream. The upstream populations of *S. dabryi* might be more vulnerable to genetic drift than those downstream. Structure analysis indicated three distinct genetic groups of *S. dabryi* in the upper Yangtze River, among which the genetic differentiation values (*Fst*) were at a high level. The genetic differentiation of *S. dabryi* exhibited a close correlation with spatial distance. We did not detect a significant correlation between isolation time and genetic differentiation, suggesting that impacts of dams on the genetic structure of *S. dabryi* can be relatively minimal on a short time scale. The results quantify the genetic diversity and population structure patterns of *S. dabryi* after habitat fragmentation caused by dams, which will provide a reference for resource protection and management of this species in the upper Yangtze River.

Keywords: *Saurogobio dabryi*, SNP, genetic diversity, population differentiation, habitat fragmentation, the upper Yangtze River

INTRODUCTION

Dam construction has damaged the continuity of natural rivers and dramatically altered riverine ecosystems, causing habitat fragmentation of fishes. This affects the connectivity and migration of fish species, leading to increased inbreeding probability, enhanced genetic drift, and blocked gene flow, thereby reducing genetic diversity and increasing genetic differentiation (Pavlova et al., 2017; Nakajima et al., 2020; Machado et al., 2021). Low genetic diversity might reduce the viability of individuals and populations in the fragmented habitats (Cook and Sullivan, 2018).

The upper Yangtze River is a hotspot of diverse fish species. However, it has been facing a serious issue of habitat fragmentation due to the construction of a cascade of hydropower dams, such as Three Gorges Dam, Xiangjiaba Dam, Xiluodu Dam, Baihetan Dam, and others (Cheng et al., 2015). Many fishes have been greatly affected in aspects, such as population size decline, spawning ground loss, and reduced reproductive success. If key habitats are lost or are no longer accessible, such destruction may have a serious negative impact on local fish species (Zhang et al., 2021), and it is particularly difficult to conduct fish conservation. Understanding the genetic effects of habitat fragmentation is a basis for conservation and management actions for fishes in the Yangtze River.

At present, studies on the genetic effects of habitat fragmentation caused by dams in the Yangtze River mostly focus on large and migratory fishes (Wang et al., 2007; Song et al., 2016; Liu et al., 2017, 2020; Dong et al., 2019), while research on small-sized species is very limited. *Saurogobio dabryi* is a small-sized fluvial fish species in the sub-family Gobioninae (Cyprinidae) and prefers to inhabit flowing waters. Its sexual maturation is early and the minimum age of sexual maturity is 1 year (Liu et al., 2019). This species produces drifting eggs, which need flowing water to be hatched (Lin et al., 2018). After dam construction, many river sections are changed from lotic water to close lentic water, which has greatly altered the habitats of *S. dabryi* and will potentially reduce its reproductive success (Xu et al., 2019). The spawning time of *S. dabryi* has become shortened and the reproductive rate has decreased (Gao, 2016). Li et al. (2016) reported the genetic structure of three populations from Chishui River, Hejiang and Yibin of the upper Yangtze River based on the mitochondrial Cyt b gene sequence, but the sample regions were near one another and not blocked by dams, thus, genetic effects caused by dam blockage were not involved. Liu (2020) studied the genetic differentiation of four populations in the Jialing River based on the mitochondrial D-loop sequence and 10 microsatellite loci, but the sample regions were limited to a tributary of the upper Yangtze River. The previous studies focused on populations of *S. dabryi* at relatively small spatial scales and included a small number of genetic markers, which yielded limited genetic information on this species.

Genome-scan approaches, such as specific locus amplified fragment sequencing (SLAF-seq), present large numbers of genome-wide single nucleotide polymorphisms (SNPs) and increase coverage throughout the genome using thousands of SNP markers, and thus can improve the capacity to unravel genetic diversity and genetic divergence within species (Zhang et al., 2020). In this study, we used SNP markers produced by the SLAF-seq method to assess the genetic diversity and genetic differentiation of seven *S. dabryi* populations in the upper Yangtze River. The aim was to reveal the genetic diversity and differentiation patterns of *S. dabryi* in fragmented habitats of the upper Yangtze River and to probe possible genetic effects of isolation by dams. The results are expected to provide a reference for natural resource protection and management of this species in the upper Yangtze River.

TABLE 1 | Sample information of *Saurogobio dabryi*.

Sample sites	Sample sizes	Elevation (m)	Sampling time	Habitat characteristics
QJ	19	642	October 2019	River habitat
SN	12	296	October 2019	Reservoir habitat
YB	10	253	October 2019	River habitat
HC	20	187	September 2019	Reservoir habitat
BN	19	160	August 2019	Fluctuating backwater area
WZ	20	115	August 2019	Reservoir habitat
TP	20	110	August 2019	Reservoir habitat
Total	120			

MATERIALS AND METHODS

Study Areas and Sample Collection

A length of the river of about 1,500 km distance from the Wudongde Dam to the Three Gorges Dam on the upper Yangtze River was selected as the research area. This zone has been fragmented by hydropower dams, such as Wudongde Dam (closure in 2016), Baihetan Dam (2015), Xiluodu Dam (2007), Xiangjiaba Dam (2008), and Three Gorges Dam (2003) from upstream to downstream. These dams are large, impassable, and power-generating dams, which lack fishways (Cheng et al., 2015). Individuals of *S. dabryi* were sampled at seven sites from August to October 2019 (**Table 1**). These sites were Qiaojia (QJ), Shaonvping (SN), Yibin (YB), Hechuan (HC), Banan (BN), Wanzhou (WZ), and Taipingxi (TP) (**Figure 1**). Sampling site QJ is between Wudongde Dam and Baihetan Dam. Sampling site SN is between Xiluodu Dam and Xiangjia Dam. Sampling sites YB, BN, WZ, and TP are in the long river section between Xiangjia Dam and Three Gorges Dam. The length of this river section is about 1,000 km with changes from river habitat (400 km) to reservoir habitat (600 km), and it is a relatively intact habitat for fishes to complete their life histories, especially for fishes producing drifting eggs. Sampling site HC is in the lower reach of the Jialing River, a tributary of the upper Yangtze River. There is Caojie hydropower station (closure in 2005) between HC and the main stream. QJ and YB are river habitats. SN, HC, WZ, and TP are reservoir habitats. BN is in fluctuating backwater area of the Three Gorges Reservoir. When the impounding height of the Three Gorges Reservoir is 145 m from June to September, the water of BN flows. When the impounding height is 175 m from October to May of the next year, the water of BN closes to lentic (**Table 1**).

Individuals were captured using trammel or gill nets. Fin clips were taken and preserved in 95% ethanol until DNA extraction.

Preparation of SLAF Sample for Sequencing, Data Processing, and SNP Calling

Twenty individuals per population were randomly selected for DNA extraction when the sample size was >20. If the population sample size was <20, all samples were taken for screening.

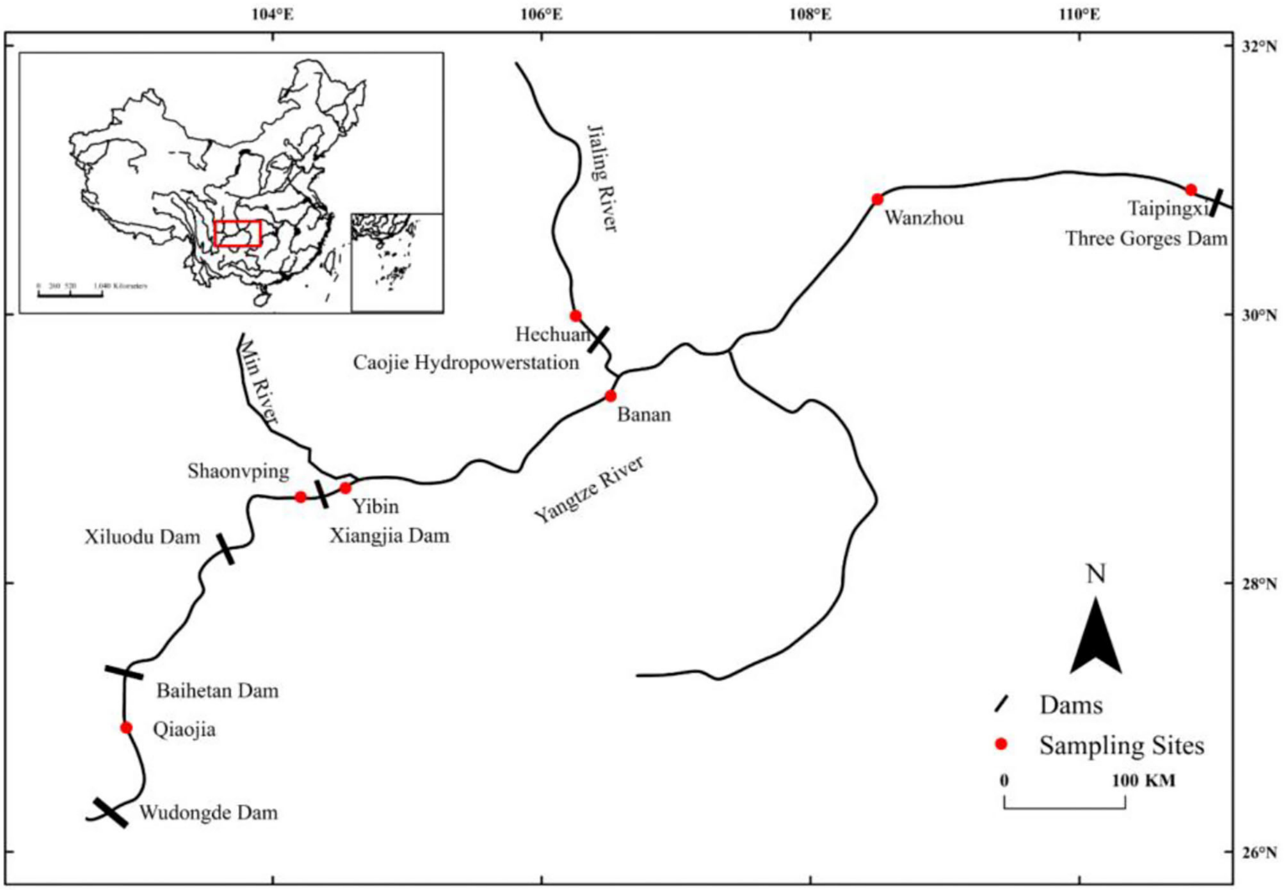

FIGURE 1 | Sampling sites in the upper Yangtze River.

Total genomic DNA was extracted from fin tissues using a phenol-chloroform protocol (Green and Sambrook, 2012), DNA quality was checked by a NanoDrop One Spectrophotometer (Thermo Scientific) and the samples were used for library construction. A total of 120 samples of *S. dabryi* were used to generate genome-wide SNP data using the SLAF sequencing methodology (Sun et al., 2013), a new sequencing technology much like RAD-seq, which targets a reduced representation of the genome. As there was no reference genome sequence for *S. dabryi*, the genome of a related species, *Cyprinus carpio* (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/951/615/GCF_000951615.1_common_carp_genome) (Xu et al., 2014) was selected to perform *in silico* digestion prediction. The restriction enzymes *RsaI* + *HaeIII* were used for digestion. SLAF tag length was selected between 314 and 364 bp. Based on the prediction, we then proceeded to library construction. Genomic DNA (about 500 ng) was digested to completion with *RsaI* (New England Biolabs, NEB), T4 DNA ligase (NEB), adenosine triphosphate (ATP), and *RsaI* adapters at 37°C. Restriction-ligation reactions were heat-inactivated at 65°C and then digested with the second restriction enzyme *HaeIII* (NEB) at 37°C. Duplex tag-labeled sequencing adapters were ligated to the A-tailed DNA with T4 DNA ligase. PCR reactions were performed using diluted restriction-ligation samples, deoxyribonucleoside triphosphates (dNTPs), high-fidelity DNA polymerase (NEB), and PCR primers. PCR products were purified and then separated by electrophoresis in a 2% agarose gel. Fragments 300–450 bp (with indexes and adaptors) in size were excised and purified using QIAquick Gel Extraction Kit (Qiagen). The gel-purified product was sequenced on the Illumina HiSeq 2500 system.

The highest depth tag in each SLAF was taken as the reference sequence tag. SLAF paired-end reads with clear index information were clustered and detected using BLAT (Kent, 2002). Sequences with over 80% identity were considered as one SLAF locus. Both GATK (McKenna et al., 2010) and Samtools (Li et al., 2009) were used to call SNPs. VCFtools (Danecek et al., 2011) was used to filter SNPs, which met all four criteria: QUALITY (QUAL) value ≥30, minor allele frequency (MAF) ≥5%, significance level of Hardy-Weinberg equilibrium (HWE) test ≥0.01, and no more than four individuals with missing genotypes at each locus among all individuals.

TABLE 2 | Genetic diversity parameters of *Saurogobio dabryi*.

Sample sites	Sequencing depth	SNP numbers	Π	*He*	*Ho*	Tajima's *D*	Fu's *FS*
QJ	21.68	9,115	0.2596	0.68	0.69	0.5835	1.7236
SN	19.34	21,651	0.3300	0.66	0.67	0.6107	4.8001
YB	19.23	26,452	0.2969	0.75	0.75	0.1551	5.2900
HC	14.74	57,362	0.3524	0.67	0.75	0.8435	4.0828
BN	14.23	57,523	0.3372	0.67	0.77	0.8326	4.5724
WZ	17.49	54,698	0.3410	0.71	0.77	0.4832	4.0184
TP	17.94	54,819	0.2972	0.67	0.67	0.4852	3.9928
Total	17.58	58,930	0.3106	0.69	0.84	1.2613	4.0686

π: nucleotide diversity index, He: expected heterozygosity, Ho: observed heterozygosity.

Genetic Analysis

VCFtools software (Danecek et al., 2011) was used to calculate the nucleotide diversity index (π), Tajima's and Fu's FS *D* value within populations, and the genetic differentiation coefficient (*Fst*) between pairs of populations. Perl scripts (Goodswen et al., 2010) were used to calculate the expected heterozygosity (*He*) and the observed heterozygosity (*Ho*). The population structure of *S. dabryi* was analyzed by Admixture software (Alexander et al., 2009), and groups with *K* values ranging from 1 to 10 were clustered, separately. The clustering results were cross-validated and the optimal number of clusters was determined by the method of minimized cross-validated error rate. A maximum likelihood (ML) tree based on the General Time Reversible model (GTR)+proportion of invariable sites (I)+gamma distributed site-to-site variation (G) model by the Bayesian inference method was constructed using RAxMLv8.1.17 software (Stamatakis, 2014) with 100,000 replicates. A principal coordinate analysis (PCA) plot was generated using Eigen software (Price et al., 2006) to visualize the population differentiation. Identity-by-state (IBS) was calculated by Plink software (Purcell et al., 2007) to assess the genetic similarity of groups.

Genetic Effect of Environmental Factors

Spatial distance between sites (measured along river channels), drainage area, and river length were measured on large-scale catchment topographic maps in ArcGIS10.5. The channel slope was the drop per unit reach length, which was calculated according to reach an elevation of 5 km upstream and 5 km downstream of the sample sites. The isolation time was calculated from the dates of dam closure. The elevation at each sample site was recorded by the Global Positioning System (GPS) (accuracy of 1 m; **Table 1**). The correlation between the parameters of genetic diversity (SNP numbers, π, and *Ho*) and the environmental factors (drainage area, river length, channel slope, and elevation) were performed using the cor.test function in the R package. The influence of environmental variables (such as spatial distance, isolation time, and channel slope) on population genetic differentiation was calculated by the Mantel test using the Vegan procedure of the R package (Dixon, 2003). The value of genetic differentiation used linearized *Fst* values [*Fst*/(1 – *Fst*)]. Significance was tested using 1,000 permutations.

RESULTS

Statistics and Evaluation of Sequencing Data

A total of 237.86 Mb of clean reads and 59.87 Gb of clean data were generated for 120 *S. dabryi* by SLAF sequencing. The average Q30 quality score was 94.98% and the average guanine-cytosine (GC) content was 42.52%. After low-quality reads, adaptors, and barcode sequences were removed, a total of 989,072 SLAF tags were obtained in which 237,400 tags were polymorphic. Population sequencing depth was from 14.23× to 21.68×, with an average sequencing depth of 17.58×. A total of 1,300,189 SNPs were obtained, among which there were 58,930 SNPs that passed the SNP filtration criteria (**Table 2**).

Genetic Diversity

The number of SNPs within the population ranged from 9,115 (QJ population) to 57,523 (BN population), exhibiting great variation among populations in the upper Yangtze River. The number of SNPs in the QJ population was significantly less than in the SN and YB populations (*t*-test, $p < 0.01$), The number of SNPs in the SN and YB populations was significantly less than in the HC, BN, WZ, and TP populations (*t*-test, $p < 0.01$), showing that the numbers of SNPs were lower in the upstream populations, which suggested that more alleles might have been lost by random genetic drift. There were no significant differences for other metrics of genetic variation (π, *He*, and *Ho*) among the seven populations (*t*-test, $p > 0.05$). All seven populations had positive values of Tajima's *D* and Fu's *FS*, indicating that *S. dabryi* in the upper Yangtze River had experienced a recent bottleneck (**Table 2**).

Population Structure

$K = 3$ had the smallest cross-validation error (**Figure 2a**), thus supporting three genetic groups. Individuals in QJ belonged to group 1 (in gray), individuals in SN and YB belonged to group 2 (in orange), and individuals in WZ and TP belonged to group 3 (in blue). Groups 2 and 3 both existed within BN and HC populations and exhibited almost equal average probabilities of membership (**Figure 2b**). This result was also indicated by the PCA, wherein the seven populations formed three distinct groups (QJ; SN\YB; HC\BN\WZ\TP), but there was one individual of the HC population that was clustered into the SN\YB group (**Figure 2c**). The ML tree also supported this arrangement (**Figure 2d**). It was clear that the upstream and downstream populations belonged to different genetic groups.

Identity-by-state analysis was conducted to assess similarity within three distinct groups. To avoid the interference, we eliminated the BN and HC populations that exchanged genes with both upstream and downstream neighbors. IBS value was the highest in group 1 (the QJ population), followed by group 2 (the SN and YB populations), and the lowest in group 3 (the WZ and TP populations), indicating that the degree of genetic similarity within groups was increased from downstream to upstream (**Figure 3**).

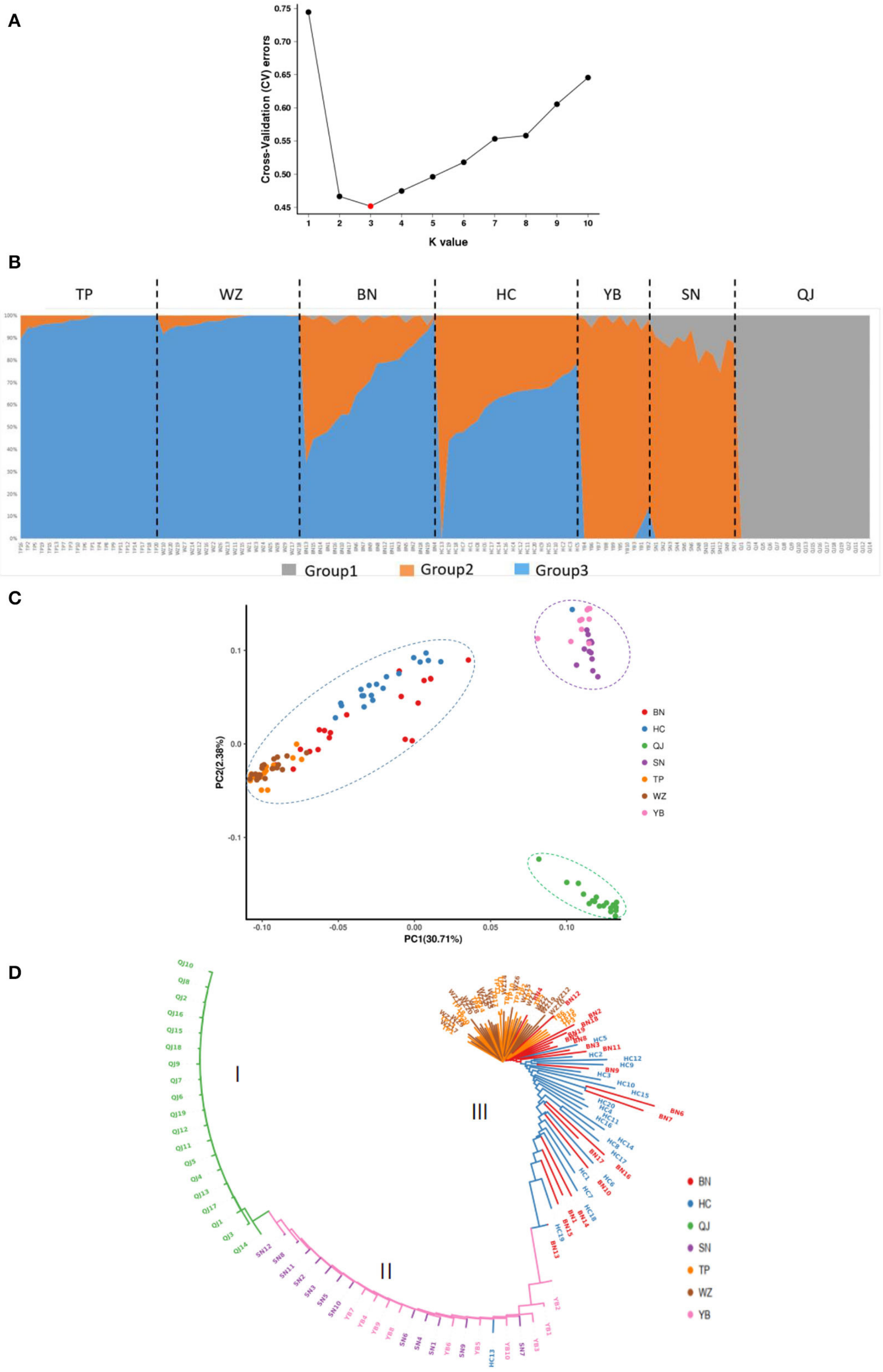

FIGURE 2 | Genetic structure of seven *Saurogobio dabryi* (*S. dabryi*) populations based on SNP markers. **(A)** The optimal number of clusters was determined by minimized cross-validated error rate. **(B)** Population structure ($K = 3$) of all individuals. Different colors represent the probability of an individual belonging to different genetic groups. **(C)** PCA plots of every individual. **(D)** ML phylogenetic tree. Cluster I included all QJ individuals, cluster II included all SN and YB individuals and one HC individual, and cluster III included all BN, WZ, and TP individuals and the rest of the HC individuals.

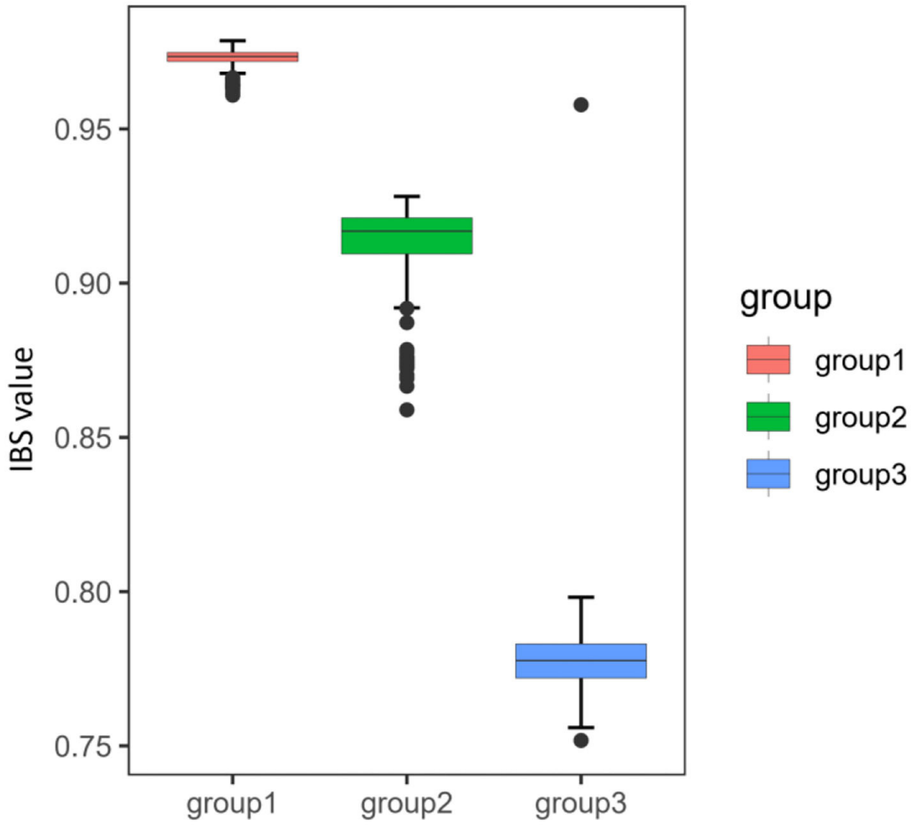

FIGURE 3 | Genetic similarity within three distinct groups by identity-by-state (IBS). The upper and lower ranges of the boxplot, with the upper limit being the upper quartile and the lower limit being the lower quartile. The horizontal line inside the box represents the median. The whiskers were extended to the 95th percentile.

TABLE 3 | Genetic differentiation (*Fst*) among seven populations of *S. dabryi* in the upper Yangtze River.

	TP	WZ	BN	HC	YB	SN	QJ
TP							
WZ	0.000						
BN	0.058*	0.057*					
HC	0.093*	0.092*	0.009				
YB	0.335*	0.335*	0.199*	0.156*			
SN	0.354*	0.353*	0.226*	0.181*	0.007		
QJ	0.416*	0.415*	0.312*	0.267*	0.179*	0.166*	

**indicates significant value.*

Population Genetic Differentiation

Fst values among the three clusters (QJ; SN\YB; HC\BN\WZ\TP) were high (*Fst* = 0.166–0.416), implying considerable genetic differentiation (**Table 3**). The QJ population most upstream had diverged highly from other populations. The *Fst* values between the SN and YB populations were very small (0.007), but they were highly differentiated from other populations. The YB, TP, WZ, and BN populations were in the same river section. However, there was high differentiation between the YB population in the upstream river habitat and the TP, WZ, and BN populations in the downstream reservoir habitat.

Genetic Effect of Environmental Factors

Correlation analysis indicated a significant negative relationship between the numbers of SNPs and the elevations of the seven populations ($r = -0.867$, $p < 0.05$). There were fewer SNPs within upstream populations in the high-elevation region than within downstream populations in the low-elevation region. The numbers of SNPs within populations had no significant correlation with drainage area, river length, and channel slope (**Figure 4**). In addition, the other genetic diversity metrics (π and *Ho*) also had no correlation with these environmental factors.

Genetic differentiation exhibited a close correlation with spatial distance among the populations ($r = 0.907$, $p < 0.01$). No significant correlation was found between genetic differentiation and isolation times. There was no correlation between genetic differentiation and channel slope (**Figure 5**).

DISCUSSION

In this study, no significant differences in the values of π, *He*, and *Ho* were observed among these populations, but the

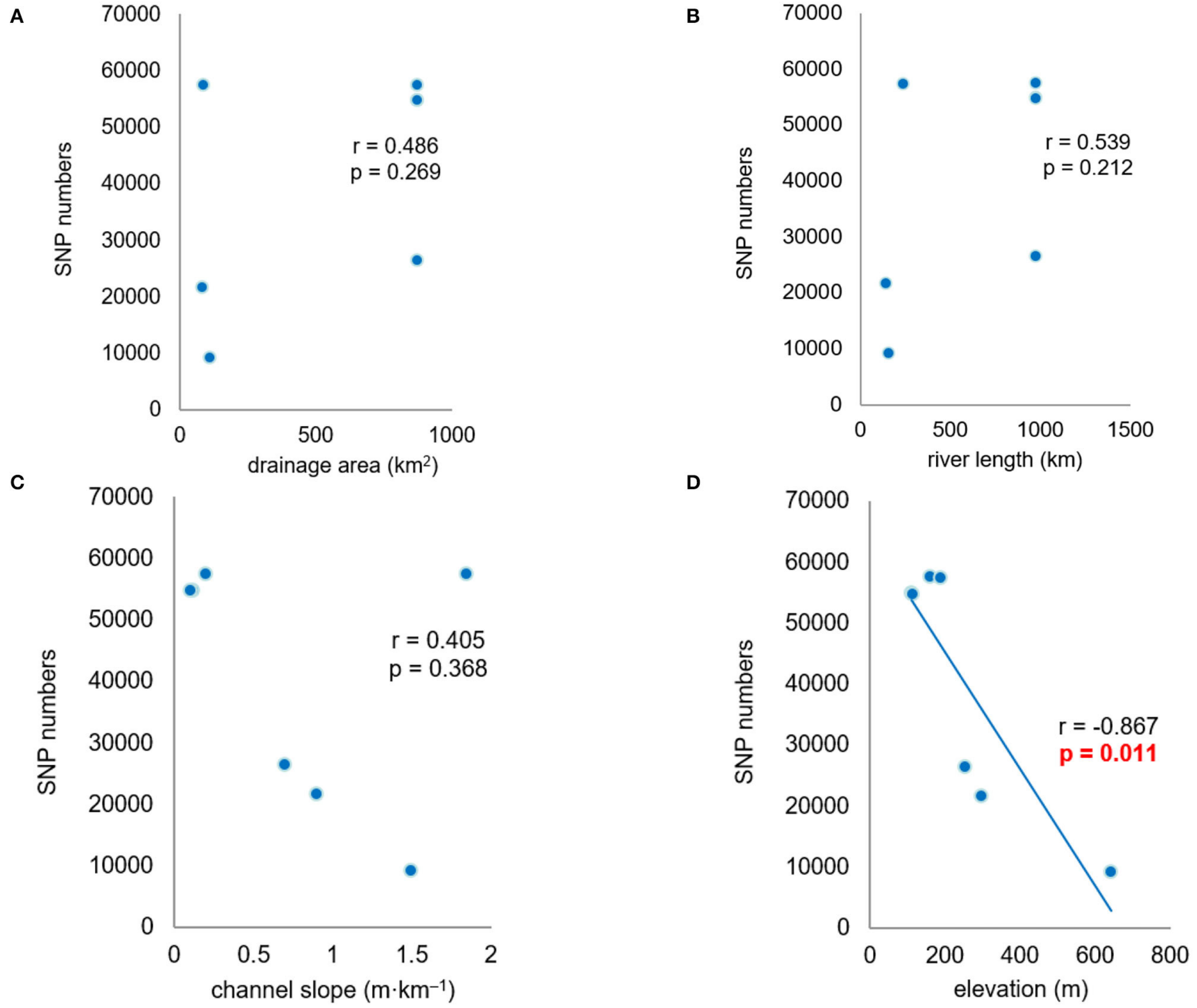

FIGURE 4 | Correlation analysis between the single nucleotide polymorphism (SNP) numbers and environmental factors. **(A–D)** r is correlation coefficient, p value is used to determine the result of hypothesis testing.

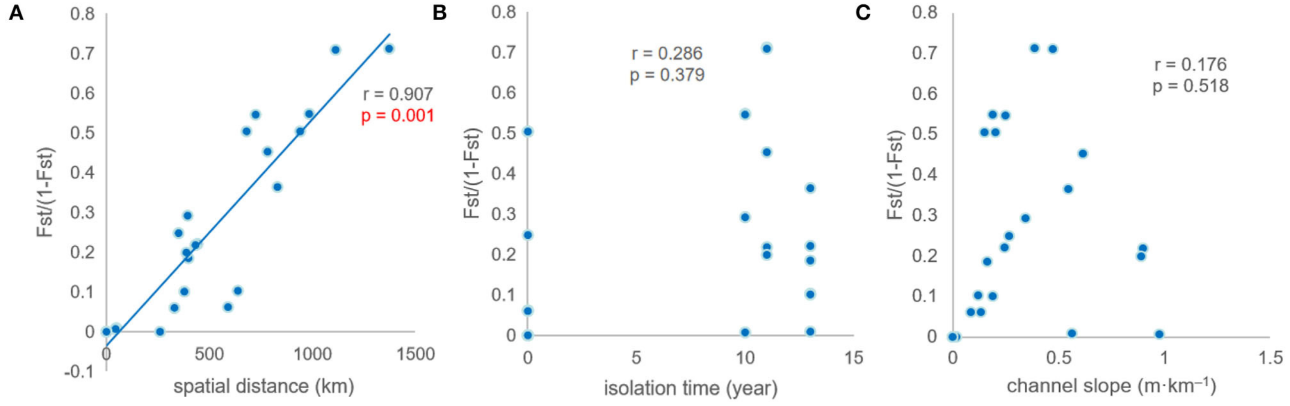

FIGURE 5 | Mantel tests between genetic differentiation and environmental factors. **(A–C)** r is correlation coefficient, p value is used to determine the result of hypothesis testing.

number of SNPs was lower in the QJ, SN, and YB populations, suggesting that more alleles might have been lost in the upstream populations due to random genetic drift. Compared with other parameters of genetic diversity, variation in the number of SNPs was more apparent. Consistently, the genetic similarity was higher within the upstream group. The two genetic values reflected that genetic diversity was lower within the upstream populations than in the downstream populations. The percentage in the abundance of *S. dabryi* was about 4.1% of the total catch in the QJ section in 2006–2012 (Tang et al., 2014), which was lower than that in the YB section, 11.3% in 2007–2009 (Xiong et al., 2015), which indicated that *S. dabryi* was historically less abundant upstream than downstream. Historically smaller populations might account for the lower genetic diversity in the upstream populations. Some other cyprinid fishes in the upper Yangtze River showed a similar trend, lower genetic diversity upstream, such as *Coreius heterodon* (Yan et al., 2008; Cheng et al., 2013), *Ancherythroculter nigrocauda* (Zhai et al., 2019), and *Megalobrama pellegrini* (Wang et al., 2019). In this study, the upstream populations of *S. dabryi* with lower allelic richness and higher genetic similarity were more vulnerable to genetic drift than downstream populations. Upstream populations should be given more conservation attention to avoid inbreeding and gene pool degradation.

We have detected a recent bottleneck event according to the positive values of Tajima's *D* and Fu's *FS*. *S. dabryi* prefers to inhabit and spawn in flowing water, but dam construction has changed the river into lentic water habitats. It has been found that the spawning time has become shortened and the reproductive rate of *S. dabryi* has decreased (Gao, 2016). In addition, environmental features in the upper Yangtze River also have changed in response to dam construction (Cheng et al., 2015). Under such conditions, the dam-induced fragmentation might lead to the decline of the fishes (Blanton et al., 2019). The recent bottleneck events may possibly have resulted in the loss of allelic richness of *S. dabryi*.

Structure analyses indicated that the seven populations of *S. dabryi* represented three distinct groups. It was clear that upstream and downstream populations presented different genetic structures. The YB, WZ, and TP populations were in the same river section with no dam barrier, but the YB population was in the riverine habitat, and the WZ and TP were in reservoir habitat. The YB population was obviously differentiated from WZ and TP, suggesting that different local flow conditions can lead to genetic differentiation (Landguth et al., 2014).

Genetic differentiation of *S. dabryi* exhibited a close correlation with spatial distance. Significant genetic differentiation was shown between populations except for YB-SN, WZ-TP, and BN-HC. It was found that the populations of YB-SN and BN-HC without differentiations were distributed very closely regardless of isolation by dams. These populations might have been genetically differentiated before the dam construction. *S. dabryi* is a small-sized fish species with a finite capacity movement (Liu et al., 2019). It tends to move short distances more frequently than long distances and thus had gene flow over a limited geographic region. Such distance-attenuated movement might give rise to population structure consistent with an isolation-by-distance (IBD) evolutionary model (Roberts et al., 2013). Interestingly, the genetic differentiation between populations WZ and TP was not significant although the distance between them is relatively long. WZ and TP are both reservoir habitats and have similar ecological environments, which might bring the similar ecological adaptation. Therefore, such genetic structure of *S. dabryi* might be attributed to spatial distance and ecological adaptation.

At present, we did not detect a significant correlation between isolation time and genetic differentiation of *S. dabryi*. It is generally considered that genetic differentiation on a short time scale is difficult to detect. No genetic change was detected in rainbow trout (*Oncorhynchus mykiss*) populations 5–10 generations or in galaxiid (*Galaxias platei*) populations 20–30 generations after dam construction (Deiner et al., 2007; Vera-Escalona et al., 2015). However, dam-induced isolation on a long time scale has had a significant impact on other fish populations. Gouskov et al. (2016) found a dam, which was built over 100 years ago and had a strong effect on population structure of the carp (*Squalius cephalus*) in the Rhine River Basin. In our study, these dams are around 20 years old or even younger (Cao, 2019) about 20 or less generations for *S. dabryi*. The effect of isolation has not obviously emerged. Hence, the genetic impacts of dams can be relatively minimal on a short time scale. Environmental changes have occurred in the upper Yangtze River after the construction of these dams. With the increase in isolation time, these changes will possibly influence the genetic structure of *S. dabryi*, and thus genetic impacts of dams need long-term monitoring.

CONCLUSION

High-throughput DNA sequencing technology has provided a large number of molecular markers and powerful tools for the study of population genetics. In this study, we identified a large number of SNP loci in populations of *S. dabryi* in the upper Yangtze River. The results showed that the numbers of SNPs were lower within the upstream populations, and more alleles might have been lost in the upstream populations. It suggests that the upstream populations are more vulnerable to genetic drift and thus should be given more attention in resource conservation. These populations of the upper Yangtze River formed three distinct groups, with significant genetic differentiation among three groups, and should be considered as three different genetic units for conservation management. Our results exemplify the genetic diversity and population structure of *S. dabryi* after habitat fragmentation caused by dams, which will provide a reference for natural resource protection and management of this species in the upper Yangtze River.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA759087.

ETHICS STATEMENT

The animal study was reviewed and approved by Jianghan University.

AUTHOR CONTRIBUTIONS

HL performed the laboratory work and wrote the manuscript. FX designed the research and revised the manuscript. DZ collected the samples and revised the manuscript. XD and DC collected the samples. YC, YW, and MX revised the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

This research was supported by the National Key R & D Program of China (2018YFD0900903), the National Natural Science Foundation of China (nos. 51979123 and 51779105), the Innovative Research Team Foundation of the Department of Education of Hubei Province, China (T2020034), and the Scientific Research Project of Jianghan University (2021kjzx006).

ACKNOWLEDGMENTS

The authors are grateful to Dr. Jinyun Luo for helping with data analysis, Jin Cai, Dong Wang, and Tianjiao Lei for assisting with the sample collecting.

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

frontiers | Frontiers in Ecology and Evolution

METHODS
published: 23 June 2022
doi: 10.3389/fevo.2022.945824

Harmonizing and Searching Macroinvertebrate Trait Information in Alpine Streams: Method and Application–A Case Study in the Three Parallel Rivers Region, China

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Specialty section:
*This article was submitted to Conservation and Restoration Ecology,
a section of the journal Frontiers in Ecology and Evolution*

Received: 17 May 2022
Accepted: 06 June 2022
Published: 23 June 2022

Citation:
*Ao S, Li X, Tian Z, Hu J and Cai Q (2022) Harmonizing and Searching Macroinvertebrate Trait Information in Alpine Streams: Method and Application–A Case Study in the Three Parallel Rivers Region, China. Front. Ecol. Evol. 10:945824.
doi: 10.3389/fevo.2022.945824*

The traits of organisms provide critical information for understanding changes in biodiversity and ecosystem function at large scales. In recent years, trait databases of macroinvertebrates have been developed across continents. Anyone using different databases to search for traits will encounter a series of problems that lead to uncertain results due to the inconsistency of the trait information. For example, traits for a particular macroinvertebrate taxon may be inconsistent across databases, coded in inconsistent ways, or cannot be found. However, most of the current studies do not clearly state their solutions, which seriously hinders the accuracy and comparability of global trait studies. To solve these problems, we collected representative databases from several continents, including the United States, Europe, South Africa, Bolivia, Australia, and New Zealand. By comparing the inconsistency of similar trait classifications in the nine databases, we harmonized 41 of these grouping features. We found that these databases differed widely in terms of the range and category of traits. And the method of coding traits also varies from database to database. Moreover, we showed a set of trait searching rules that integrate trait databases from different regions of the world, allowing traits to be identified more easily and uniformly using different trait databases worldwide. We also applied this method to determine the traits of 155 macroinvertebrate taxa in the Three Parallel Rivers Region (TPRR). The results showed that among a total of 155 macroinvertebrate taxa, the 41 grouping features of all genera were not fully identified, and 32 genera were not recorded (thus using family-level data). No trait information was found at all for two families, which contain two genera. This suggests that many macroinvertebrate taxa and their traits have not been fully studied, especially in those regions, including China, where macroinvertebrate trait studies are lagging. This inadequacy and unevenness have seriously hindered the study and development of macroinvertebrate trait and functional diversity worldwide. Our results complement the information on stream macroinvertebrate traits in the TPRR, a global biodiversity hotspot, and greatly promote the uniformity of global trait research and the accuracy and comparability of trait research in different regions.

Keywords: macroinvertebrate, the Three Parallel Rivers Region, stream ecology, trait, trait harmonization, trait determination

INTRODUCTION

The study of functional traits has a long history. Cummins (1973) was the first to apply the functional trait approach to stream biology. Kerans and Karr (1994) proposed a benthic index of biotic integrity (B-IBI) that can be effectively applied to riverine environmental monitoring, incorporating macroinvertebrate traits. After that, many countries began to develop macroinvertebrate trait information for biological monitoring and biodiversity conservation. The earliest development was in Europe. Usseglio-Polatera et al. (2000) started to compile information on macroinvertebrate traits in 2000. Europe uses a fuzzy coding method to code traits, considering the uncertainty of macroinvertebrate traits, which indicates how strongly a taxon expresses a particular trait based on affinity (Chevenet et al., 1994; Usseglio-Polatera et al., 2000). At the same time, the United States began to synthesize the traits of its macroinvertebrate. The trait database of USGS (Vieira et al., 2006) comprehensively summarized the traits of macroinvertebrates in North America for the first time. After that, Twardochleb et al. (2021) and the US EPA (2012) released more comprehensive databases of macroinvertebrate traits by integrating relevant domestic studies. However, unlike Europe, the United States applied a binary approach in which each taxon was assigned to only one trait. These two methods have been used in Australia, New Zealand, and South Africa.

With the development of trait-based ecology, researchers have demonstrated the efficiency of analyzing functional traits and functional diversity in ecological studies (McGill et al., 2006; Alahuhta et al., 2019). A trait is a well-defined and measurable property of organisms at the individual level (McGill et al., 2006; Mouillot et al., 2013). There are several advantages of using trait data for analysis compared to species data: (1) Traits directly reflect the behavior of organisms and control their interrelationships with the environment and other organisms, so that traits can deepen the mechanistic understanding of species-environment and even interspecies relationships (Thornhill et al., 2018; Desrosiers et al., 2019); (2) Traits vary less across geographic regions and can be analyzed across a wide geographic range or even across species (Bernhardt-Roemermann et al., 2011; Heino et al., 2013); (3) Trait data have low requirements for taxonomy and are less susceptible to taxonomic ambiguities or inconsistencies in long-term data (US EPA, 2012) for that genus or family-level traits have long been used in biological assessments (Vieira et al., 2006; Schaefer et al., 2011; Rodil et al., 2013); (4) Traits can be used to calculate a range of functional diversity indices, to detect changes in the functional characteristics of communities, or as indicators to estimate ecosystem function (Carmona et al., 2016; de Bello et al., 2017; Laughlin et al., 2018). With increased human disturbance and warming, the study of biological traits will allow us to better cope with the effects of such changes on ecosystems.

Although trait-based research has developed rapidly in the past few years, trait information of organisms is still the foundation of research. However, it is often difficult to obtain the trait information of species, and the collection and collation of trait data are time-consuming and laborious (US EPA, 2012). This difficulty is especially true for the small and diverse macroinvertebrates in alpine streams. To date, descriptions of macroinvertebrate traits have been scattered among numerous studies. The development of trait databases that focused on aquatic organisms has progressed the most in Europe and North America compared to other regions. The European database contains 8586 taxa (Schmidt-Kloiber and Hering, 2015), making it the largest database. Researchers have recently collated a new database on disperse-related traits of European macroinvertebrates (Sarremejane et al., 2020). Twardochleb et al. (2021) developed a North American database of freshwater insect traits by referring to the research of Poff et al. (2006), the US Geological Survey (USGS; Vieira et al., 2006), the US EPA (2012), and other studies on North American freshwater insects. There are 3,791 macroinvertebrate taxa, which is second only to Europe in terms of data volume. In addition, similar trait databases are available for South Africa (Odume et al., 2018), Australia (Kefford et al., 2020), New Zealand (Phillips and Smith, 2018), and Bolivia (Tomanova and Usseglio-Polatera, 2007). These databases are not unique, but rather describe the same taxa from different traits, forming a complementary.

Unfortunately, there are three obvious problems when using these different databases: (1) There is no uniform way to describe traits information in these databases, and the ways used to describe traits may vary considerably. For example, US EPA (2012) classified macroinvertebrate sizes into 3 categories: small (<9 mm), medium (9–16 mm), and large (>16 mm), whereas Sarremejane et al. (2020) classified them into 7 categories, with the smallest < 0.25 mm and the largest > 8 mm. This inconsistency hampered the development of comparative studies on macroinvertebrate traits worldwide and may lead to erroneous results (Schmera et al., 2015). Kunz et al. (2021) harmonized seven grouping features from six databases in four regions, greatly facilitating the study of traits among different regions. However, many grouping features are scattered in various databases that need to be harmonized, such as rheophily and thermal preference etc., which are all important to macroinvertebrates. Moreover, recent trait-based studies have highlighted the importance of standardizing traits and trait terminology to ensure that data can be integrated more easily in the future (Schmera et al., 2015; Kunz et al., 2021). In this study, we follow the suggestion of Schmera et al. (2015). We use "grouping feature" to refer to the general properties of related traits (rather than "trait"), and "trait" refer to traits that can be measurable at the individual level (rather than "trait state," "modality" or "trait modality"). (2) These databases contain many duplicate species, but there are inconsistent descriptions of similar traits in these same species. They may all be accurate, but this has caused great confusion for researchers in other regions to determine the macroinvertebrate traits. (3) There are still many macroinvertebrates whose traits have not been described so far. Many species are found in areas with poorly studied macroinvertebrate traits, including some biodiversity hotspots.

The Three Parallel Rivers Region (TPRR) in China has long been recognized as a global biodiversity hotspot (Myers et al., 2000; Xing and Ree, 2017), but studies on macroinvertebrate traits in this region are still scarce (Liu et al., 2021). Three international rivers, including the Yangtze, Mekong, and Salween rivers, flow parallel from north to south in a 100 km wide area and are recognized by UNESCO as a World Natural Heritage Site and a priority area for biodiversity conservation (Myers et al., 2000). In our six study regions, the northernmost Three-River Headwaters region has an average altitude of over 4,000 m and a cold and dry climate. The Dulong River, Zhubaluo River, and Gangqu River in the center are distributed parallel to each other from west to east and are located in the area where the three rivers are closest to each other. And due to the blockage of the summer monsoon by the mountains, precipitation gradually decreases from west to east (Xing and Ree, 2017). These regions and the Cangshan Mountains region have a great altitude span with a distinct vertical climate classification (Wu et al., 1988; Zeitler et al., 2015). The southernmost region of Dehong is below 1,000 m above sea level and has a much warmer climate.

By harmonizing the trait information of macroinvertebrates from different world regions, we proposed a set of trait searching rules with clear steps to help people identify macroinvertebrate traits using different trait databases. At the same time, we applied this set of rules to the TPRR and determined the macroinvertebrate traits in this region. Our aim is not only to extend the seven grouping features harmonized by Kunz et al. (2021) to more grouping features and to extrapolate this trait information to the TPRR in China, but also to try to find an accurate and comprehensive method for trait determination using different databases. This will provide a methodological basis for future research on macroinvertebrate traits in China and even the world. At the same time, it will help us improve the uniformity of research on stream macroinvertebrate traits in China and around the world and further understand the unique and rapidly changing alpine stream ecosystems.

MATERIALS AND EQUIPMENT

Study Area

The study area is located in the TPRR of Yunnan, China. The north-south-oriented Hengduan Mountains located at the southeastern rim of the Tibetan Plateau, separate Asia's major rivers in the TPRR, including the Yangtze, Mekong, and Salween rivers. The region has high mountains and deep valleys, with snowcapped mountains over 6,700 m above sea level, most of which are in the national nature reserves and belong to global biodiversity hotspots and priority conservation areas (Myers et al., 2000; Xing and Ree, 2017). The average annual temperature in this region is only 6°C. The average annual precipitation is 624.7 mm and decreases from west to east due to the blocking effect of the Hengduan Mountains on the summer monsoon with abundant water vapor, and 73% of the precipitation is concentrated from June to September (Yu et al., 2018).

This study was conducted mainly in six regions of the TPRR, which are the Three-River Headwaters, Dulong River, Zhubaluo River, Gangqu River, Cangshan Mountain, and Dehong Prefecture region (**Supplementary Figure 1** in **Supplementary Material**). The Three-River Headwaters region has the highest average altitude of more than 4,000 m, the lowest annual precipitation of less than 300 mm, and the lowest average annual temperature below 0°C (Shi et al., 2016). The Dehong Prefecture region has the lowest average altitude of fewer than 1,000 m and the highest average annual temperature of 20°C.[1] Furthermore, the Dulong River has the highest average annual precipitation, once recording average annual precipitation of up to 4,800 mm (Li, 1996). Due to this huge difference in altitude, precipitation, and temperature, the TPRR can be a good microcosm of entire China to some extent.

Macroinvertebrate Sampling

A total of 213 sampling sites were collected from six regions, most of which were located at the headwaters of the streams and were wadable. The sites in Dehong were sampled in September 2020, Three-River Headwaters in September 2018, Dulong River in November 2018, Zhubaluo River in November 2017, Cangshan Mountain in November 2012, and Gangqu River in May 2005. All the samplings were carried out in the dry season with stable hydrological conditions. Macroinvertebrates were collected using a 500 μm mesh surber net (30 × 30 cm) within a 100 m river reach. All samples were preserved in 95% ethanol concentrations and brought back to the laboratory for identification (Morse et al., 1994; Merritt et al., 2019). In our sampling, we only focused on aquatic insects, which were identified to genus level, except Chironomidae to subfamily level.

Trait Databases

A total of 213 sampling sites were collected from six regions, most of which were located at the headwaters of the streams and were wadable. The sites in Dehong were sampled in September 2020, Three-River Headwaters in September 2018, Dulong River in November 2018, Zhubaluo River in November 2017, Cangshan Mountain in November 2012, and Gangqu River in May 2005. All the samplings were carried out in the dry season with stable hydrological conditions. Macroinvertebrates were collected using a 500 μm mesh surber net (30 × 30 cm) within a 100 m river reach. All samples were preserved in 95% ethanol concentrations

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[1] http://data.cma.cn/

and brought back to the laboratory for identification (Morse et al., 1994; Merritt et al., 2019). In our sampling, we only focused on aquatic insects, which were identified to genus level, except Chironomidae to subfamily level.

METHODS

Trait Harmonization

In this study, we chose the binary approach to determine macroinvertebrate traits. The fuzzy coding method is applied in the trait database in Europe and other regions. However, we used the binary method (i.e., each taxon was assigned to only one trait) for the following reasons: (1) The development of macroinvertebrate trait research is lagging in China, and there is not much detailed trait information available. This is one of the critical reasons why Poff et al. (2006) used a binary approach to build a database of macroinvertebrate traits in the first place. (2) There are still many studies on macroinvertebrate traits in China and the world using the binary method (Krynak and Yates, 2018; McKie et al., 2018; Wang et al., 2020). (3) The advantage of this approach is that it simplifies functional and even phylogenetic analyses, thus allowing an exploration of the dominant relationships between traits and macroinvertebrate taxa (Poff et al., 2006).

The main taxonomic resolution we considered was the genus level (except for some Chironomidae where the tribe was used). There are several reasons for this: (1) Aquatic insect larvae have great taxonomic uncertainty at the species level, but it is more likely that congeneric species have similar functional traits (Gayraud et al., 2003; Poff et al., 2006). Using genus-level traits dramatically simplifies the identification work of macroinvertebrates for researchers and reduces the error rate of identification. (2) Trait assignment is inevitably more difficult at higher taxonomic resolution (e.g., species). In contrast, trait information is relatively more abundant and complete at lower taxonomic resolutions (e.g., genus or family; Kefford et al., 2020). (3) Trait information at the genus and family level has long been successfully used in biological assessments in many countries (Vieira et al., 2006; Schaefer et al., 2011; Rodil et al., 2013). Moreover, we ignored the unique grouping features in each database (including some grouping features that look similar but are challenging to harmonize. For example, in the freshwaterecology.info database, there is the grouping feature "dietary preference," which includes specialist, moderate, and generalist. While another grouping feature "food" specifically describes whether macroinvertebrates eat detritus, animals, or plants. Although these two grouping features look similar, they are difficult to harmonize.) and only harmonized those that appear in at least two databases because these grouping features are unavailable for most of the taxa.

In the process of finding traits, we mainly have the following problems:

(1) When there are multiple sources of trait data for a particular macroinvertebrate, which one should we choose to use?

(2) How can different trait classifications from different sources be harmonized?

(3) If only species-level trait information is available, how should we determine genus-level trait information? What if congeneric species have different traits?

(4) What if a taxon fits multiple traits within a grouping feature?

(5) What if the taxon cannot be found in any of these databases?

Almost anyone searching multiple macroinvertebrate trait databases will encounter these problems. However, many studies do not provide a clear solution, especially those studies where the development of macroinvertebrate trait databases is lagging and need to refer to trait databases in other regions. These studies only provide references to databases or relevant literature and may incorporate expert advice (Addo-Bediako, 2021; Li et al., 2021; Shrestha et al., 2021). Although macroinvertebrate trait databases are described in reports and papers including their process of acquiring trait information, the use of these databases can still be confusing. For example, while the Australian database states its traits as originating from multiple published articles, the database provided simply integrates all the traits from these articles. There are both fuzzy coding data and binary data in this database, and the trait classification methods differ. For a given species, the same grouping feature also has different results. While all this information is valuable, this undoubtedly causes great confusion to database users and may lead to uncertainty in trait studies.

However, the EPA database is an exception. It integrated trait databases from 14 sources in the United States and gave specific harmonization rules. This rule can be summarized as follows: (1) Use the Poff, USGS (Vieira et al., 2006), and other databases in sequence; (2) If traits vary among species within the genera, use the most frequently recorded trait [Twardochleb et al. (2021) also used this rule]; (3) If different traits occur with the same frequency, select the more typical traits that mentioned by other sources (e.g., medium size, univoltine), or select a trait at random. However, these three rules can only solve part of the first three of the five problems mentioned above. Additional rules are needed to determine more traits using databases from multiple regions worldwide.

When we encountered the above problems, we developed a set of rules for determining taxon traits in combination with the US EPA's method to harmonize trait classification to ensure consistency in the treatment of all traits. For question one ("When there are multiple sources of trait data for a particular macroinvertebrate, which one should we choose to use?"), we consider mainly the zoogeographic realm and the similarity of regional climate. Ecological and historical factors shape species assemblages and thus their trait composition (Southwood, 1977; Cavender-Bares et al., 2009). For example, as the oldest fauna in the world, Australia experienced a long period of isolation from the Mesozoic to Pliocene, making the creature more unique (Holt et al., 2013). And many macroinvertebrate traits may vary with altitude and climate (Magiafoglou et al., 2002; Corbet et al., 2006; Bonada and Dolédec, 2018). Thus regions with similar climates are more likely to have similar traits, especially those that are evolutionarily stable (Poff et al., 2006; Nussey et al., 2007). Based on latitudinal, climatic similarities (Beck et al., 2018) and the zoogeographic realm (Holt et al., 2013), we considered the trait databases of North America (The Nearctic Realm), Europe (The Palearctic realm), South Africa (The Ethiopian Realm), South America (The Neo-Tropical Realm), and Australia and New Zealand (The Australian Realm) in turn. In North America, traits from the latest CONUS database were given priority, followed by the Poff and EPA databases (Since EPA chose the Poff database as the first, we consider the Poff database prior to the EPA.). In Europe, traits from the latest Sarremejane database were given priority, followed by the freshwaterecology.info database. For question two ("How can different trait classifications from different sources be harmonized?"), we determined the harmonization result of many similar grouping features and traits based on the databases mentioned above (**Table 1**). For question three ("If only species-level trait information is available, how should we determine genus-level trait information? What if congeneric species have different traits?"), consistent with the US EPA (2012), we use the majority rule to determine genus-level traits. For question four ("What if a taxon fits multiple traits within a grouping feature?"), we have two cases: for binary data, we used the trait with the most records; for fuzzy-coded data, we used the trait with the highest affinity (Except for the secondary habit, secondary feeding style, and emerge season two, which used traits with the second-highest affinity). We do not select less special traits or randomly select a trait following the EPA method to reduce the uncertainty of the results. For question five ("What if the taxon cannot be found in any of these databases?"), we tried to replace genus-level traits with family-level traits. Many species within the same family have similar traits (Gayraud et al., 2003; Poff et al., 2006), and most databases also provide traits at the family level. Even so, there may still be undetermined traits. Therefore, like most databases, we use NA to indicate indeterminate traits.

Trait Searching Rules

The following are the specific rules we developed for determining traits within a grouping feature at the genus level (**Figure 1**):

(1) Sequentially, using the CONUS, Poff, EPA, Sarremejane, freshwaterecology.info, South African, Bolivian, Australian, and New Zealand databases. (The Australia and freshwaterecology.info databases contain many similar or identical grouping features. When we search these two databases, we combine similar grouping features and use them as one. For example, use the result when the results of multiple similar grouping features are consistent or when only one grouping feature can determine the results. Otherwise, this grouping feature is considered in an uncertain state.) It is worth noting that when the study area changes, the search order of the database should be changed according to the similarity of the area. And the local study of macroinvertebrate traits should be prioritized.

(2) When using different trait classifications from different databases, the trait classifications are determined according to the harmonization results in **Table 1**. The selection of standard grouping features was determined primarily by their ability to include as many trait categories as possible within all other grouping features and by the extensiveness of their use. And other similar grouping features from different databases were harmonized according to the classification of the standard grouping features.

(3) When the genus that to be found available in the database, find the trait information of the genus. If the genus fits more than one trait under a grouping feature, i.e., the trait varies within the grouping feature. Use the most frequently occurring trait (binary data) or the highest affinity trait (fuzzy coded data). For example, suppose the respiration mode is both tegument and gills. The number of tegument respiration records is three and gills is one (binary data), or the affinity of tegument respiration is three and gills is one (fuzzy coded data). In that case, the respiration mode of this genus is tegument.

(4) If the database has the genus but no information on a particular grouping feature or multiple traits has the same frequency of occurrence (binary data), or multiple traits have the same affinity (fuzzy coded data), then use the next database.

(5) If only species-level trait information is available in the database but not genus-level, use the trait that occurs most frequently among species. The number of the same trait should be at least three, and only calculate the trait with the highest affinity when encountering the fuzzy-coded data). If the rule cannot determine the trait, search the trait information at the family level of the genus. For example, if three or more of the five species within a genus have an identical trait, the genus is considered to have this trait. Otherwise, use the next database.

(6) When a grouping feature of the genus could not be determined after searching the last database, search the trait information at the family level from the first database.

(7) Use the information when a family-level grouping feature needs to be searched available in the database. If the traits within the grouping feature vary, then the most frequently occurring trait (binary data) or the one with the highest affinity (fuzzy coded data) within the grouping feature was used as in step 3 above.

(8) If the family could not be found, or different traits have the same frequency of occurrence (binary data), or multiple traits have the same affinity (fuzzy coded data), then use the next database.

(9) If the grouping feature cannot be determined after using the last database, the grouping feature is considered currently undetermined (noted as NA).

TABLE 1 | Detailed information contained in the databases.

Database	Code method	Number of taxa	Number of grouping features
Twardochleb et al., 2021	Binary/Fuzzy	3791	11
Kefford et al., 2020	Binary/Fuzzy	2292	≈80
Odume et al., 2018	Binary	3604	40
Poff et al., 2006	Binary	311	20
US EPA, 2012	Binary	3857	≈50
Sarremejane et al., 2020	Fuzzy	480	9
Schmidt-Kloiber and Hering, 2015	Fuzzy	8586	40
Phillips and Smith, 2018	Fuzzy	495	16
Tomanova and Usseglio-Polatera, 2007	Fuzzy	82	8

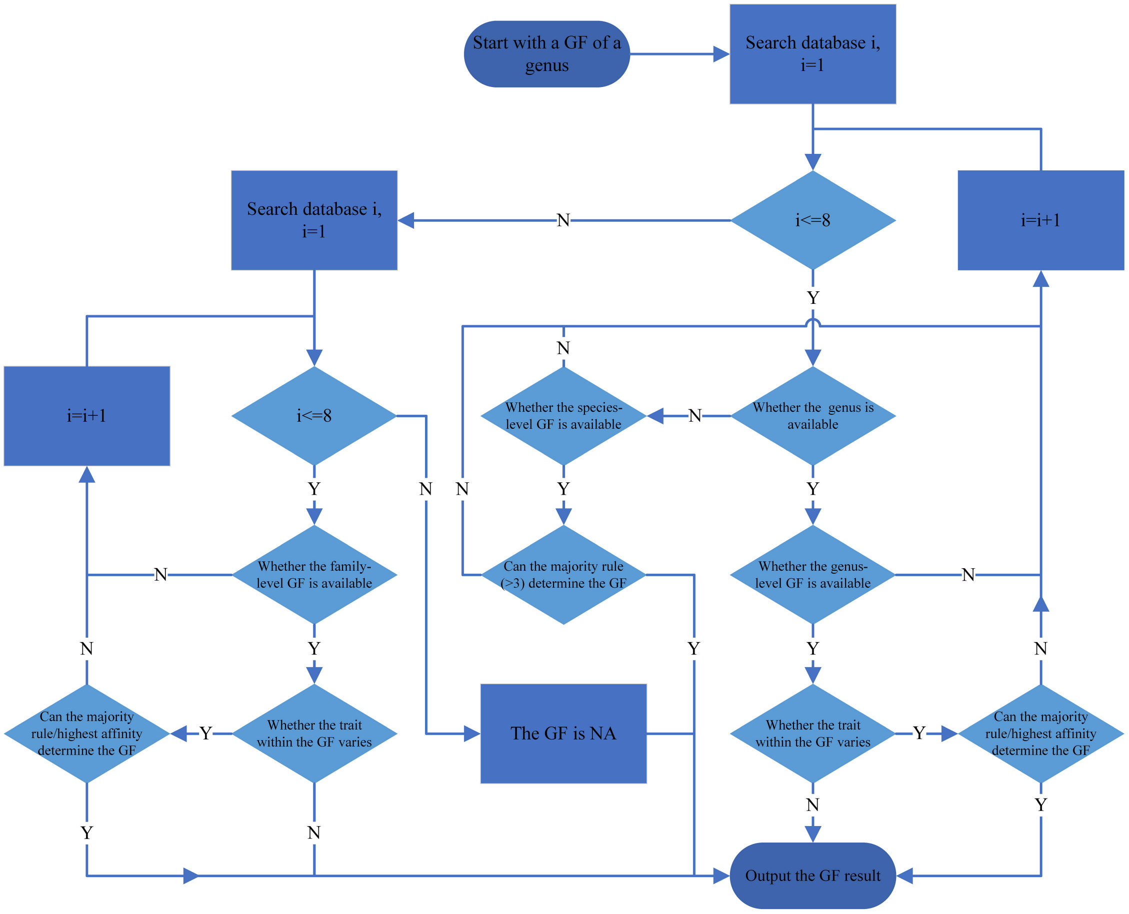

FIGURE 1 | Flowchart for determining genus-level grouping features (GF) from the databases. The oval represents a start or end point, the rectangle represents a process, and the diamond represents a decision. According to the databases searching sequence, the databases are considered as databases one to nine, respectively.

RESULTS

In the table of harmonization results for different trait classifications we identified, 41 grouping features with 154 traits were harmonized (**Table 2**). Of the 41 grouping features, seven standard grouping features from Kunz et al. (2021) were selected as standard grouping features, six in the CONUS database, nine in the Poff database, nine in the EPA database, two in the Sarremejane database, one in the freshwaterecology.info database, three in the South African database, three in the Australian database, and one in the New Zealand database. All other grouping features are harmonized with the standard grouping features mentioned above. More detailed trait harmonization results are presented in **Supplementary Material** for convenient use by researchers.

In our process of harmonizing traits, there are roughly 3 scenarios: (1) Harmonizing grouping features with specific ranges. Examples include size, thermal preference, and adult life span. These traits are relatively easy to harmonize due to their clear ranges. (2) Harmonizing entirely categorical grouping features. It is easy to harmonize if the trait categories are consistent within the grouping features. Examples include voltinism, emergence season, and female dispersal. Conversely, if inconsistent, it is difficult to harmonize. For example, in the freshwaterecology.info database, reproduction type includes both the location of eggs (terrestrial or aquatic) and the type of eggs (free isolated. cemented isolated, fixed clutches, or free clutches). While in the South African database, the location of eggs and the type of eggs are two grouping features. (3) Harmonizing grouping features containing both categories and specific ranges. These grouping features are relatively the most difficult to harmonize, even impossible to harmonize. For example, in the EPA database, salinity tolerance is classified as fresh, brackish, and saline, while the Australian database classifies salinity tolerance according to the magnitude of electrical conductivity. Examples also include rheophily and development speed.

A total of 153 genera, 71 families, and 10 orders (Ephemeroptera, Odonata, Plecoptera, Hemiptera, Neuroptera, Megaloptera, Trichoptera, Lepidoptera, Coleoptera, and Diptera) of macroinvertebrates were found in our study (including three subfamilies of Chironomidae). When we applied the rules to determine the traits, the results showed that the grouping features of all genera were not fully determined. Furthermore, 32 genera had no records (thus using family-level traits), accounting

TABLE 2 | Harmonization results of different trait classification (partial).

Grouping feature	Combined grouping features	Trait	Combined traits
Maximum body size	Max Size, Size at maturity, Maximal potential size	Small (<9 mm)	<10 mm
		Medium (9–16 mm)	10–20 mm
		Large (>16 mm)	>20 mm
Locomotion	Habit, Morphology, Mobility, Locomotion type, Attachment to substrate of aquatic stages, Mobility and attachment to substrate	Burrower	Burrow, Epibenthic burrower, Endobenthic burrower, Burrowing/boring
		Crawler	Sprawler, Walking, Climber, Climb, Clinger, Cling, Sprawl, Sprawler
		Sessile	Attach_perm, (Semi)Sessile, Attached, Temporarily attached
		Swimmer	Swim, Diving, Full water swimmer, Swimming/diving, Skate, Skater, Surface swimmer, Swimming/skating
Female dispersal		Weak	
		Strong	

The first and third columns represent the standard grouping features and traits, respectively. The second and last columns represent the grouping features and traits combined for harmonization (empty means no combining is required). Only partial results are shown, considering the table is too big. The complete results are shown in ***Supplementary Material****.*

for 20.9% of the total taxa. These genera belong to 10 orders and 22 families, which indicates that all families in this region contain genera for which no records could be found. No trait information was found for two families (Deuterophllobldae and Phryganopsychidae), which contain two genera. (See **Supplementary Material** for detailed results.)

DISCUSSION

Worldwide, there are approximately 50,000 species of aquatic insects (Balian et al., 2008). However, there are fewer than 20,000 species in the United States, European, Australian, South African, New Zealand, and Bolivian databases without considering the same taxon. It is evident that the current study of macroinvertebrates is still far from adequate in terms of species numbers. This problem is particularly evident in the TPRR. Our results showed that a total of 34 genera were not recorded, accounting for approximately 22% of the total. Moreover, two families have no records. This reflects not only the inadequacy of global studies on macroinvertebrate traits but also unevenness. Many developing countries, including China, often do not have enough macroinvertebrate studies to establish a trait database. However, at the same time, numerous endemic macroinvertebrates are scattered in various regions of the globe. As a result, many studies have to exclude species without trait information (Krynak and Yates, 2018), which seriously hinders the development of comparative studies on macroinvertebrate traits worldwide and may lead to erroneous results (Schmera et al., 2015).

The trait classifications used in current research are often predefined by scientists. However, studies using such predefined and variable numbers of traits undoubtedly impact many research results. The negative impact is mainly reflected in the following points: (1) How macroinvertebrate traits are described and their numbers vary significantly from database to database. For example, the Poff database described a total of 59 traits for 20 grouping features, while the freshwaterecology.info database described nearly 40 grouping features with hundreds of traits. (2) Several similar traits of macroinvertebrates have been described in different ways. For example, dispersal is defined in the European and South African databases as aquatic active, aquatic active, aquatic passive, aerial active, and aerial passive. In contrast, the dispersal traits in the EPA and New Zealand databases are described as the level of dispersal distance before female spawning, the strength of adult flight ability, and the frequency of drift occurrence. (3) Many species have different trait descriptions in different databases. They may all be true for reasons such as differences in geographic regions and the presence of many interference factors, but the reason for this is not apparent. (4) Insufficient research on some specific species. For example, many Coleoptera adults and larvae live in water, and their morphology is entirely different, so their traits should be different. However, only the Australian and Bolivian databases classified this condition, and many other databases do not take this into account. In addition, synonymy is one of the factors leading to uncertain results. However, these problems should be left to professional taxonomists and are not the focus of this paper. (5) An unstated assumption of trait-based analyses is that characterizing traits for late instars are adequate to account for organism-environment relations, but this may not always be the case (Poff et al., 2006). Although recently Kunz et al. (2021) harmonized traits within six databases in four regions, it is undeniable that different traits provide valuable information on different aspects of trait research. Therefore, we harmonized the traits that appeared in at least two of the eight databases to promote the use of more traits.

Although we assigned most macroinvertebrate grouping features to only one trait, trait values are usually characterized by their uncertainty. The uncertainty is because macroinvertebrate traits may change at different life stages (e.g., feeding groups, respiration) and to our lack of knowledge of most taxa (Kefford et al., 2020). Some databases, including those in Europe, use the fuzzy coding method to solve this problem. However, for the reasons stated in the method section, we applied the binary method. We do not deny that the fuzzy coding method provides precious information for describing different taxon traits. This method takes into account some intraspecific variations of macroinvertebrate traits. Perhaps fuzzy coding of different traits or multi-state binary approaches should be considered in the future as research progresses. For example, Li et al. (2020) found that the voltinism of the *Drunella submontane* (Ephemeroptera: Ephemerellidae) varies with altitude: univoltine below 1,600 m and semivoltine above 2,100 m. Therefore, in future studies, the voltinism of the species should be determined to be univoltine or semivoltine, depending on the sampling elevation.

It is worth noting that the method we provide has its limitations. Although it is generally believed that the traits vary less across geographic regions (Bernhardt-Roemermann et al., 2011; Heino et al., 2013). And many studies have used trait information from other regions without validation. For example, studies in China referenced traits from North America and Europe (Addo-Bediako, 2021; Shrestha et al., 2021), and studies in Nepal and South Africa referenced traits from North and South America (Addo-Bediako, 2021; Shrestha et al., 2021). However, the use of trait information from other regions may not be appropriate for evolutionary labile traits, i.e., traits that are not constrained by phylogeny and thus respond to environmental change (Poff et al., 2006). Examples include traits describing body size, feeding type, and voltinism (Bonada and Dolédec, 2018; Wilkes et al., 2020). Consequently, individuals of the same species may have different traits when they inhabit habitats with different environmental conditions. In contrast, for phylogenetically constrained traits like respiration or locomotion type, it is possible to use trait information from different regions (Poff et al., 2006; Bonada and Dolédec, 2018). However, the lability of macroinvertebrate traits has not been sufficiently studied (Bonada and Dolédec, 2018; Wilkes et al., 2020). This important work will not only enable us to use trait databases more accurately but also improve our understanding and prediction of trait-environment relationships.

Despite the rapid development of trait-based ecological research, the trait-based approach has its limitations. First, traits do not evolve independently in response to single environmental stress (de Bello et al., 2017) but are related because of mechanistic relationships or phylogenetic constraints (Verberk et al., 2013; Hamilton et al., 2020), and thus appear as specific combinations of traits (Poff et al., 2006). For example, multivoltine species necessarily have rapid development and small size (Resh et al., 1988; Hamilton et al., 2020). Consequently, a causal mechanism by which a trait appears to influence a species' persistence may be related to another correlated trait (Hamilton et al., 2020). And spurious causality may seriously confound trait-based applications (Poff et al., 2006; Pilière et al., 2016; Hamilton et al., 2020). Second, although a large number of functional diversity indices have been developed to explore the relationship between traits and the environment, the current use of functional diversity indices is confusing (Mammola et al., 2021). And the number of traits to be included in the initial trait matrix for functional diversity calculation to adequately reflect the relationship between organisms and their environment is still unresolved (Maasri, 2019). Some studies used approximately a dozen traits (Statzner et al., 2005; Wang et al., 2020), while others used more than sixty (Feld et al., 2014). This significantly hinders relevant comparative studies and meta-analyses.

A large number of macroinvertebrate trait databases have been developed worldwide, yet data usage remains challenging due to inconsistencies in trait classification. By comparing inconsistencies in trait classification in nine trait databases across six continents worldwide, we harmonized 41 common grouping features. Moreover, we try to propose a set of methods to help researchers determine traits using a unified procedure. These results link macroinvertebrate trait databases from different regions of the world, laying a solid foundation for the establishment of a globally unified trait database, and may contribute to the accuracy and comparability of trait studies in different regions in the future. However, the current macroinvertebrate trait studies are still far from adequate, especially in areas where macroinvertebrate trait studies are lagging with many macroinvertebrate taxa and their traits remain undetermined. Before reaching the goal of global harmonization, vigorous development of local macroinvertebrate trait studies in each region of the world is still needed. Therefore, this paper advocates the development of macroinvertebrate trait research in China, a region where macroinvertebrate traits are currently poorly studied and lack integration.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Our research does not involve any regulated animals, including all live vertebrates and higher invertebrates, but only benthic macroinvertebrate. And we believe that this type of research does not require an ethics statement and has not previously been required for this type of research (Luo et al., 2021).

AUTHOR CONTRIBUTIONS

SA and QC conceived the ideas and designed the methodology. SA, XL, ZT, and JH sampled the macroinvertebrate. SA, XL, and

ZT identified the macroinvertebrate. SA and QC analyzed the data and led wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

FUNDING

This study was funded by the Second Tibetan Plateau Scientific Expedition and Research Program (2019QZKK0402), the National Natural Science Foundation of China (U1602262), and the State Key Laboratory of FEBL Research Grant (2019FBZ01).

ACKNOWLEDGMENTS

We give special thanks to Wen Xiao and Shuoran Liu from the Institute of Eastern Himalaya Biodiversity Research, Dali University, for their generous help in the field sampling.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.945824/full#supplementary-material

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

frontiers | Frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 01 July 2022
doi: 10.3389/fevo.2022.913279

Similarities and Differences in Fish Community Composition Accessed by Electrofishing, Gill Netting, Seining, Trawling, and Water eDNA Metabarcoding in Temperate Reservoirs

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Edited by:
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Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *05 April 2022*
Accepted: *02 June 2022*
Published: *01 July 2022*

Citation:
Golpour A, Šmejkal M, Čech M, dos Santos RA, Souza AT, Jůza T, Martínez C, Bartoň D, Vašek M, Draštík V, Kolařík T, Kočvara L, Říha M, Peterka J and Blabolil P (2022) Similarities and Differences in Fish Community Composition Accessed by Electrofishing, Gill Netting, Seining, Trawling, and Water eDNA Metabarcoding in Temperate Reservoirs. Front. Ecol. Evol. 10:913279. doi: 10.3389/fevo.2022.913279

It is difficult to understand the composition and diversity of biological communities in complex and heterogeneous environments using traditional sampling methods. Recently, developments in environmental DNA metabarcoding have emerged as a powerful, non-invasive method for comprehensive community characterization and biodiversity monitoring in different types of aquatic ecosystems. In this study, water eDNA targeting fish (wf-eDNA) and four traditional fish sampling methods (electrofishing, gill netting, seining, trawling) were compared to evaluate the reliability and efficiency of wf-eDNA (vertebrate mitochondrial 12S ribosomal RNA (rRNA) as an alternative approach to assess the diversity and composition of freshwater fish communities. The results of wf-eDNA showed a consistency between the traditional sampling methods regarding species detection. However, some fish species detected using wf-eDNA assay were not detected using traditional sampling methods and vice versa. Comparison of wf-eDNA and traditional sampling methods revealed spatial homogeneity in fish community composition in all reservoirs. Ordination analysis showed that the wf-eDNA approach covers all traditional sampling methods and occupies an intermediate position. In addition, based on the Shannon diversity index, we found that in one reservoir the wf-eDNA method yielded similar fish community diversity to traditional sampling methods. However, in other reservoirs, the calculated Shannon diversity index of the wf-eDNA method was significantly higher than traditional sampling methods. In general, significant positive correlations were found between the wf-eDNA method and almost all traditional sampling methods. We conclude that wf-eDNA seems to be a reliable and complementary approach for biomonitoring and ecosystem management of freshwater ichthyofauna.

Keywords: biomonitoring, environmental DNA, reservoir, sampling method, species detection

INTRODUCTION

Assessing fish community composition is central to provide information on species structure and characteristics of whole aquatic ecosystems (Facey and Grossman, 1990; McElroy et al., 2020). Despite the implementation of the EU Water Framework Directive (2000/60/EC), the ecological status of natural water bodies requires a broader collection of information on the composition of the fish community. Reservoirs are considered widespread examples of heterogeneous aquatic ecosystems that are intermediate between rivers and lakes (Banerjee et al., 2017). Therefore, monitoring ecological indicators such as fish composition in reservoirs based on traditional sampling methods provides fragmented information and is challenging because they represent a transitional environmental system between lakes and rivers (Irz et al., 2002; Straškraba, 2005). Reliable assessment of fish species diversity in these systems is difficult to achieve using traditional sampling methods. Different fish species utilize different depths, flows, and water temperature habitats due to which, multi-gear sampling approaches require high demand for know-how and professional labor (Eggleton et al., 2010; Mueller et al., 2017). Information on fish abundance and composition has traditionally been obtained using electrofishing, fish traps, gill nets, and seine nets (Fischer and Quist, 2014; Merz et al., 2021). However, the limitations in commonly used sampling methods have hampered standardization and comparison of monitoring in a range of freshwater habitats (Revenga et al., 2005; Jurajda et al., 2009). Furthermore, the implementation of traditional sampling methods in the field is relatively laborious, expensive, and destructive (Iknayan et al., 2014; Thomsen and Willerslev, 2015; Blabolil et al., 2021a), resulting in low precision in fish community monitoring (Kubečka et al., 2009). Therefore, to standardize an accurate and reliable monitoring method for fish communities in aquatic ecosystems we need to implement a universal and affordable non-invasive tool that characterizes the richness, relative abundance, and presence of taxa throughout a water body.

Recently, environmental DNA metabarcoding (eDNA) has been considered as a promising non-invasive tool for monitoring the spatial and temporal distribution of species and communities in aquatic ecosystems (Deiner et al., 2017; Bylemans et al., 2018; Lawson Handley et al., 2019; Crane et al., 2021). The eDNA technique is based on the molecular identification of organisms whose genetic material has entered the water (Olds et al., 2016), soil (Epp et al., 2012), and sediment (Evrard et al., 2019). Water eDNA targeting fish (hereafter), wf-eDNA based on the two-level terminology proposed by Pawlowski et al. (2020) and Rodriguez-Ezpeleta et al. (2021) has recently been suggested as a complementary biomonitoring strategy for the European Union Water Framework Directive (2000/60/EC) to detect elusive and rare species (Seymour et al., 2020). It is particularly useful for detecting cryptic species and identifying new taxa (Grey et al., 2018; McElroy et al., 2020; Seymour et al., 2020). This approach has also facilitated remote sampling in diverse water bodies, such as rivers, ponds, reservoirs, and marine environments (Yamanaka and Minamoto, 2016; Harper et al., 2019; Djurhuus et al., 2020; Hayami et al., 2020). However, sampling strategies, laboratory protocols, and analytical pipelines can affect wf-eDNA performance (Bylemans et al., 2018). The main advantage of traditional sampling methods is that biometric data (e.g., length, weight, sex, maturity stage, etc.) can be obtained during sampling, whereas the wf-eDNA approach can only identify samples at genus or species level. Most comparisons of the wf-eDNA approach with traditional sampling methods to characterize ecological communities have been conducted at the local scale and show how wf-eDNA and traditional sampling methods represent community composition in lakes, reservoirs, and rivers (Murienne et al., 2019; Czeglédi et al., 2021; Gehri et al., 2021). Several studies have also shown that wf-eDNA is a complementary approach to traditional sampling methods to assess fish community composition in lakes and rivers (Shaw et al., 2016; Doi et al., 2019; Sard et al., 2019; Antognazza et al., 2021; Ritterbusch et al., 2022). In general, wf-eDNA may characterize a large number of taxa with less sampling effort than traditional sampling methods for detecting fish in low-diversity freshwater systems (McElroy et al., 2020). In this context, Hänfling et al. (2016) showed that water samples collected for wf-eDNA along gillnet sampling sites detected 14 of the 16 fish species previously detected in a lake in the United Kingdom. Several studies have also shown that wf-eDNA can detect more taxa than traditional capture methods (Civade et al., 2016; Yamamoto et al., 2017; Sard et al., 2019; Zou et al., 2020).

In the present study, the wf-eDNA approach was used to assess fish communities in three reservoirs in the Czech Republic for two consecutive years. This study was conducted to compare the ability of wf-eDNA with data from traditional survey methods (day and night electrofishing, multiple mesh gill nets, day and night seine nets, and night trawling) to identify differences and/or similarities regarding fish community composition. Specifically, we aimed to (1) characterize and estimates of fish community composition between traditional and wf-eDNA sampling methods in each reservoir (2) compare and assess fish species diversity and richness across sampling methods in each reservoir, and (3) examine the relationships between wf-eDNA and site occupancy (i.e., number of positive samples divided by total number of samples) and scores derived from all traditional sampling methods in each reservoir.

MATERIALS AND METHODS

Site Description

This study was conducted in three reservoirs (Římov, Klíčava and Žlutice) in the Czech Republic, which were constructed as drinking water reservoirs and are not accessible to the public. All reservoirs possess canyon-shape morphology with one main inflow (**Supplementary Figure 1**), resulting in longitudinal gradients of nutrients and water quality from the inflow to the dam. All studied reservoirs serve as drinking water storages and are therefore restricted to public access. The reservoirs have different trophic conditions but similar canyon-shaped morphologies with the main inflow and a side bay (see **Supplementary Table 1**). Římov and Žlutice reservoirs are eutrophic reservoirs (average year total phosphorus concentration, TP: 22 and 24 μg/L), while Klíčava possesses mesotrophic condition (TP: 17 μg/L). The largest one is the Římov (surface area 2.1 km^2), middle size Žlutice (1.1 km^2) and the smallest Klíčava (0.54 km^2). The maximum depth in Klíčava, Římov, and Žlutice was 31, 41, and 22 m, respectively.

Traditional Sampling

Traditional fish sampling was conducted during summer (August) 2018 and 2019 using gill nets, day and night electrofishing, seine nets, and trawls. Benthic and pelagic gillnets with 12 mesh sizes following the protocol of CEN (2015) [hereafter standard benthic (SBG) and pelagic gillnets (SPG)] and additional gillnets with four mesh sizes (70, 90, 110, and 135 mm) to capture larger fish (Šmejkal et al., 2015); [hereafter benthic (LBG) and pelagic (LPG) large-mesh gillnets] were distributed across the sampling area of each reservoir to sample benthic and pelagic habitats, respectively (**Supplementary Table 17**; Blabolil et al., 2021b). All gillnets were deployed 2 h before sunset and lifted 2 h after sunrise to capture the maximum peaks of fish activity. The total length of the standard gillnets was 30 m (12 m × 2.5 m panels) and that of large-mesh gillnets was 40 m (4 m × 10 m panels). Electrofishing from a boat was limited to nearshore areas (i.e., less than 2 m deep) to capture species during the day (DE) and night (NE) (Miranda and Kratochvíl, 2008). Electrofishing (electrofisher EL 65 II GL DC, Hans Grassel, Schönau am Königsee, Germany, 13 kW, 300/600 V) was conducted in transects at a distance of 100 m (**Supplementary Table 18**). Seining was conducted at night (NAS) to capture adult fishes using a 50 m long and 4 m high net with a mesh size of 10 mm (Říha et al., 2008), and also during the day (DFS) and at night (NFS) for juveniles using a 10 m long and 2 m high net with a mesh size of 1.7 mm (**Supplementary Table 19**; Blabolil et al., 2016). In addition, a fixed-frame pelagic trawl (FT) with a mouth opening of 3 m × 3 m, length of 5.4 m, and a mesh size of 6 mm at the belly and 3 mm at the cod end was used for fry night trawling. Trawling was conducted throughout the reservoir at depths of 0–3 m and based on the maximum depth in the depth strata of 3–6 and 6–9 m (**Supplementary Table 20**).

Catches from each net or transect were processed separately. All fish caught were identified to the species level, measured (standard length in mm) and weighted (in g). To compare the different sampling methods and obtain comparable data sets, catch data were standardized to an area of 1,000 m^2 for gillnets, 100 m^2 for seine, the trajectory of 100 m for electrofishing, and a volume of 1,000 m^3 for trawling. Data were quantified using two quantitative methods: catch estimation rate, biomass per unit effort (BPUE), and catch per unit effort (CPUE) for species caught. All calculations and statistical analyses were performed using the calculated BPUE and CPUE datasets.

wf-eDNA Sampling, Extraction, and Library Preparation

Water samples were collected for two consecutive years (2018 and 2019) during the same weeks as traditional fish sampling following Blabolil et al. (2021a). The total number of samples collected during the summer in each reservoir were 38, 29, and 28 in Římov, Klíčava, and Žlutice respectively (**Supplementary Table 21**). Eight localities across the longitudinal gradient from the dam toward the tributary were sampled in Římov and five sites in Klíčava and Žlutice (**Supplementary Figure 1**). At each locality, water samples were collected from different depth layers (surface, 5, 10, and 20 m) to capture different ecological gradients (Prchalová et al., 2008). A Friedinger sampler was used for water collection. In the field, water samples (2 L) were pre-filtered to prevent clogging by excessive seston, stored in sterile, labeled bottles, and kept in an icebox until laboratory processing. Within 24 h of sampling, 1 L of each sample was filtered individually through open filters in the laboratory. The Mu-DNA water protocol (Sellers et al., 2018) was used to extract the DNA. Primers (forward 12S-V5-F, 5′-ACTGGGATTAGATACCCC-3′ and reverse 12S-V5-R, TAGAACAGGCTCCTCTAG) were used to amplify a 73–110 bp fragment of the vertebrate mitochondrial 12S ribosomal RNA (rRNA) gene (Riaz et al., 2011). Field blanks (purified water passed through the sampler and sterile plankton net), negative PCR controls (molecular grade water) and positive PCR controls (tissue of *Maylandia zebra* DNA 0.05 ng μl^{-1}) were included in each set of the individual sampling campaign to detect possible contamination and inhibition. All PCRs were performed with individually attached caps in a UV and bleach sterilized laminar flow hood in the eDNA laboratory to minimize the risk of contamination. The sequencing library was generated from the PCR amplicons and run on an Illumina MiSeq sequencer.

Raw sequences were demultiplexed and processed using the metaBEAT v0.97.11 pipeline[1]. Average read quality was assessed in 5-bp sliding windows starting at 3′ of the read, and reads were clipped until the average quality per window was above Phred score of 30. Reads shorter than 90 bp were discarded. The filtered sequences were clustered with 100% identity. Clusters represented by less than three sequences were excluded from further analysis. Non-redundant sequences were compared with a reference database using BLAST (Zhang et al., 2002). The reference database was developed at the University of Hull, England[2] and supplemented with *Leuciscus aspius* (GenBank accession numbers: MT163435, MT163450, MT163449) and *Coregonus maraena* (GenBank accession numbers: MT163451, MT163458, MT163460) to represent all fish species in the study catchment. The BLAST output was interpreted using a custom Python function that implements the lowest common ancestor approach for taxonomic assignment, and the hits were only considered if they had a minimum identity of 99% and query coverage of 90%. Taxa with a read frequency below 0.1% were excluded as possible false-positive detections. Compared to Blabolil et al. (2021a), the assigned species pairs presented identical sequences in the amplified regions of *Leuciscus aspius* + *Scardinius erythrophthalmus*, *Sander lucioperca* + *Perca fluviatilis*, and *L. leuciscus* + *L. idus* were retained in the downstream analyses. On the other hand, detection of lampreys

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[1] https://github.com/HullUni-bioinformatics/metaBEAT

[2] https://github.com/HullUni-bioinformatics/Curated_reference_databases/tree/master/12S_Fish

(*Lamperta* spp.) in wf-eDNA was excluded as the taxa are not the target of any of the traditional methods used. To follow the wf-eDNA data consistency, the six species determined by the traditional methods were merged into two species pairs. Similarly, uncertain species (species with identical sequences in amplified regions creating natural and/or artificial hybrids) were assigned to genus level *Coregonus*, *Hypophthalmichthys*, and *Acipenser*.

Data Analysis

Prior to statistical analysis and comparison of all traditional sampling methods with eDNA, Hellinger's transformation was employed on datasets (Laporte et al., 2021). Beta diversity (non-metric multidimensional scaling) was calculated using the vegdist function from the vegan package with Jaccard and Bray-Curtis dissimilarity matrices (Oksanen et al., 2019). Permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis function to test whether species composition showed statistically significant differences between the methods. Alpha diversity, including Shannon ($H = -\Sigma[(pi) \times \ln(pi)]$) and richness indices, was calculated using the diversity function from the vegan package (Oksanen et al., 2019). Analyses of variance and *post hoc* Tukey honest significant difference tests were performed to test whether the difference in alpha diversity between groups was statistically significant and to compare the diversity of each group. The aov, ANOVA, and TukeyHSD functions from the package stats were used to perform the analysis of variance and Tukey's tests (R Core Team, 2020). We calculated sample-based site occupancy estimates for each taxon (calculated detection rates and relative rank abundance of species detected in both eDNA and traditional approaches). Spearman's rank correlation coefficient was used to test the relationship between eDNA-based site occupancy and the taxon score derived from traditional sampling methods (R Core Team, 2020). All statistical analyses were performed using R software version v4.0.3 (R Core Team, 2020).

RESULTS

The 12S libraries generated 22.06 million raw sequence reads, with 17.63 million reads passing bioinformatic filters. During the quality filtering and removal of chimeric sequences 9.9 million reads were discarded.

Comparisons of Traditional Capture and wf-eDNA Data

The number of taxa captured/detected by traditional sampling methods and their assigned wf-eDNA sequences in each reservoir is listed in **Table 1**. Of the traditional sampling methods, only DE recorded more taxa than the other methods in all the reservoirs. More taxa were detected with wf-eDNA than with traditional methods in all the reservoirs (**Table 1**). A total of 31 fish taxa were identified in all reservoir samples. The Římov reservoir had the highest number of taxa (28), while the Klíčava reservoir had the lowest number (13 each) (**Supplementary Tables 2–4**). In the Klíčava reservoir, the wf-eDNA method detected all listed species, except for *Hypophthalmichthys* sp. (**Supplementary Table 2**). Two taxa (*Carassius gibelio* and *Gobio gobio*) were only identified using the wf-eDNA method and were not captured using traditional sampling methods (**Supplementary Table 2**). Cyprinids were the dominant taxa, with nine species (**Supplementary Table 2**).

In the Římov reservoir, almost all taxa were identified by the wf-eDNA method, except for three taxa that were captured only by traditional sampling methods (*Coregonus* sp., *Pseudorasbora parva*, and *Squalius cephalus*) (**Supplementary Table 3**). Indeed, the number of taxa detected or captured between the wf-eDNA and traditional sampling methods did not show significant differences (**Supplementary Table 3**). The dominant taxa were cyprinids, with 14 species (**Supplementary Table 3**).

In the Žlutice reservoir, a total of 20 taxa were detected by combining all traditional sampling methods and the wf-eDNA assay (**Supplementary Table 4**). Of the taxa identified by the traditional sampling methods, 12 species belonged to the cyprinid family as the dominant taxa (**Supplementary Table 4**). All taxa listed in **Supplementary Table 4** were identified using the wf-eDNA assay, except for four fish species (*Anguilla anguilla*, *Carassius carassius*, *Hypophthalmichthys* sp., and *Lota lota*).

Comparing Fish Communities Between Traditional Capture and wf-eDNA Data

In the Klíčava reservoir, NMDS analysis of BPUE datasets showed that fish communities varied slightly between gillnet methods and wf-eDNA, although there were similarities in fish composition between wf-eDNA and the other traditional methods (**Figures 1A,B**). PERMANOVA analysis (**Supplementary Tables 5**, **6**) revealed significant similarity between all methods, but statistically no significant similarity was found between some methods, such as DE and NE ($F = 5.60$, $R^2 = 0.02$, $p = 0.640$), LBG and FT ($F = -2.07$, $R^2 = 1.00$, $p = 1.0$), SPG and FT ($F = 2.69$, $R^2 = 0.02$, $p = 0.681$), SBG and wf-eDNA ($F = 2.42$, $R^2 = 0.05$, $p = 0.065$), FT and wf-eDNA ($F = 7.72$, $R^2 = 0.02$, $p = 0.597$), and DE and FT ($F = 4.44$, $R^2 = 0.28$, $p = 0.069$). There were slight differences in community composition between wf-eDNA, LPG and LBG in the CPUE datasets, however, there were similarities between wf-eDNA and all traditional sampling methods (**Figures 2A,B**). Furthermore, PERMANOVA analysis

TABLE 1 | Number of taxa (species) caught or detected in sampled reservoirs using different sampling methods in 2018 (before slash) and 2019 (after slash).

Reservoir	DE	NE	LBG	SBG	LPG	SPG	NAS	DSF	NFS	FT	wf-eDNA
Římov	15/15	10/10	2/2	11/11	3/3	8/9	11/11	10/9	11/10	5/5	20/19
Klíčava	7/7	10/9	1/4	8/8	3/2	4/5	-	-/5	-/8	2/4	12/14
Žlutice	12/8	11/9	7/6	10/10	3/2	8/8	-	-/7	-/8	8/2	12/15

Values indicate the number of species captured or detected.
Žlutice sampling.
DSF and NFS were not applied in 2018 sampling.
DE = day electrofishing, NE = night electrofishing, SBG = standard benthic gillnets, SPG = standard pelagic gillnets, LBG = large mesh-size benthic gillnets, LPG = Large mesh-size pelagic gillnet, NAS = Night-time adult seining, DFS = Day-time fry seining, NFS= Night-time fry seining, FT = Fry Trawl and wf-eDNA = eDNA from water targeting fish.

revealed no significant similarity between DE and NE ($F = 1.87$, $R^2 = 0.07$, $p = 0.098$), DE and FT ($F = 3.64$, $R^2 = 0.24$, $p = 0.161$), FT and SPG ($F = 2.03$, $R^2 = 0.01$, $p = 0.801$), FT and wf-eDNA ($F = 6.45$, $R^2 = 0.02$, $p = 0.686$), FT and SBG ($F = 1.30$, $R^2 = 0.06$, $p = 0.305$), LBG, and FT ($F = -2.07$, $R^2 = 1.00$, $p = 1.0$) in CPUE 2018 (**Supplementary Tables 7**, **8**). However, in CPUE 2019, no statistically significant similarity (**Supplementary Table 8**) was observed between DE and NE ($F = 1.70$, $R^2 = 0.07$, $p = 0.223$) and DFS and NFS ($F = 1.52$, $R^2 = 0.05$, $p = 0.146$).

In Římov reservoir, NMDS in the BPUE dataset showed similarities in fish community between wf-eDNA and almost all traditional methods, however, community composition revealed slight differed between methods such as LBG, DE, SBG, and wf-eDNA (**Figures 1C,D**). The PERMANOVA test showed statistically significant similarity between all sampling methods in the 2018 sampling year, except between DE and SBG ($F = 0.77$, $R^2 = 0.01$, $p = 0.552$). In 2019, BPUE datasets showed that fish communities varied slightly between gillnet LBG, SPG, DFS and wf-eDNA. PERMANOVA analysis revealed statistically significant similarity between all sampling methods, but no statistically significant similarity was found between LPG and LBG ($F = 0.40$, $R^2 = 0.01$, $p = 0.617$) (**Supplementary Tables 9**, **10**). For the CPUE datasets in 2018, NMDS ordinations of fish communities were partially differed between the wf-eDNA and LBG, SPG, and DE methods (**Figure 2C**). Only PERMANOVA analysis revealed no statistically significant similarity between LPG and LBG ($F = 0.50$, $R^2 = 0.02$, $p = 0.784$) (**Supplementary Table 11**). In the 2019 CPUE datasets, NMDS ordinations of fish communities differed in part between the wf-eDNA and LBG, SPG, and DE methods (**Figure 2D**). In addition, PERMANOVA test showed no statistically significant similarity between wf-eDNA and NAS ($F = 2.31$ $R^2 = 0.06$, $p = 0.083$), wf-eDNA and FT ($F = 1.53$, $R^2 = 0.03$, $p = 0.162$), DE and FT ($F = 1.53$, $R^2 = 0.06$, $p = 0.172$), SBG and FT ($F = 2.01$, $R^2 = 0.038$, $p = 0.101$), SPG and FT ($F = 2.20$, $R^2 = 0.08$, $p = 0.107$), DFS and FT ($F = 2.44$, $R^2 = 0.08$, $p = 0.083$), and DFS and NFS ($F = 2.43$, $R^2 = 0.06$, $p = 0.059$) (**Supplementary Table 12**).

In the Žlutice Reservoir, community composition analysis of BPUE records with NMDS showed slight difference between wf-eDNA, LBG and LPG (**Figures 1E,F**). The PERMANOVA analysis showed significant similarities among most methods, however, no statistically significant similarity was found between DE and NE ($F = 2.16$, $R^2 = 0.08$, $p = 0.080$), NE and SPG ($F = 2.35$, $R^2 = 0.08$, $p = 0.073$), and SPG and wf-eDNA ($F = 2.03$, $R^2 = 0.05$, $p = 0.097$) in the 2018 (**Supplementary Table 13**). No statistically significant similarity was also observed between DE and NE ($F = 1.36$, $R^2 = 0.05$, $p = 0.245$), DE and SPG ($F = 1.77$, $R^2 = 0.06$, $p = 0.171$), DE and DFS ($F = 0.73$, $R^2 = 0.02$, $p = 0.534$), SBG and SPG ($F = 1.77$, $R^2 = 0.06$, $p = 0.632$), DFS and SPG ($F = 1.92$, $R^2 = 0.05$, $p = 0.139$) (**Supplementary Table 14**). For the CPUE datasets, NMDS ordinations of fish communities were partially differed between the wf-eDNA, LBG and SPG methods (**Figures 2E,F**). Moreover, PERMANOVA analysis revealed no statistically significant similarity between wf-eDNA and DE ($F = 2.22$, $R^2 = 0.05$, $p = 0.079$), wf-eDNA and NE ($F = 1.69$, $R^2 = 0.60$, $p = 0.086$), DE and NE ($F = 1.36$, $R^2 = 0.05$, $p = 0.264$), NE and SPG ($F = 2.17$, $R^2 = 0.07$, $p = 0.079$), NE and FT ($F = 2.01$, $R^2 = 0.07$, $p = 0.131$), and LPG and LBG ($F = 1.35$, $R^2 = 0.07$, $p = 0.276$) in the 2018 CPUE dataset (**Supplementary Table 15**). In contrast, in the 2019 CPUE dataset, no statistically significant similarity was observed between DE and NE ($F = 0.21$, $R^2 = 0.01$, $p = 0.919$), wf-eDNA and NE ($F = 2.26$, $R^2 = 0.05$, $p = 0.053$), DE and DFS ($F = 2.14$, $R^2 = 0.07$, $p = 0.096$), DE and SPG ($F = 0.95$, $R^2 = 0.03$, $p = 0.378$), NE and SBG ($F = 1.91$, $R^2 = 0.05$, $p = 0.112$), NE and SPG ($F = 1.61$, $R^2 = 0.05$, $p = 0.187$), NE and DFS ($F = 1.73$, $R^2 = 0.06$, $p = 0.139$), and DFS and NFS ($F = 0.83$, $R^2 = 0.02$, $p = 0.481$) (**Supplementary Table 16**).

Fish Species Diversity Between Traditional Sampling and wf-eDNA Methods

Fish species diversity in the Klíčava reservoir showed a statistically significant difference in the Shannon diversity index. The value was significantly higher ($p < 0.05$) for NE than for other traditional sampling methods and wf-eDNA in the BPUE dataset of 2018 (**Figure 3A**). However, in the sampling year 2019, the value of wf-eDNA was significantly higher ($p < 0.05$) than that of other traditional sampling methods (**Figure 3B**). In addition, the Shannon index in NE was significantly higher ($p < 0.05$) than that of the other methods in the 2018 CPUE dataset, while wf-eDNA had a significantly higher ($p < 0.05$) than the traditional sampling methods in 2019 (**Figures 4A,B**). Based on the BPUE datasets of Římov reservoir in 2018 and 2019, the Shannon diversity index was significantly higher in NAS ($p < 0.05$) (**Figures 3C,D**). The highest value of the index in the NAS followed by wf-eDNA and NFS sampling approaches. A similar trend was also observed in the CPUE dataset of the 2018 sampling year, as it was significant in NAS sampling ($p < 0.05$) (**Figure 4C**), while in the 2019 sampling year the values were significantly higher in wf-eDNA, NE and DE ($p < 0.05$) (**Figure 4D**). In the Žlutice reservoir, the Shannon index for NE was significantly higher ($p < 0.05$) than that of other traditional methods and wf-eDNA in the BPUE dataset of 2018, while the value for wf-eDNA in 2019 was significantly higher ($p < 0.05$) than that of the traditional sampling methods (**Figures 3E,F**). In addition, the Shannon index in wf-eDNA in the CPUE dataset was statistically higher ($p < 0.05$) than that of the traditional methods in both sampling years (**Figures 4E,F**).

In the Klíčava reservoir, there was also a significant difference in richness value ($p < 0.05$) in NE compared to other traditional sampling methods and wf-eDNA in the 2018 AND 2019 BPUE dataset, (**Figures 5A,B**). A similar trend was observed in the CPUE datasets (**Figures 6A,B**). There was also a significant difference in richness value ($p < 0.05$) in NAS method compared to other traditional methods and wf-eDNA in BPUE dataset (**Figures 5C,D**) in the Římov reservoir. Based on the CPUE dataset, there was a significant difference in richness between the traditional methods and wf-eDNA. The values were significantly different ($p < 0.05$) in DFS compared to other traditional sampling methods and wf-eDNA (**Figures 6C,D**). In addition, the value of species richness in the Žlutice reservoir in DE was significantly higher ($p < 0.05$) than traditional methods and wf-eDNA in the 2018 BPUE dataset (**Figures 5E,F**), while wf-eDNA

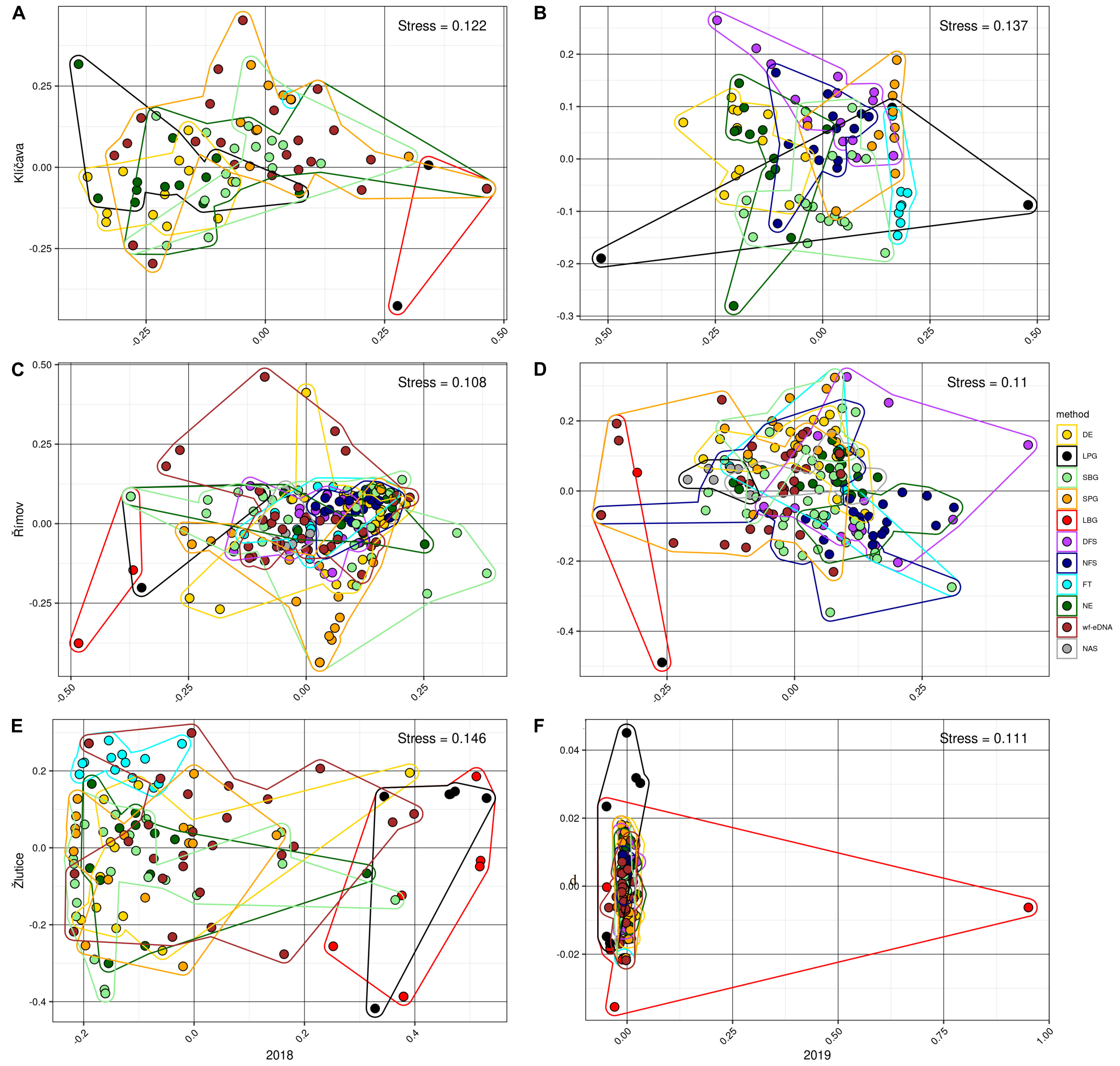

FIGURE 1 | Beta- diversity visualized using non-metric multidimensional scaling (NMDS) based on BPUE data in 2018 (left plots) and 2019 (right plots) with Bray-Curtis dissimilarity distances of community compositions for the wf-eDNA assay and traditional sampling methods in Klíčava, Římov and Žlutice reservoirs. NMDS plots on rank order using Bray-Curtis distances were used to evaluate relationships based on intersample variation in estimated fish community composition between sampling methods. Plot ellipses represent the 95% confidence regions for the group clusters. Traditional sampling methods are abbreviated as follows: DE = day electrofishing, NE = night electrofishing, SBG = standard benthic gillnets, SPG = standard pelagic gillnets, LBG = large mesh-size benthic gillnets, LPG = large mesh-size pelagic gillnets, NAS = night-time adult seining, DFS = day-time fry seining, NFS= night-time fry seining, FT = fry trawl and wf-eDNA = eDNA from water targeting fish.

had significantly higher values than traditional methods in 2019. A similar pattern of species richness was also found in the CPUE dataset (**Figures 6E,F**).

Correlations Between Methods

In the Klíčava reservoir, Spearman's rank correlation coefficient between wf-eDNA and all traditional sampling methods was highly significant in the 2018 BPUE dataset, except that the correlation between wf-eDNA and LBG was not found. Similarly, a moderately significant Spearman's rank correlation coefficient was found between wf-eDNA site occupancy and all traditional sampling methods in the 2019 BPUE dataset, but the correlation between wf-eDNA and LPG was not observed (**Supplementary Figures 1A,B**). In addition, similar to the 2018 BPUE dataset, a moderately significant Spearman's rank correlation coefficient was observed between wf-eDNA and all traditional sampling methods in the 2018 CPUE dataset. However, Spearman's rank correlation coefficient was highly significant between wf-eDNA and all traditional sampling methods in the 2018 BPUE dataset, except that the correlation between wf-eDNA, DE, and LPG was not observed in the 2019 CPUE dataset (**Supplementary Figures 2A,B**). Similar to

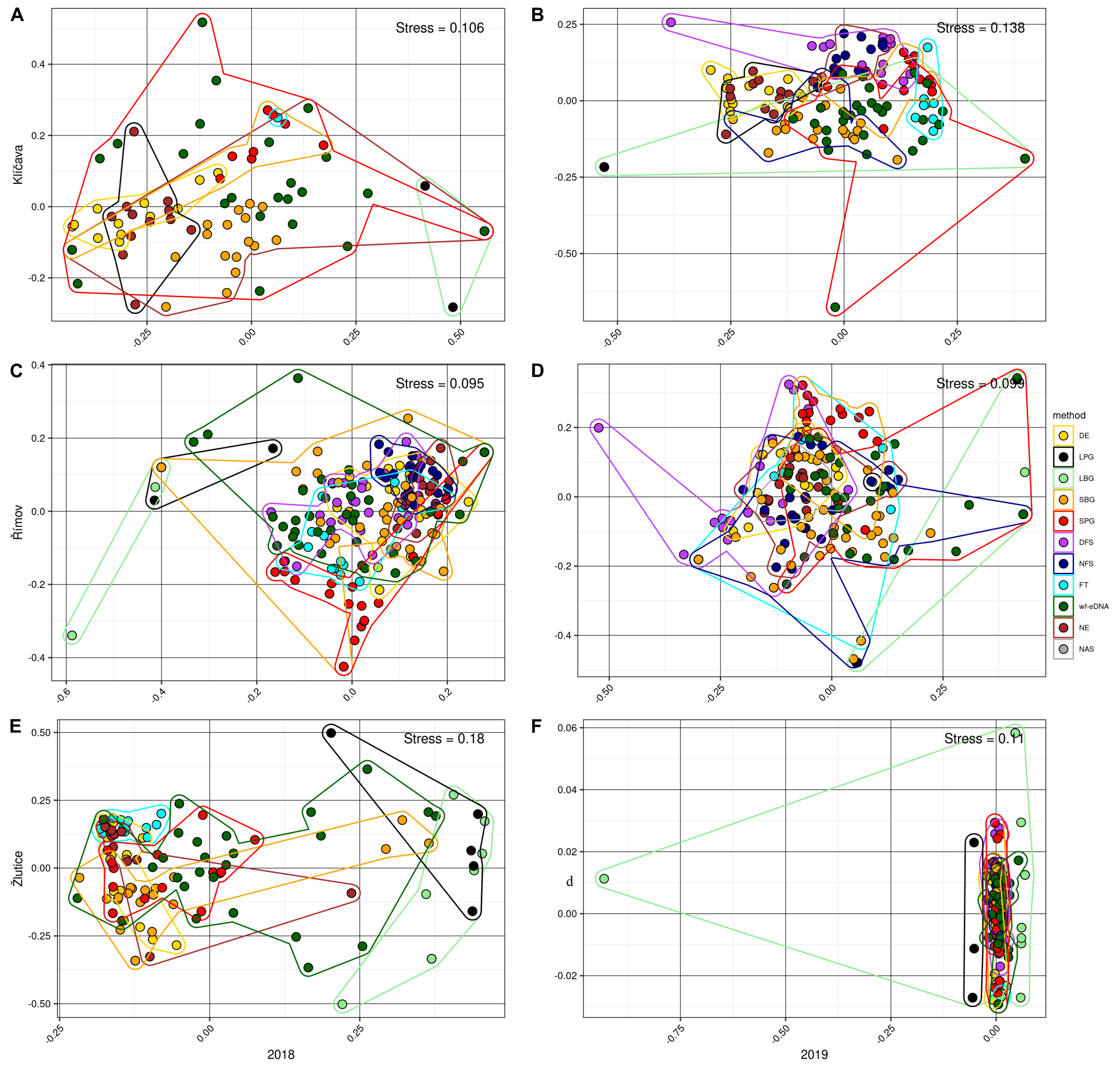

FIGURE 2 | Beta-diversity visualized using non-metric multidimensional scaling (NMDS) based on CPUE data in 2018 (left plots) and 2019 (right plots) with Bray-Curtis dissimilarity distances community compositions for wf-eDNA assay and all traditional sampling methods in Klíčava, Římov and Žlutice reservoirs. NMDS plots on rank order using Bray-Curtis distances were used to assess relationship based on intersample variation in estimated fish community composition between sampling methods. Plot ellipses represent the 95% confidence regions for group clusters. Traditional sampling methods are abbreviated as follow: DE = day electrofishing, NE = night electrofishing, SBG = standard benthic gillnets, SPG = standard pelagic gillnets, LBG = large mesh-size benthic gillnets, LPG = large mesh-size pelagic gillnets, NAS = night-time adult seining, DFS = day-time fry seining, NFS= night-time fry seining, FT = fry trawl and wf-eDNA = eDNA from water targeting fish.

the Klíčava reservoir, the 2018 Římov BPUE dataset showed a highly significant Spearman's rank correlation coefficient between wf-eDNA and almost all traditional sampling methods; however, there was no significant Spearman's rank correlation coefficient between wf-eDNA, NE, and LBG (**Supplementary Figures 3A,B**). A similar relationship was also observed in the CPUE dataset (**Supplementary Figures 4A,B**). In addition, Spearman's rank correlation coefficients between wf-eDNA and all traditional sampling methods were highly significant in the BPUE dataset in Žlutice reservoir in 2018 and 2019 (**Supplementary Figures 5A,B**), although no correlation was found between wf-eDNA, LBG, LPG, and DE. A similar trend in Spearman's rank correlation coefficient was also observed between wf-eDNA and all traditional sampling methods in the CPUE dataset in 2018 and 2019 (**Supplementary Figures 6A,B**).

DISCUSSION

This study is the first to compare multiple traditional sampling methods and wf-eDNA in three independent lentic ecosystems. To date, few studies have used multiple sampling methods (different types of nets and electrofishing) and wf-eDNA to investigate the composition and diversity of biological

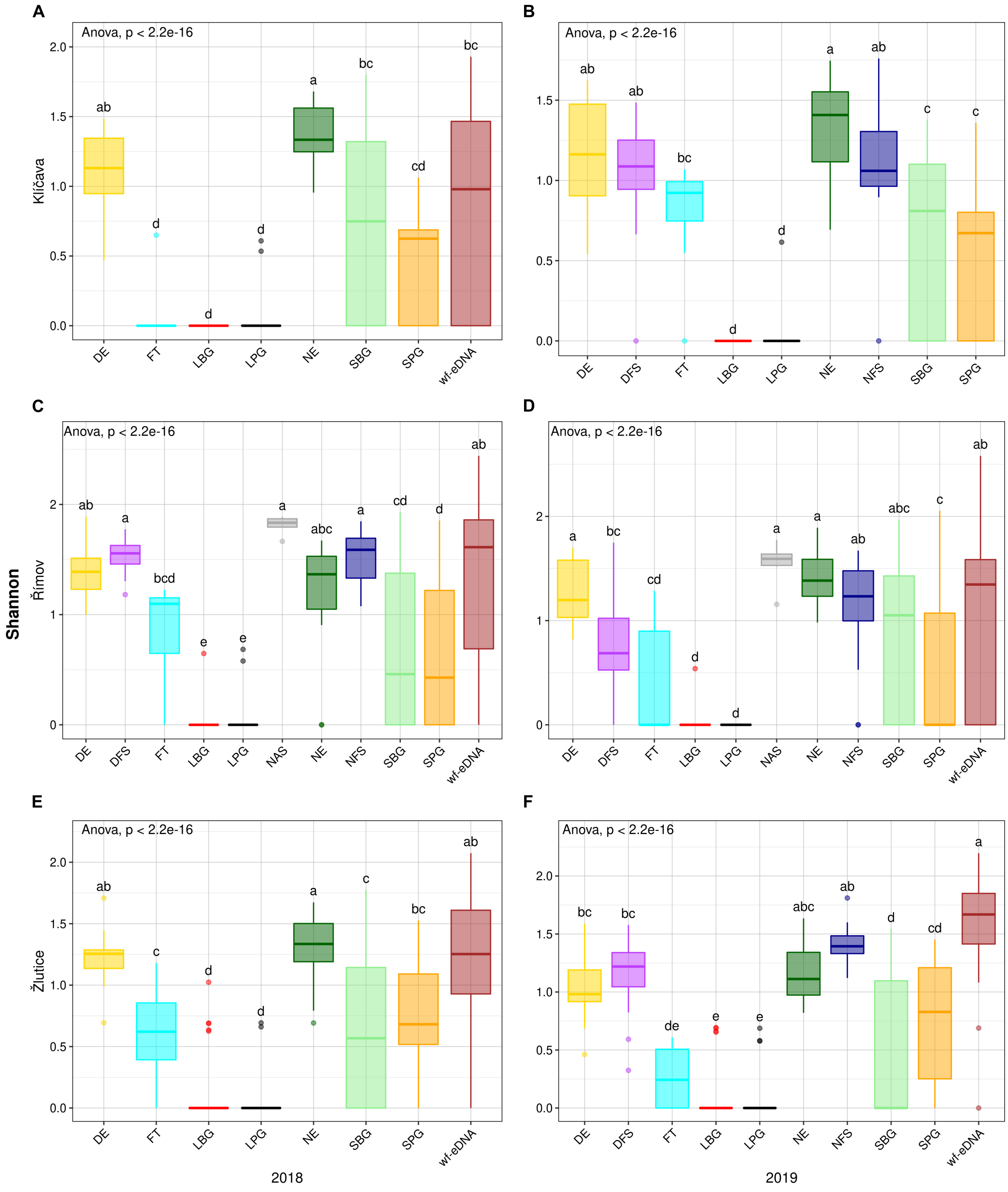

FIGURE 3 | Box plots comparison of alpha diversity (Shannon) based on BPUE data in 2018 (left plots) and 2019 (right plots) between wf-eDNA assay and traditional sampling methods in Klíčava, Římov and Žlutice reservoirs. Significant differences are indicated by different upper superscripts. Horizontal line inside the box indicates the calculated median, box 1st and 3rd quartile, whiskers data range and dots outliers of Shannon value. Traditional sampling methods are abbreviated aS follow: DE = day electrofishing, NE = night electrofishing, SBG = standard benthic gillnets, SPG = standard pelagic gillnets, LBG = large mesh-size benthic gillnets, LPG = large mesh-size pelagic gillnets, NAS = night-time adult seining, DFS = day-time fry seining, NFS= night-time fry seining, FT = fry trawl and wf-eDNA = eDNA from water targeting fish.

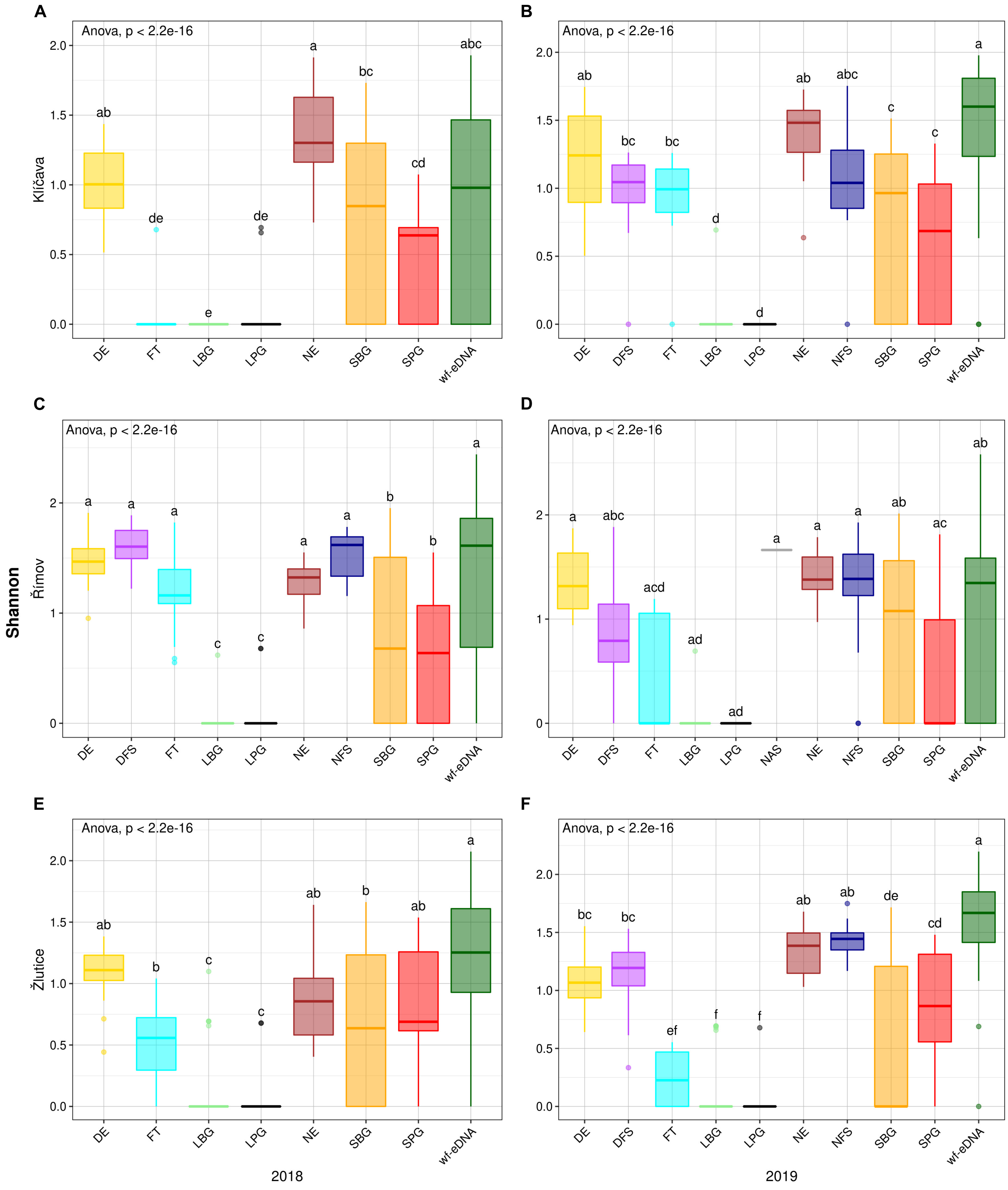

FIGURE 4 | Box plots comparison of alpha diversity (Shannon) based on CPUE data in 2018 (left plots) and 2019 (right plots) between wf-eDNA assay and traditional sampling methods in Klíčava, Římov and Žlutice reservoirs. Significant differences are indicated by different superscripts. Horizontal line inside the box indicates the calculated median, box 1st and 3rd quartile, whiskers data range and dots outliers of Shannon value. Traditional sampling methods are abbreviated as follow: DE = day electrofishing, NE = night electrofishing, SBG = standard benthic gillnets, SPG = standard pelagic gillnets, LBG = large mesh-size benthic gillnets, LPG = large mesh-size pelagic gillnets, NAS = night-time adult seining, DFS = day-time fry seining, NFS= night-time fry seining, FT = fry trawl and wf-eDNA = eDNA from water targeting fish.

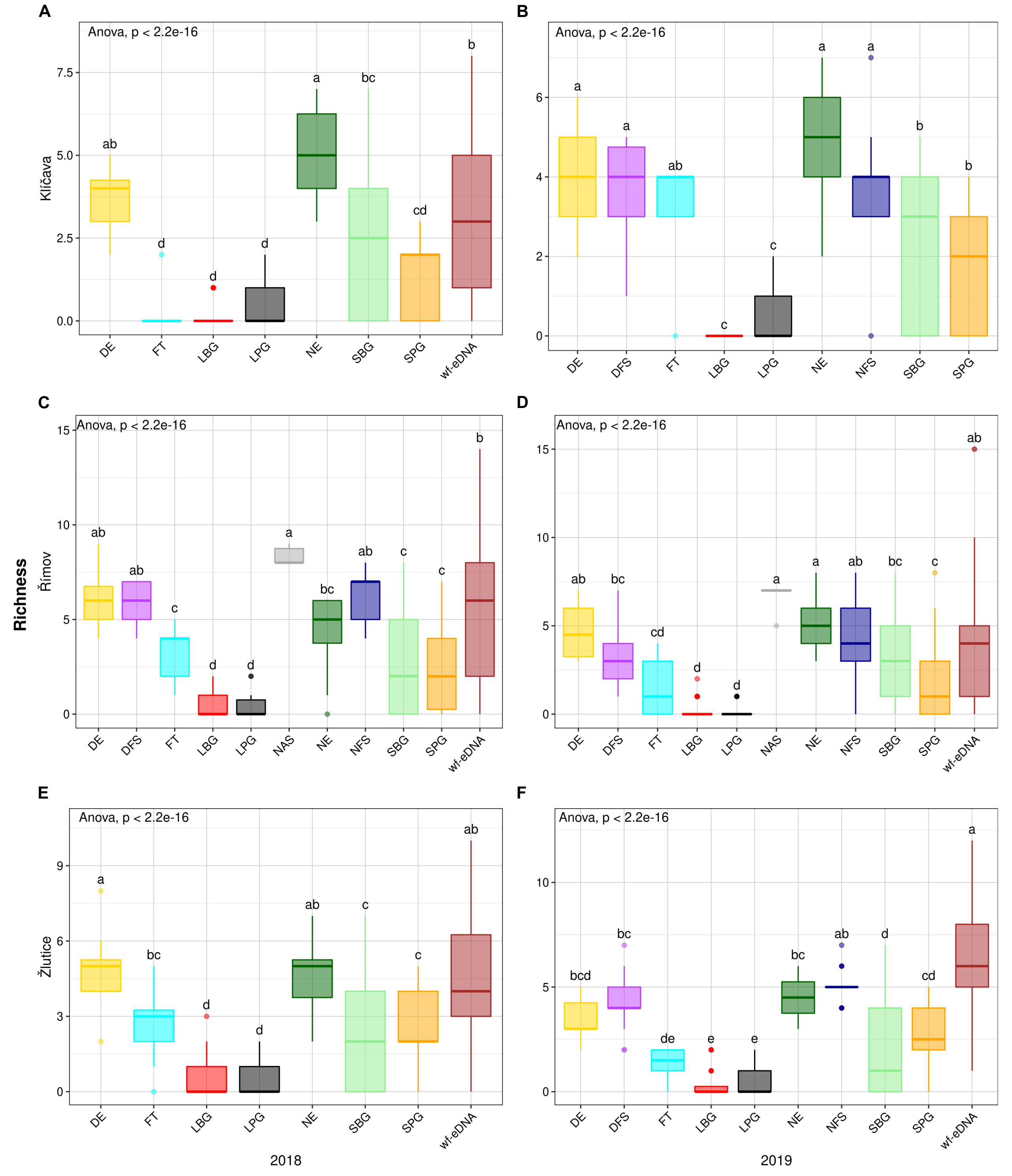

FIGURE 5 | Box plots comparison of alpha diversity (Richness) based on BPUE data in 2018 (left plots) and 2019 (right plots) between wf-eDNA assay and traditional sampling methods in Klíčava, Římov and Žlutice reservoirs. Significant differences are indicated by different superscripts. The horizontal line inside the box indicates calculated median, box 1st and 3rd quartile, whiskers data range and dots outliers of Richness value. Traditional sampling methods are abbreviated as follow: DE = day electrofishing, NE = night electrofishing, SBG = standard benthic gillnets, SPG = standard pelagic gillnets, LBG = large mesh-size benthic gillnets, LPG = large mesh-size pelagic gillnets, NAS = night-time adult seining, DFS = day-time fry seining, NFS= night-time fry seining, FT = fry trawl and wf-eDNA = eDNA from water targeting fish.

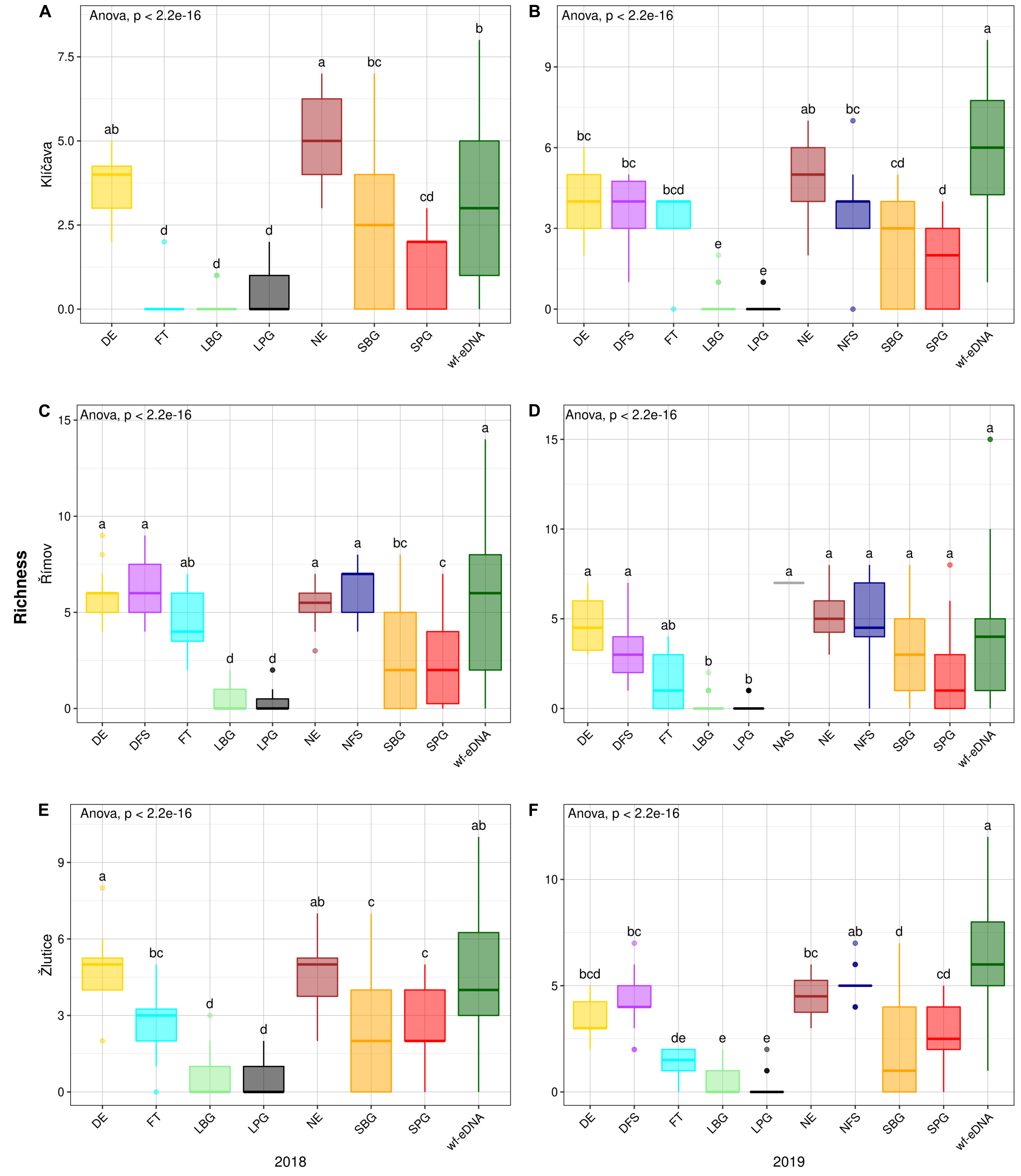

FIGURE 6 | Box plots comparison of alpha diversity (Richness) based on CPUE data in 2018 (left plots) and 2019 (right plots) between wf-eDNA assay and traditional sampling methods in Klíčava, Římov and Žlutice reservoirs. Significant differences are indicated by different superscripts. The horizontal line inside the box indicates the calculated median, box 1st and 3rd quartile, whiskers data range and dots outliers of Richness value. Traditional sampling methods are abbreviated as follow: DE = day electrofishing, NE = night electrofishing, SBG = standard benthic gillnets, SPG = standard pelagic gillnets, LBG = large mesh-size benthic gillnets, LPG = large mesh-size pelagic gillnets, NAS = night-time adult seining, DFS = day-time fry seining, NFS= night-time fry seining, FT = fry trawl and wf-eDNA = eDNA from water targeting fish.

communities in aquatic environments. Compared to our study, previous studies have used only one or relatively few established traditional methods compared to wf-eDNA to investigate fish community composition and diversity in heterogeneous temperate waters. Most studies have used single or relatively fewer traditional sampling methods, such as electrofishing and gill netting (Lawson Handley et al., 2019; Czeglédi et al., 2021), fyke net and gill netting (Sard et al., 2019; Gehri et al., 2021), cast netting, gill netting, and stationary netting (Hayami et al., 2020) to compare the performance of wf-eDNA with traditional sampling methods and to characterize the ecological communities of an aquatic ecosystem at a regional scale. Fuji et al. (2019) used multiple and complex established traditional sampling methods (traps, longline fishing, gill nets, cast nets, dip nets, trawls, and electrofishing) for community analysis in reservoirs in Japan.

Species Composition Assessment

In our study, NMDS ordination was used to characterize fish community composition between established traditional sampling methods and the wf-eDNA assay. Despite most overlap of most methods in all reservoirs examined in this study, fish communities showed partial difference between traditional methods and wf-eDNA (electrofishing, multiple gillnets, and wf-eDNA). This pattern is confirmed by the results of Boivin-Delisle et al. (2021), who showed that wf-eDNA and fish caught with gillnets provide significantly different portraits of fish communities. Previous studies have also confirmed that the greatest discrepancy in community patterns is based on electrofishing and gill netting (Goffaux et al., 2004; Sutela et al., 2008; Czeglédi et al., 2021). However, in support of our findings, Fuji et al. (2019) found no significant difference between NMDS ordinations using traditional sampling methods and wf-eDNA at different study sites. In the present study, it was found that the similarities in the composition of detected fish between wf-eDNA and traditional sampling methods in the Římov were relatively low compared to other reservoirs studied. This result could be due to the different trophic statuses of the studied reservoirs, where eutrophication-tolerant species (common carp, bream, and roach) are more abundant. This shows some spatial structuring that accurately reflects the ecology of the species (Lawson Handley et al., 2019). In agreement with our results, significantly fewer genera and families of the macroinvertebrate community were found with the traditional sampling method than with wf-eDNA (Erdozain et al., 2019). This is likely due to a combination of limitations associated with primer biases, incompleteness of the reference database, or possible stochasticity in low biomass species. The slight heterogeneity in fish community composition between traditional methods and wf-eDNA observed in the current study is consistent with the congruence between electrofishing, gillnet, and wf-eDNA sampling methods in characterizing fish community structure in oxbow lakes in Hungary (Czeglédi et al., 2021). It has been shown that differences in fish community structure may be due to sampling methods rather than actual differences in fish communities between habitats (Eros et al., 2009a,b). Nevertheless, several studies support the research showing that wf-eDNA provides a practical and comprehensive characterization of fish communities (Hänfling et al., 2016; Port et al., 2016; Valentini et al., 2016; Yamamoto et al., 2017; Lawson Handley et al., 2019; Li et al., 2019). Several authors have shown that capturing actual fish community structure and monitoring using either established traditional methods or wf-eDNA alone cannot provide robust and accurate data, although their combination as complementary approaches can reliably provide a true picture of communities in dynamic freshwater habitats (Lacoursière-Roussel et al., 2016; Hering et al., 2018; Lecaudey et al., 2019; García-Machado et al., 2021), which was evident in the present study. Therefore, we propose that wf-eDNA can be used in the assessment of fish communities in aquatic ecosystems with different trophic statuses to complement traditional sampling methods rather than replace them.

Diversity and Site Occupancy of Fish Communities

Biodiversity estimation is the most fundamental key to ecosystem function through the measurement of Shannon and species richness indices (Gotelli and Colwell, 2011; Goutte et al., 2020). Limited information is available on the effectiveness and performance of wf-eDNA compared to traditional sampling methods for measuring biodiversity indices. However, wf-eDNA has been shown to detect more species than traditional survey methods, particularly in freshwater systems with fewer than 100 species (McElroy et al., 2020). In Římov reservoir, Shannon and richness indices between methods were statistically significant; however, the degree of difference between wf-eDNA and other traditional surveys (DE, NE, and NAS) was negligible. Accordingly, a congruency was found in the regional estimation of richness by comparing wf-eDNA with traditional sampling methods, confirming the potential of wf-eDNA for biodiversity assessment in aquatic ecosystems. Consistent with our results, similarity was found between wf-eDNA and electrofishing for estimating richness in the St. Joseph River, United States (Olds et al., 2016). This shows several advantages, such as identification of species by wf-eDNA without increased effort and habitat disturbance for biomonitoring of fish fauna (Adamson and Hurwood, 2016). The Shannon index calculated from wf-eDNA was significantly higher than traditional sampling methods in the present study in the Klíčava and Žlutice Reservoirs. In contrast, congruence in the richness index was observed in the present study, regardless of the statistically significant differences between the approaches, especially between wf-eDNA and NAS. This discrepancy in the richness index could be related to the less abundant species composition and methods used in the Klíčava and Žlutice reservoirs, suggesting that the wf-eDNA method is sensitive to very rare species that occur at low abundance. In support of our data, species richness was significantly different across methods and consistently higher for the wf-eDNA method than for traditional sampling methods (electrofishing, seining, trawling), despite extensive sampling using traditional methods (Hallam et al., 2021). Recent evidence also confirms that wf-eDNA outperforms gillnet and fyke net methods in measuring species richness in Boardman Lake (Gehri et al., 2021). Correlations between the detection sensitivity and relative abundance of wf-eDNA and conventional survey methods are essential to obtain a comprehensive overview of the distribution of freshwater species in different aquatic environments. Several studies have shown that semi-quantitative abundance estimates of wf-eDNA in reservoirs (i.e., site occupancy) are consistent with data from traditional sampling methods (Evans et al., 2016; Hänfling et al., 2016; Andruszkiewicz et al., 2017; O'Donnell et al., 2017; Lawson Handley et al., 2019; Blabolil et al., 2021a). In the present study, a positive and significant correlation was found between eDNA and BPUE and CPUE data in all the reservoirs studied. Several studies have reported a positive correlation between eDNA and biomass or abundance data in freshwater (Evans et al., 2016; Lacoursière-Roussel et al., 2016; Sard et al., 2019; Czeglédi et al., 2021) and marine aquatic environments (Port et al., 2016; Thomsen et al., 2016; Andruszkiewicz et al., 2017; Afzali et al., 2021). Takahara et al. (2012) highlighted that data on species biomass in natural environments can be obtained more easily and quickly with wf-eDNA than with traditional sampling methods. Nevertheless, a weak quantitative relationship was found between wf-eDNA, and the biomass of marine fish caught with trawls (Knudsen et al., 2019). Lamb et al. (2019) also conducted a meta-analysis and showed a weak quantitative relationship between biomass and wf-eDNA. The insufficient or lack of correlation between abundance and wf-eDNA observed in different natural systems could be attributed to variations associated with environmental factors (Yates et al., 2019; Czeglédi et al., 2021). However, the importance of the environmental factors considered is likely to depend on the species (e.g., body size, age distribution, temperature, time of day, season, etc.) and the system used to measure wf-eDNA (Wilcox et al., 2016).

Utility of wf-eDNA Versus Traditional Survey Methods

In the present study, we demonstrated that wf-eDNA generally covers the broadest taxon range, indicating a more comprehensive characterization of community species diversity and composition than traditional sampling methods. Our results are consistent with those studies that have shown that wf-eDNA surveys provide taxa-detection data and, in many cases, outperform traditional survey methods (Fuji et al., 2019; Lawson Handley et al., 2019; Goutte et al., 2020; Zou et al., 2020; Czeglédi et al., 2021). It can be noted that in the present study, large-mesh gillnets and trawling provided the least information on community composition and diversity compared to other traditional sampling methods. Of the traditional methods used in the present study, daytime electrofishing and standard benthic gill netting surveys provided more comprehensive data in the evaluated reservoirs. Together with the above-mentioned traditional methods and wf-eDNA, we were able to capture and detect most species in each reservoir. Consistent with our results, there is further evidence of higher fish species detection rates with wf-eDNA compared to traditional survey methods in freshwater ecosystems (Civade et al., 2016; Hänfling et al., 2016; Valentini et al., 2016). More species were also detected in the marine ecosystem using wf-eDNA than bottom trawls, with mainly rare species with low abundance (Afzali et al., 2021). In the present study, some fish species collected using traditional methods were not detected with wf-eDNA in any of the reservoirs. These discrepancies are likely due to inconsistent species distribution or different detection methods for species in the three reservoirs studied. This could also be attributed to the fact that the lower detection of species with wf-eDNA and using other traditional methods is due to the different sampling efforts in each reservoir. In Římov reservoir, *Coregonus* sp., *Pseudorasbora parva* and *Squalius cephalus* as well as *Hypophthalmichthys* sp. in Klíčava reservoir and *Carassius carassius* and *Lota lota* in Žlutice reservoir were caught only by traditional methods. This is probably due to the limited amount of eDNA in the water samples released by rare species or the larger spatial structure in the reservoir during the summer months, which failed to detect wf-eDNA. This limitation has also been reported in other studies (Takahara et al., 2012; Dougherty et al., 2016; Doi et al., 2019; Lawson Handley et al., 2019). Numerous studies have also quantitatively assessed the cost-effectiveness of wf-eDNA compared to traditional sampling methods and have shown that the wf-eDNA approach is relatively more cost-effective than traditional methods (Biggs et al., 2015; Davy et al., 2015; Huver et al., 2015; Sigsgaard et al., 2015)., although some technical challenges remain, such as target taxa, site-specific detection rates and budgets (Smart et al., 2016). For instance, a quantitative comparison of financial costs and sampling effort to assess the distribution of native brook trout *Salvelinus fontinalis* in the Namekagon River watershed in northern Wisconsin found that wf-eDNA analysis required less sampling effort and was 67% cheaper than triple-pass electrofishing (Evans et al., 2017). Similarly, Smart et al. (2016) showed that eDNA analysis is relatively inexpensive compared to traditional sampling methods. Sigsgaard et al. (2015) found that wf-eDNA for the detection of European weather loach (*Misgurnus fossilis*) was 1.9 times cheaper than a combination of traditional methods. Davy et al. (2015) showed that the cost of detecting nine sympatric freshwater turtles using capture-based methods was 2–10 times higher than that using eDNA. The major drawbacks of wf-eDNA analysis includes the inability of mitochondrial eDNA to distinguish "pure" species from hybrids and the movement of eDNA from site to site both in water and in the organism (Evans et al., 2017; Evans and Lamberti, 2018). However, the latter can be managed through careful wf-eDNA sampling plans and collections. Furthermore, the trade-offs between the accuracy, cost, coverage, and speed of each method in biodiversity monitoring are strongly recommended for managers to assess biodiversity in aquatic environments (Qu and Stewart, 2019).

CONCLUSION

In summary, the wf-eDNA and traditional sampling methods used in the present study should not be considered as alternative methods for biodiversity assessment and monitoring because they can provide different information. To maximize the number of species detected and obtain a complete overview of species composition and diversity estimates, while reducing the cost, time, and effort associated with sampling, the combination of day electrofishing, benthic gill nets, and wf-eDNA is the most appropriate strategy in aquatic systems, especially for science-based policy decisions. On the other hand, large-mesh gillnets and trawling added little additional information. In addition, how the combination of wf-eDNA and traditional sampling methods is proposed in this study may be influenced by practical considerations, such as the availability of resources (including funding, time, and the knowledge and skills of the individuals conducting the research) and the issues addressed in the study.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: PRJNA611963).

ETHICS STATEMENT

The study was conducted in accordance with all legal requirements of the Czech Republic. All sampling procedures and experimental protocols used in this study were performed in accordance with the guidelines of and with permission from the Experimental Animal Welfare Commission under the Ministry of Agriculture of the Czech Republic (Ref. No. CZ 01679).

AUTHOR CONTRIBUTIONS

AG and PB: conceptualization, data curation, writing—original draft, review, and editing. DB, MŘ, and MŠ: review and editing, and sampling. MČ, MV, VD, LK, and TK: sampling. RS and TJ: conceptualization and sampling. CM: Statistical data analysis. AS: conceptualization, review and editing, and sampling. JP: conceptualization and review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by project QK1920011 “Methodology for quantification of predators in drinking water reservoirs for optimization of aquatic ecosystem management,” MSM200961901 “The true picture of eDNA,” ERDF/ESF project “Biomanipulation as a tool for improving the water quality of reservoirs” (no. CZ.02.1.01/0.0/0.0/16_025/0007417) and by the PPLZ project L200962102 “Fish community assessment using environmental DNA metabarcoding and traditional fishing methods; a promising tool for fisheries management.”

ACKNOWLEDGMENTS

We thank the Vltava River State Enterprise (www.pvl.cz) for facilitating the fieldwork and our colleagues at FishEcU (www.fishecu.cz) for the assistance in the field.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.913279/full#supplementary-material

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

frontiers | Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 14 July 2022
DOI 10.3389/fevo.2022.915787

EDITED BY
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SPECIALTY SECTION
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

RECEIVED 08 April 2022
ACCEPTED 27 June 2022
PUBLISHED 14 July 2022

CITATION
Li D, He P, Liu C, Xu J, Hou L, Gao X, Wang D and Wang J (2022) Quantitative relationship between cladocera and cyanobacteria: A study based on field survey. *Front. Ecol. Evol.* 10:915787. doi: 10.3389/fevo.2022.915787

Quantitative relationship between cladocera and cyanobacteria: A study based on field survey

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Cyanobacteria are a widely distributed phytoplankton that can bloom and produce algal toxins in the eutrophicated water bodies. Large cladocerans are a group of zooplankton that presents higher grazing efficiency on algae. Studying the quantitative relationship between cyanobacteria and cladocera, especially in unmanipulated and unpredictable natural ecosystems in the wild, provides the key to revealing the mechanism of cyanobacterial blooms and finding effective control and prevention methods. This paper proposes a research method to detect the threshold for cladocera to control cyanobacteria by using the path of “edge scatter-segment regression.” Based on the field survey data from 242 sample sites in shallow and slow-flowing rivers in North China, the quantitative relationship between the main groups of zooplankton and phytoplankton was analyzed, and the standard deviation and coefficient of variation were used to test the threshold. This paper finally compares the roles of body size and cladocera abundance in cyanobacteria control. The results showed that in natural ecosystems, cladocera were the best group for controlling the abundance of cyanobacteria among zooplankton. The control effect of cladocera on the abundance of cyanobacteria is not linear but non-linear, and cladocera can only have a stable control effect under certain conditions. The total phosphorus concentrations and water temperatures did not interfere with the analysis results in this paper. In wild ecosystems, the predation process of cladocera on cyanobacteria basically follows the “size-efficiency” hypothesis, but when cladocera successfully control cyanobacterial abundances, it is often due to “win by quantity” rather than “win by size.” The phenomenon of non-linear variation in the cladocera density-cyanobacteria density relationship fits well with the description of the transition from a stable to chaotic state in chaos theory. This paper reveals the complex quantitative relationships of plankton food chains in wild aquatic ecosystems. The ecological threshold detection of the cladocera-cyanobacterial abundances provides a quantitative basis for early warning, control and prevention of cyanobacteria blooms. The non-linear variations in cladocera density-cyanobacteria density revealed in this paper provide insight and evidence for understanding the complex changes in aquatic ecosystems.

KEYWORDS

cladocera, cyanobacteria blooms, eutrophication control, ecological threshold, wild ecosystem, aquatic ecosystems

Introduction

Eutrophication is one of the major water environmental problems facing the world (Pick, 2016; Hayes and Vanni, 2018). Severe eutrophication and its accompanying cyanobacteria blooms can be devastating to healthy aquatic ecosystems (Postel and Carpenter, 1997; Huisman et al., 2018). The massive growth of algae and production of algal toxins also seriously threaten water security for humans (Shen et al., 2003; Jing et al., 2017; Czyewska et al., 2020). How to control the abundance of cyanobacteria and inhibit the growth of cyanobacteria has become an important topic in water research (Paerl et al., 2011; Huertas and Mallén-Ponce, 2021).

Biomanipulation based on the predation of zooplankton on phytoplankton is an important approach for eutrophication governance and cyanobacterial bloom control (Shapiro et al., 1975; Peretyatko et al., 2012). Biomanipulation theory affirms the status and role of cladocera in zooplankton and attempts to restore the abundance of cladocera as a major goal (Bernardi et al., 1987). In addition, biomanipulation theory suggests that it follows the “size-efficiency” hypothesis when zooplankton graze on phytoplankton; that is, the higher the number of large zooplankton is, the greater the reduction in phytoplankton abundance (Hall et al., 1976; Gliwicz, 1990). Thus, in specific cases of biological manipulation, higher proportions of large cladocera such as *Daphnia* are commonly pursued.

However, a large number of eutrophication governance practices show that cyanobacterial abundances are not easily controlled (Michalak et al., 2013). Moreover, some studies based on field monitoring data show that cyanobacterial abundances are difficult to predict (Cha et al., 2017). The stable, two-dimensional, quantitative relationships of cyanobacteria-total phosphorus (TP), cyanobacteria-N/P, cyanobacteria-residence time, and cyanobacteria-temperature obtained in laboratory experiments often fail in natural ecosystems in the wild. Some lakes or rivers are at high risk of cyanobacteria blooms even under conditions (e.g., very low TP concentrations and lower temperatures) that laboratory studies consider unlikely to occur (Liang et al., 2020; Zhu et al., 2020). The experiences of failure that were gained from controlled experiments in practice prompts us to turn to natural ecosystems to find more real and general laws. This is important for successful lake management.

Although zooplankton predation is generally understood to be the main top-down effect on cyanobacteria, few studies have used zooplankton to predict and warn of changes in cyanobacterial abundances. In the context that some environmental factors cannot accurately predict and control cyanobacterial abundances (Cha et al., 2017), it is important to find other effective factors. At present, the research on the quantitative relationships between zooplankton and cyanobacteria is not deep enough, especially the quantitative relationship between the two in unmanipulated wild ecosystems. In addition, the roles of the abundances and body sizes of cladocera in the process of controlling cyanobacterial abundances are still unclear. In biomanipulation practice, we need to understand whether abundance or body size should be the focus when restoring cladocera communities.

Among the types of aquatic systems that are prone to cyanobacteria blooms, the shallow and slow-flowing reaches that are formed by gentle terrain and dams are important systems that cannot be ignored. The river network in North China is dense, with many dams but less water flow, which forms a large number of shallow and slow-flowing waters. These rivers provide the water source for more than 100 million people. Severe eutrophication and cyanobacteria blooms have occurred in these rivers in recent years, and these problems have seriously threatened the water security of North China (Shan et al., 2012; Zhang et al., 2015). These shallow, slow-flowing reaches are widely distributed, with different nutritional statuses and aquatic habitat conditions and are present in mountain rivers in their natural state, urban rivers and agricultural irrigation rivers that are disturbed by high-intensity human activities. Accordingly, we conducted *in situ* surveys of the aquatic organisms and environmental conditions in these shallow and slow-flowing reaches during summer to understand the quantitative relationships between zooplankton and phytoplankton in these waters to try to confirm the useful control effect of cladocera on cyanobacteria in wild ecosystems and to reveal the roles of cladocera abundances and body sizes. Based on the observations of field ecosystems, this paper analyzes the quantitative relationships between cladocera and cyanobacteria, provides a scientific basis for the early warning and prevention of cyanobacterial blooms in rivers, and provides preliminary insights and evidence for revealing the non-linear changes of complex systems.

Methods

Sampling method

We surveyed 242 sample sites on rivers in North China from 2018 to 2020. The river network in North China is dense, and there are many dams on the rivers. This survey is mainly aimed at the high-risk areas for cyanobacteria blooms in North China. All sampling sites are located in the shallow and slow-flowing waters of rivers and reservoirs to ensure that the surveyed waters have long residence times. These sample sites cover not only the reaches that cross central cities (e.g., Beijing, Tianjin, and Jinan) that are strongly disturbed by humans but also the upper reaches that are rarely affected by human activities. The environments in which the sample points are located have large gradients. The sampling time in each reach is in the peak season of cyanobacteria blooms (e.g., June to September), and the spatial distribution of the sampling sites is shown in **Figure 1**.

The water temperatures at 0.2 m below the water surface were directly measured on site. The water samples were collected in 500-mL polyethylene plastic bottles and stored in an incubator filled with ice packs. After the sampling for each day was completed, the samples were sent back to the laboratory for analysis and determinations. The total phosphorus (TP) concentrations in the water samples were determined with the ammonium molybdate spectrophotometric method.

Water samples with volumes of 1,000 mL were taken from the middle depths of the reaches and were placed in polyethylene plastic bottles, shaken well, and 10–15 mL of Lugol's iodine was added to fix the organisms. The water samples were left to settle for more than 48 h, the organisms that settled to the bottoms of the bottles were collected, and the volumes was adjusted to 50 mL. For the identification and counting of phytoplankton cells, 0.1 mL from a constant-volume sample was injected into a 0.1 mL chamber and counted with an optical microscope (CX21FS1, Olympus, Hatagaya, Tokyo, Japan) at 400 × resolution. The Protozoa and rotifers were identified and counted at 100–400 × resolution. The Protozoa were counted using a 0.1-mL chamber, and the rotifers and nauplii were counted using a 1-mL chamber. Each sample was counted twice, and the average was taken.

The identification and counting of cladocera and copepods were performed under a low-resolution microscope. Water sample with volumes of 10 L were collected and poured into a No. 25 plankton net for filtration, the concentrates were placed into 20-mL glass bottles, and 4% formalin was added to fix the organisms. The collected water samples were precipitated for 24 h, and the volumes were then adjusted to 30 mL. The identification and counting of copepods and cladocera were performed using a 5-mL chamber (Wang, 2011).

Analytical method

Piecewise regression

Piecewise regression is a regression estimation method that is applicable when two variables obey different linear relationships within different independent variable ranges (Toms and Lesperance, 2003; Toms and Villard, 2015). If the two variables (e.g., independent variable X and dependent variable Y) obey a simple linear relationship, the model is shown in Formula (1):

$$Y = \beta_0 + \beta_1 X \tag{1}$$

When $X < X_1$, Y and X obey the simple linear relationship shown in Equation 1 and when $X > X_1$, Y and X obey another linear relationship, its intercept and slope are significantly different from before, but Y is continuous at X_1. Therefore, a dummy variable D is used to conform to the conditions of Formula (2):

$$D = \begin{cases} 0, (X < X_1) \\ 1, (X \geq X_1) \end{cases} \tag{2}$$

The piecewise regression model between Y and X is shown in Formula (3):

$$Y = \beta_0 + \beta_1 X + \beta_2 (X - X_1) D \tag{3}$$

According to Equation 3, when X is in different ranges, the expected values of Y are:

when

$$X < X_1, E(Y) = \beta_0 + \beta_1 X;$$

when

$$X \geq X_1, E(Y) = (\beta_0 - \beta_2 X_1) + (\beta_1 + \beta_2)X.$$

Segmented regression is a statistical method that is suitable for detecting breakpoints in "broken stick" models. Breakpoint detection was performed by using the Segmented package in R4.0.2. The working principle of Segmented package is described in the literature (Muggeo, 2008). The Segmented package can automatically provide the Akaike information criterion (AIC) values for evaluating the effect of segmented regression analysis, which is a standard used to measure the goodness of model fitting (Akaike, 1974). For the case of the same number of samples, the lower the AIC value is, the better the model fitting effect. In addition, to compare the reliability of the breakpoints that are determined by the segmented regression analysis, this paper uses the standard error (SE) and SE/Est. (the ratio of the standard error to the abscissa

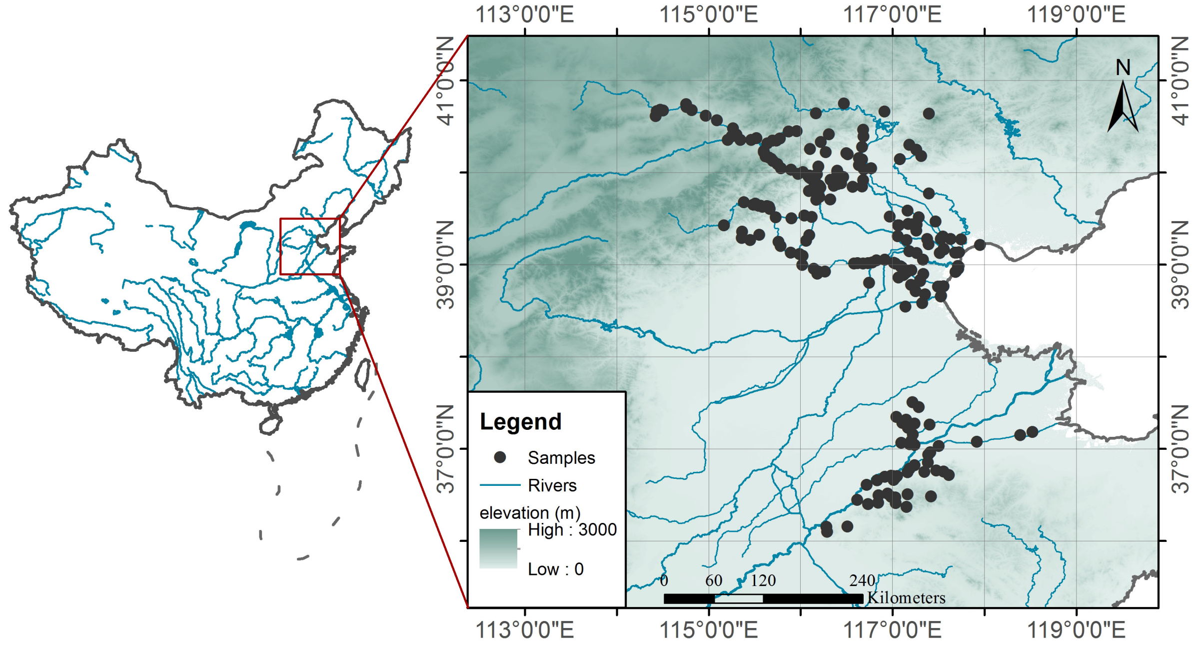

FIGURE 1
Spatial distribution of sampling sites.

value of the breakpoint), which are automatically calculated by the Segmented package in R4.0.2 (R Development Core Team, Auckland, New Zealand). The lower the SE and SE/Est. values, the more reliable the breakpoint evaluation. If both the AIC value and the SE/Est. value are relatively low at the same time, it can indicate that the reliability of the threshold detection is relatively high.

Ecological threshold analysis path of "finding edge scattered points-piecewise regression"

An "L"-shaped scatter is a common type of bivariate scatter observed in the quantitative relationship analyses of aquatic ecosystems (Cade and Schroeder, 1999; Wang et al., 2007), as shown in **Figure 2A**. The distribution of this type of scatter has obvious change characteristics, that is, it is similar to the "broken stick" threshold type, but it cannot be determined by piecewise regression.

For this type of scatter, the boundary of the scatter is often determined, and then the abrupt point of the scatter distribution is estimated according to the breakpoint of the boundary (Brenden et al., 2008). The mainstream method that is used to determine the boundaries of scatter points is the quantile method, that is, using an appropriate cutting quantile to separate the edge scatter points that can describe the variations in the scatter distribution so that these scatter points can be fit for piecewise regression (Brenden et al., 2008; Qiu et al., 2021). However, for an "L" shaped scatter, as shown in **Figure 2A**, the right branch of the scatter plot is close to the horizontal axis, and the edge scatter cannot be obtained through the quantile cut as for the left.

In this paper, the maximum value is used as the upper boundary, that is, the horizontal axis is divided into several segments at equal intervals, and the scatter point with the largest ordinate value in each segment is taken as the edge scatter point (**Figure 2B**). These edge scatter points effectively reflect the change characteristics of the scatter point distribution and can indicate the changes in the maximum value of the ordinate when the abscissa moves from left to right or from right to left. The Segmented package is used to perform piecewise regression fitting on these selected edge scatter points to determine the threshold position of the "L" shaped scatter points.

Threshold validation

Polynomial regression fitting analysis was used to investigate the change trends of the averages, standard deviations and coefficients of variation and to verify the validity of the threshold detection method. The average and standard deviation describe the concentrated characteristics of the scatter points. The smaller the standard deviation is, the more concentrated the scatter points are. In contrast, the larger the standard deviation is, the more scattered the scatter points are, which indicate a greater degree of scatter fluctuation. The coefficient of variation is the ratio of the standard deviation to the average, which offsets the influence of different average values and can more objectively reflect the degree of dispersion of the scatter points.

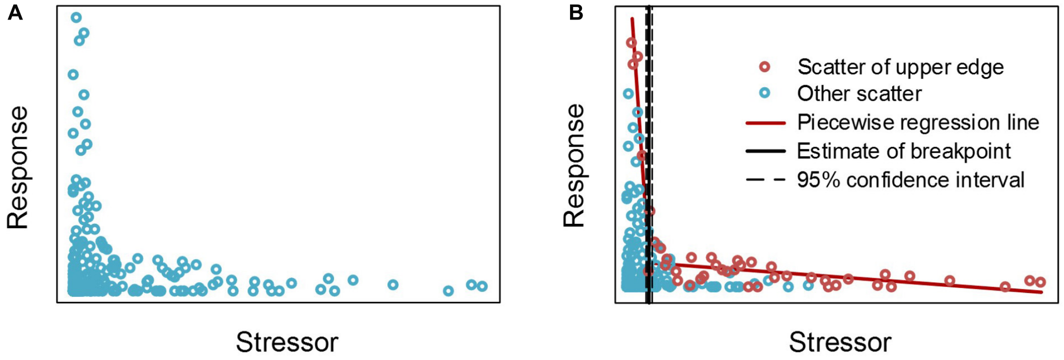

FIGURE 2
Segmented analysis results of "L" shaped simulated scattered points after selecting the edge scatter. Panel **(A)** shows simulation of "L" shaped scattered points, panel **(B)** shows the selection of the edge scatter points and the segmented analysis results of edge scatter points.

The larger the coefficient of variation is, the greater the degree of fluctuation of the scatter points.

For the convenience of observation, we examined whether the amplitudes of the fluctuations in the cyanobacteria densities changed when the cladocera densities were close to the threshold and less than the threshold as the cladocera density changed from the maximum value to minimum value. The calculation process for the average, standard deviation, and coefficient of variation are as follows: starting from the first sample on the far right, calculate the average, standard deviation, and coefficient of variation for all samples on the right side of the abscissa as each integer bit, and record them at the corresponding abscissa as the integer bit. If there are no samples from n to n+k on the abscissa, no calculation is performed between them.

Quantitative relationship analysis of zooplankton and phytoplankton

Before performing the threshold analysis, a preliminary observation was made of the distribution characteristics of each group of scatterplots. If the scatter distribution has a typical "L" shape, the analysis path of "finding edge scattered points-piecewise regression" can be used for threshold detection. If the scatter distribution do not have the characteristics of an "L" shape, threshold detections were not performed for these groups. To confirm whether cladocera is the key group for cyanobacterial control in zooplankton, the analysis was carried out using the screening idea of "whole to part."

The effect of cladocera size on cyanobacteria control

All sites are grouped according to the cladocera density and cyanobacterial density. The averages of the density and dominance among groups were compared by using one-way ANOVA followed by an LSD *post hoc* test. The assumptions of ANOVA were met because the homogeneity and normality were confirmed by Levene's test and by the Kolmogorov–Smirnov test or Shapiro–Wilk test, respectively. The statistical analyses were performed using the Statistical Product and Service Solution (SPSS) 20 statistical package (International Business Machines Corporation, New York City, NY, United States).

Results

Quantitative relationship between zooplankton and phytoplankton

According to the observations of the scattered points, (a), (b), (c), (d), (e), (f), and (g) in **Figure 3** conform to this feature; therefore, threshold detection is performed on these seven groups of scatter points. However, the scatter plots in (h) and (i) do not have the characteristics of an "L" shape, so threshold detections were not performed for these two groups.

Figures 3A–C demonstrate the relationships between the densities of three dominant groups of phytoplankton (e.g., chlorophyta, diatoms, and cyanobacteria) and the overall density of zooplankton. From the AIC values, the piecewise regression fitting effect for (a) and (b) is slightly better than that for (c) and from the SE/Est. values, the breakpoint estimation effects of (b) and (c) are better. Judging from the fit between the fitting results of piecewise regressions and the scatter distribution characteristics, (b) and (c) have a high degree of fit, while (a) has a low degree of fit. In addition, the densities of the three phytoplankton groups indicate that cyanobacteria have the highest density and predominate among the phytoplankton. The relationship between zooplankton density and diatom density seems to have a relatively clear non-linear relationship, but the advantage that is occupied by diatoms in phytoplankton is limited, so cyanobacteria are mainly considered next.

Figures 3D–G show the quantitative relationships between the cyanobacteria densities and the four groups of zooplankton (e.g., protozoa, rotifer, copepoda, and cladocera). From the

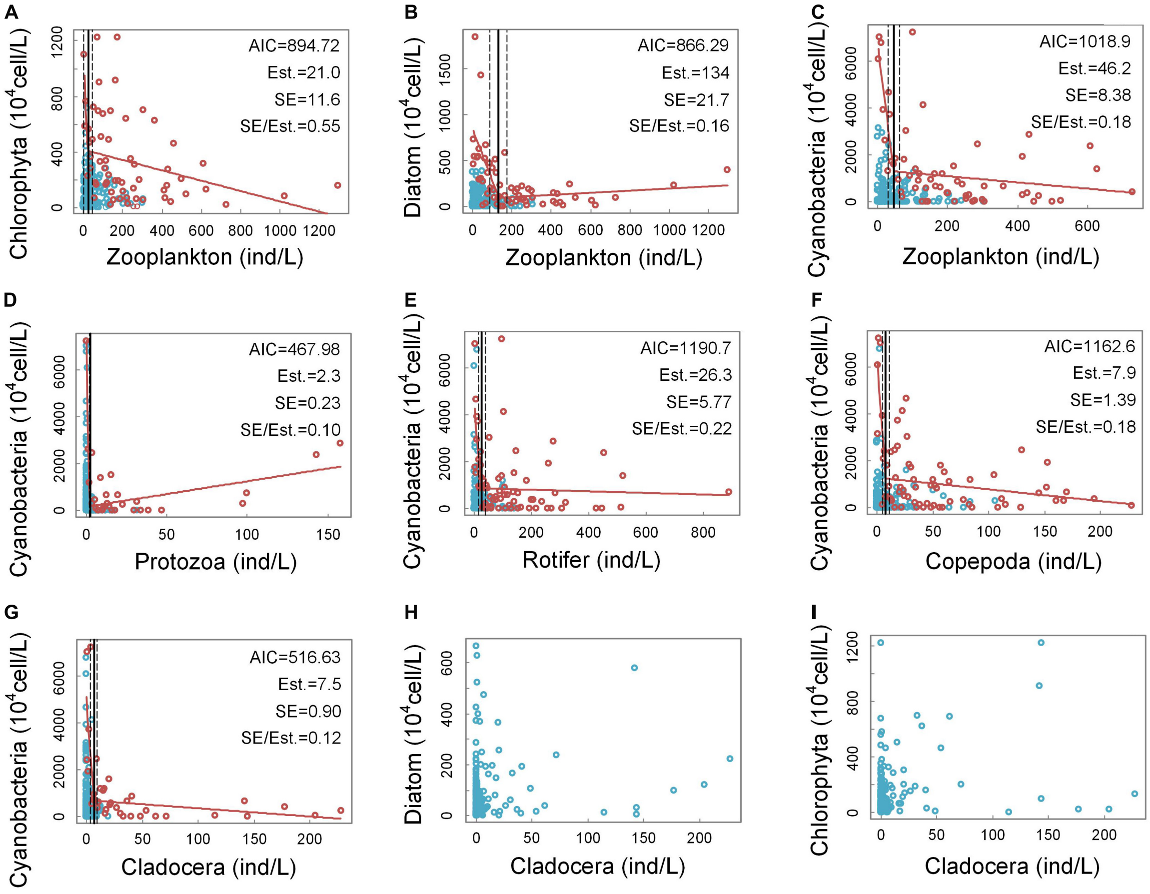

FIGURE 3

Quantitative relationships between the densities of the groups of zooplankton and phytoplankton. In panels **(A–G)**, the horizontal axis intervals used in the selection of the edge scatter points are 5 ind/L, 5 ind/L, 5 ind/L, 1 ind/L, 2 ind/L, 2 ind/L, and 1 ind/L, respectively. In panel **(D)**, a singular point with extremely high protozoan density is excluded. This sample point is located in Xinglin Reservoir, Jinan. At this site, the density of protozoa reached 888 ind/L, and the density of cyanobacteria was 723.8·10^4 cell/L. Panels **(H–I)** cannot select edge scatter points.

AIC values, the piecewise regression fitting effects of (d) and (g) are the best, while (d) and (g) have the relative lower SE/Est. values, which indicate that the breakpoint estimation effects are also the best. Judging from the fit between the fitting results of piecewise regression and the scatter distribution characteristics, the fits in (d), (e), (f), and (g) are all good, which indicate that the cyanobacteria densities have a clear non-linear relationship with the densities of the four zooplankton groups. Among them, (d) and (g) have the best fits. It can be seen from (d) that when the protozoan density is low, the values of the cyanobacteria density fluctuate greatly, and the scatter points with high cyanobacterial densities are all distributed in this range. The highest possible cyanobacterial density decreases rapidly as the protozoan density increases. However, as the density of protozoa continued to increase, the density of cyanobacteria began to increase significantly. From (e) and (f), it can be seen that the scatter points with higher cyanobacterial densities are located in the lower densities of rotifers and copepods. When the densities of rotifers and copepods were high, the maximum cyanobacterial densities decreased steadily but still maintained high levels. According to (g), the amplitudes of the cyanobacteria density fluctuations change when the cladocera density is approximately 7.5 ind/L. When the cladocera densities were lower than 7.5 ind/L, the amplitudes of the fluctuations in the cyanobacteria densities were very large, and the maximum value that the cyanobacteria density could reach dropped sharply from a very high level to a low level. When the cladocera density was greater than 7.5 ind/L, the cyanobacteria density decreased slowly with increasing cladocera density, and the amplitudes of the fluctuations in the cyanobacterial densities were small. The above results show that cladocera may be the most effective zooplankton group for controlling the abundance of cyanobacteria.

Figures 3H,I show the relationships between the cladocera density and the densities of diatoms and chlorophyta. According to (h) and (i), the scatter plots of diatom density-cladocera density and chlorophyta density-cladocera density did not have obvious "L"-shaped characteristics, so threshold detection was not performed.

Threshold test and reanalysis

The gray vertical solid line is the threshold position for cladocera controlling cyanobacteria detected in **Figure 3G**.

According to **Figure 4**, all three parameters have a common feature, namely, they all tend to increase as the cladocera density increases from large to small. When the cladocera densities reached different levels of low values, they all increased rapidly as the cladocera densities continued to decrease. Among them, the average growth rate began to increase significantly when the cladocera density was less than 50 ind/L. The standard deviation began to increase significantly when the cladocera density was less than 25 ind/L, and the standard deviation remained at a low level when the cladocera density was greater than 25 ind/L.

Figure 4D shows that there are many locations where the coefficient of variation changes with changes in the cladocera density, but the location of the rapidly increasing change point when its value remains high is consistent with the location of the threshold detection shown in **Figure 3G**.

The effect of cladocera body size

Figure 5 shows the body lengths of 37 cladocera that appeared in all samples. According to the body length order of all species, cladocera are classified as medium-large sized and micro-small sized (**Figure 5**). According to the threshold detection results shown in **Figure 3G**, all of the samples were divided into three groups (**Figure 6**). The average densities (**Figure 6A**) and average dominance (**Figure 6B**) of the medium-large and micro-small cladocera in each group were compared.

According to the comparison of the average cladocera densities (**Figure 6A**), the results of the comparisons among groups showed that the average density of the GC group (30.3 ind/L, 18.3 ind/L) was highest for both medium-large and micro-small cladocera and was significantly ($P < 0.01$) greater than that of the GB group (1.24 ind/L, 1.46 ind/L) and GA group (1.16 ind/L, 1.04 ind/L). The difference between the GA and GB group was not significant ($P > 0.05$). The results of the comparisons of the inner groups showed that in the GA and GC groups, the average density of medium-large cladocera was greater than that of micro-small cladocera, but the difference was not significant ($P > 0.05$).

According to the comparison of the average dominance of cladocera (**Figure 6B**), the results of the comparisons among groups showed that for the medium-large cladocera, the average dominance in the GC group was largest (63.5%), which was significantly ($P < 0.05$) greater than that of the GB group (46.4%) and greater (51.5%) than that of the GA group but was not significant ($P > 0.05$). For micro-small cladocera, the average dominance of the GB group was largest (53.6%), which was significantly ($P < 0.05$) greater than that of the GC group (36.5%) and slightly greater than that of the GA (48.5%) group, but this difference was not significant ($P < 0.05$). The results of the comparisons of the inner groups showed that there were no significant differences ($P > 0.05$) in the average dominance of the medium-large and micro-small groups regardless of whether it was the GA or GB group. The average dominance of the medium-large cladocera in the GC group was significantly ($P < 0.01$) greater than that of micro-small cladocera.

Interference effects of total phosphorus and water temperature

In the threshold analysis, it is not enough to only consider differences in abundance and dominance of cladocera. It needs to be considered whether the lower cyanobacteria density in GC group is due to lower temperature or TP concentration, and whether the higher cyanobacteria density in GA group is due to higher temperature or TP concentration. The differences in water temperature and TP concentration among the three groups were examined below (**Figure 7**).

According to the comparison of average TP concentrations (**Figure 7**), the average TP concentration of GC group was the largest (454 μg/L), and was significantly ($P < 0.01$) greater than that of GA group (72 μg/L) and GB group (186 μg/L). The average TP concentration in GA was less than that in GB group but not significant ($P > 0.05$). This suggests that the lower cyanobacteria density in the GC group was not caused by the lower TP concentration, instead, the TP concentration was greater in the GC group.

According to the comparison of average water temperature (**Figure 7**), the average water temperature of GB group was the lowest (23.4°C), and was significantly ($P < 0.05$) lower than that of GA group (26.5°C) and GC group (26.0°C). The average water temperature of the GA group was very close to that of the GC group ($P > 0.05$). This suggests that in the GC group, the lower cyanobacteria density was not due to temperature differences.

Discussion

Effectiveness of the methods

Detecting the abrupt changes in the quantitative relationships between pressure-response variables in the scatters of "L"-shaped distributions has always been a difficult problem in aquatic ecosystems. In this paper, we propose a

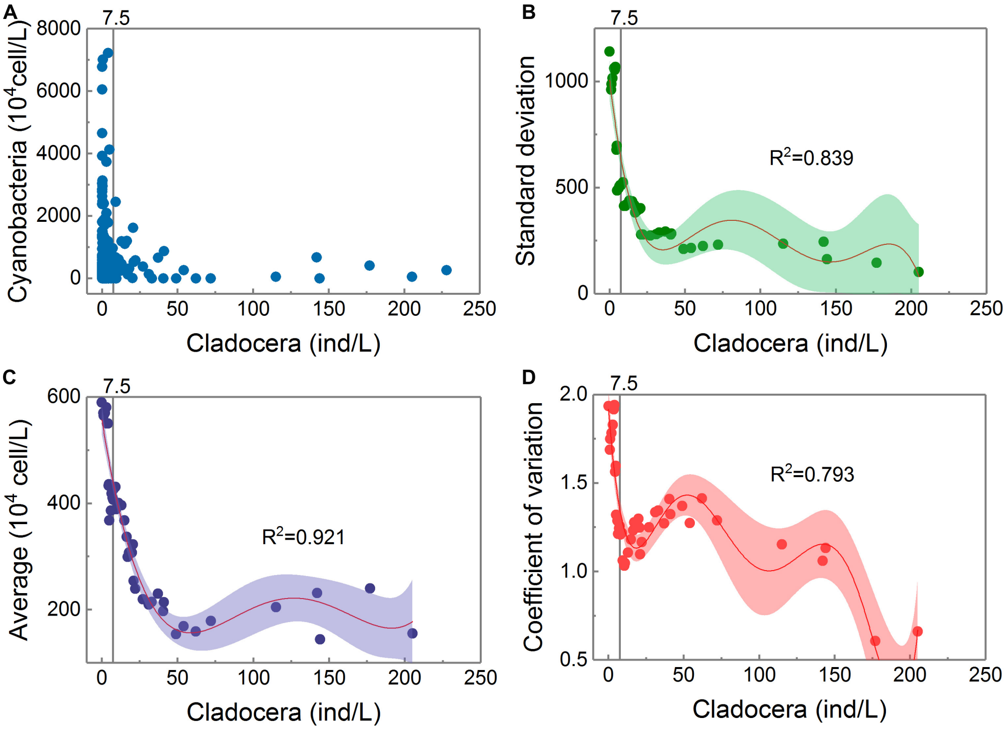

FIGURE 4
Panel **(A)** shows the scatter points of the Cladocera Density-Cyanobacteria Density. The trends of the dispersion degrees of the scatter points with changes in cladocera density. Panels **(B–D)** all adopt the polynomial fitting method, while panel **(B)** uses a 5th degree polynomial, panel **(C)** uses a 4th degree polynomial, and panel **(D)** uses a 6th degree polynomial. There is only one sample on the right side of the abscissa to 206 ind/L, and calculations of the average, standard deviation, and coefficient of variation are not performed.

threshold detection path of "finding edge scattered points-piecewise regression" to explore the non-linear relationship between zooplankton density and phytoplankton density. We confirm the accuracy of our adopted threshold detection method by observing the changes in the coefficient of variation. The results show that the position of the breakpoint that is obtained by the threshold detection analysis is consistent with the position of the change in the coefficient of variation, which demonstrates that the detection path of "finding edge scattered points-piecewise regression" is reliable in this study. In fact, in addition to the scatter of zooplankton-phytoplankton densities, scatter plots showing this "L"-shaped distribution are common in aquatic ecosystems (Cade and Schroeder, 1999; Wang et al., 2007). This type of scatter plot clearly shows a non-linear relationship between two variables. When the number of samples is sufficient, the analysis path proposed in this paper is an important threshold detection method that is worth considering.

This paper mainly studies the control effect of zooplankton on the phytoplankton abundance (especially cyanobacteria), and other important factors that affect cyanobacterial abundances need to be excluded during the analysis. Water temperatures, TP concentrations, and residence times are considered to be the main environmental factors that affect cyanobacterial abundances (Cha et al., 2017). In this paper, the sampling is limited to slow-flowing waters, so the effect of residence time on cyanobacteria abundance is not considered. We analyzed the interference effects of water temperatures and TP concentrations on the cyanobacterial control of cladocera. The results (**Figure 7**) showed that the average TP concentration in the GC group, with a lower cyanobacterial abundance, was significantly higher than that in the GA group with a high cyanobacterial abundance, while the average water temperature of the GC group was not significantly different from that of the GA group. This indicated that the lower cyanobacteria density in the GC group was not caused by water temperature or TP, which ruled out the interference of temperature or TP in this study. Some literatures indicate that bioavailability of nitrogen and phosphorus have different effects on the density of phytoplankton (Tarapchak and Moll, 1990). For example, the density of phytoplankton was highly related to the concentrations of nutrients of reactive nitrogen and phosphorus (Crossetti et al., 2013). The concentrations of various forms of nitrogen and phosphorus were not tested for this survey. The effect of reactive nitrogen and phosphorus on phytoplankton deserves consideration in subsequent studies.

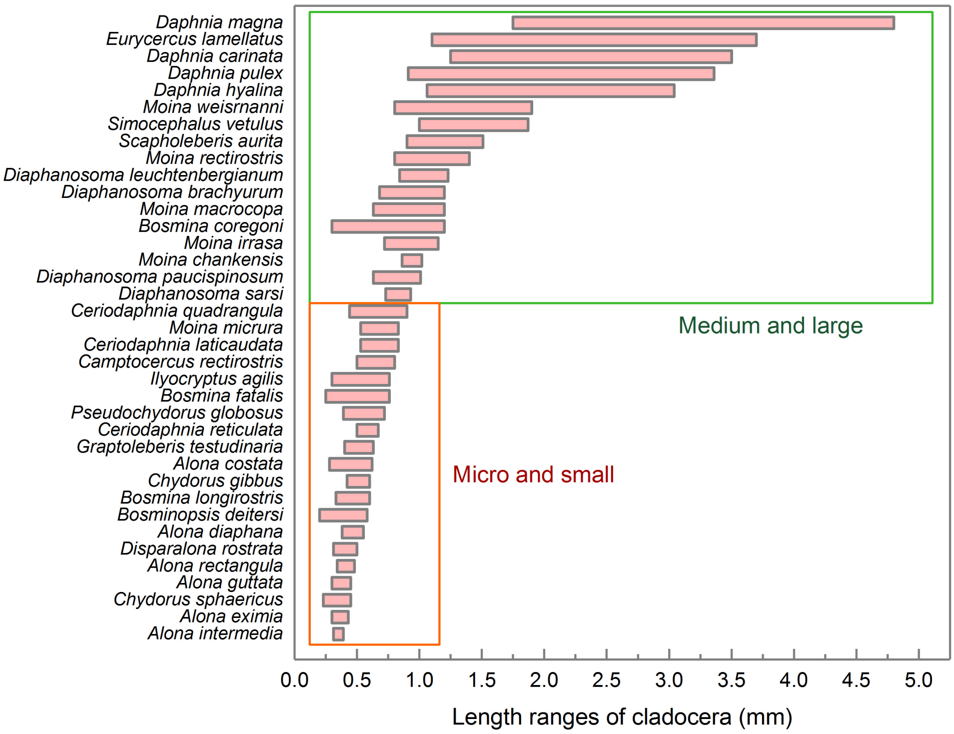

FIGURE 5

Length ranges and classification of various cladocera. The statistics for the body length range included both female and male individuals. The data for the cladocera body lengths come from the China Animal Scientific Database (http://www.zoology.csdb.cn/), which was jointly constructed by the Institute of Zoology, Chinese Academy of Sciences, Kunming Institute of Zoology, Chinese Academy of Sciences, Chengdu Institute of Biology, Chinese Academy of Sciences, Shanghai Entomological Museum, Chinese Academy of Sciences, and Institute of Hydrobiology, Chinese Academy of Sciences.

The quantitative relationship between cyanobacterial abundance and cladocera density may not only be the result of predation by cladocera, but the adaptation of cladocera to cyanobacteria may also have an important impact on this relationship. Some studies suggest that high abundance of cyanobacteria and cyanobacterial toxins will threaten the survival of cladocera (Jiang et al., 2013; Amorim and Moura, 2021), and the density of cladocera may be reduced. At the same time, under the pressure of the harsh external environment, the reproduction and growth strategies of cladocera have changed significantly, and the cladocera will tend to be dominated by small individuals (Li et al., 2017). These could be another explanation for the inverse correlation of cyanobacterial abundance in cladocera densities, as well as differences in cladocera size among groups (i.e., GA, GB, GC groups).

Groups with the best cyanobacteria control ability in zooplankton

Zooplankton is recognized as a key biological group for algae control (Bernardi et al., 1987; Amorim and Moura, 2020), and it is very important for the ecological restoration of eutrophic lakes to identify the most effective groups for cyanobacteria control in zooplankton. In addition, since most of the previous studies focused on the abundance or biomass of zooplankton or phytoplankton as a whole, such binary quantitative relationship analysis is often difficult to identify non-linear characteristics in complex systems. Based on the threshold detection and the screening of groups, the analysis results in this paper support that in wild ecosystems, cladocera may be the group with the strongest ability to control the abundance of cyanobacteria in zooplankton.

Although the reliability of threshold detection in protozoa-cyanobacteria is good, the cyanobacteria density eventually increases with the increase of protozoa density, which indicated that the potential control effect of protozoa on cyanobacteria was unstable. Both rotifer and copepod seem to have some control effect on cyanobacteria density, but the larger AIC and SE/Est. values suggest that the potential control effect is not very significant. Cladocera seem to have the best control ability on cyanobacteria abundance. When the cladocera density was greater than 7.5 ind/L, the cyanobacteria density was maintained at a very low level, and the maximum cyanobacteria density decreased slowly with the increase of cladocera density. When the cladocera density changes from large to small, and

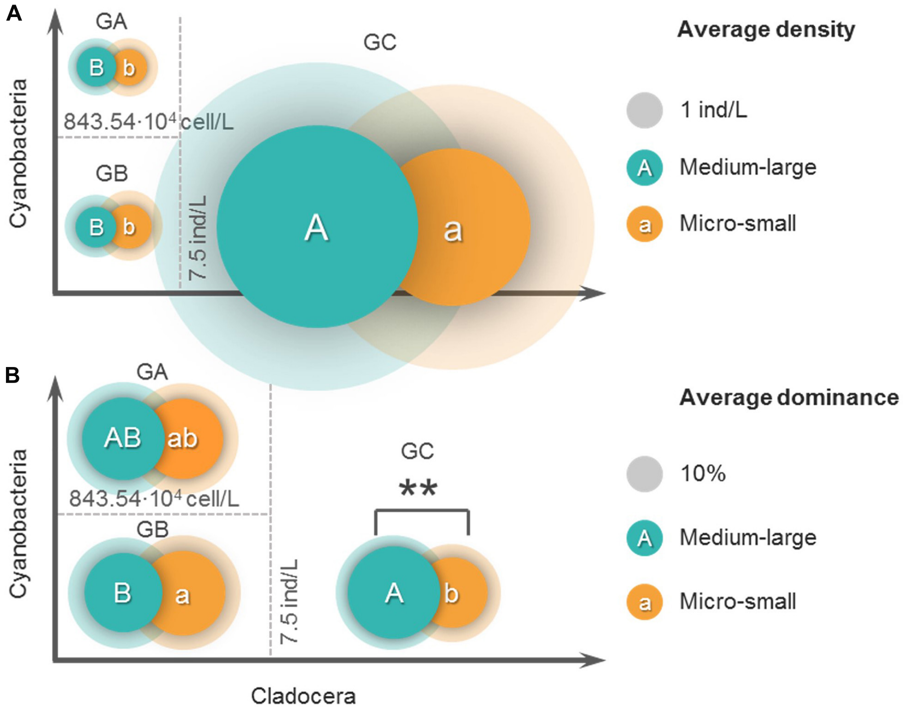

FIGURE 6
Comparisons of the average density **(A)** and average dominance **(B)** of the two sizes of cladocera in the three groups. Before grouping, the samples with zero cladocera density in the original samples were eliminated. All samples are divided into three groups based on the threshold value. GA: cladocera density < 7.5 ind/L (cladocera density corresponding to the breakpoint position of piecewise regression), and cyanobacteria density > 843.54 10^4 cell/L (cyanobacteria density corresponding to the breakpoint position of piecewise regression). GB: cladocera density < 7.5 ind/L and cyanobacteria density < 843.54 10^4 cell/L. GC: cladocera density > 7.5 ind/L. The capital letters A and B in the circles represent the results of the LSD *post hoc* test for the comparison among the groups of medium-large cladocera, and the lowercase letters a and b in the circles represent the LSD *post hoc* test results for the comparison among the groups of micro-small cladocera. The overlapping of two circles indicates that a comparison of the inner groups was carried out, ** indicates that the result of the comparison of the inner groups is extremely significant ($P < 0.01$), and those without ** indicate that the differences in the inner groups are not significant ($P > 0.05$). The opaque circles represent the average values, and the transparent rings represent the standard deviations. The areas of the circles and rings represent the averages and standard deviations, respectively.

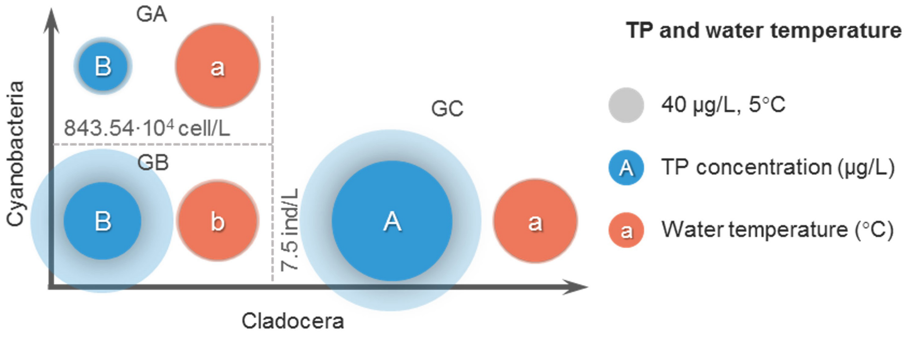

FIGURE 7
Average comparison of TP and water temperature among the three groups. No intragroup comparisons were made in this comparison.

the density is close to or less than 7.5 ind/L, the fluctuation amplitude of the cyanobacteria density begins to increase rapidly. This shows that the control effect of cladocera on cyanobacteria is non-linear as the cladocera abundance changes.

In fact, similar non-linear changes may exist in many forms in aquatic ecosystems. Through the quantitative study of these non-linear changes, it may be easier to reveal the mechanism of complex change in the whole system.

The role of body size and abundance in the cyanobacterial control of cladocera

Through the comparative analysis of grouped samples based on thresholds, the results of this study show that in wild ecosystems, the cladocera-cyanobacteria predation relationship generally follows the "size-efficiency" hypothesis. However, the role of cladocera body size in cyanobacteria control is not very prominent. In the GC group (the samples in which the cyanobacteria abundances were effectively controlled), the average dominance of medium-large cladocera was significantly greater than that of micro-small cladocera ($P < 0.01$). The average dominance of medium-large cladocera in the GC group was greater than that in the GA group (the samples in which cyanobacteria abundance was uncontrolled), but the difference was not significant ($P > 0.05$). In addition, the average density of medium-large cladocera in the GC group was higher than that of micro-small cladocera, and the difference was not significant ($P > 0.05$). This result indicates that when cladocera control the abundance of cyanobacteria, the role of medium-large cladocera is indeed greater than that of micro-small cladocera, but this gap is not overwhelming, and micro-small cladocera also play an important role. This result suggests that the benefits of ecological governance strategies by pursuing the proportion of large cladocera among cladocera may be limited.

The overall abundances of cladocera in wild ecosystems may have a greater impact than individual body sizes on the control efficiency of cyanobacteria abundances. In fact, the average densities of both medium-large cladocera and micro-small cladocera were much larger in the GC group than in the GA group (**Figure 6A**). This indicates that the higher density of cladocera may be the overwhelming reason for the effective control of cyanobacteria abundances in the GC group. This means that in wild ecosystems, cladocera can effectively control cyanobacteria abundances and depend on sufficient densities, regardless of the proportions of large cladocera. That is, the strategy that cladocera may adopt when controlling cyanobacteria is "win by quantity" rather than "win by size."

Significance of the non-linear quantitative relationship between cladocera and cyanobacteria

The above analysis suggests that increasing the abundances of cladocera should be considered a priority when restoring zooplankton communities to reduce the risk of cyanobacteria blooms in temperate freshwater ecosystems. Increasing the density of cladocera above 7.5 ind/L can be an important goal of water environment management. In slow-flowing waters, the greater the abundance of cyanobacteria is, the higher the risk of cyanobacteria blooms. According to the results of analyzing 242 sampling points in North China (**Figure 3G**), when the cladocera density is greater than 7.5 ind/L, cladocera can effectively control the cyanobacteria abundance to a low level, which significantly reduces the risk of cyanobacteria blooms. When the cladocera densities fell below this threshold, the likelihood of cyanobacteria blooms rose rapidly. Therefore, cladocera densities that are close to or below the threshold can be used as an important signal for early warnings of cyanobacterial blooms. It should be noted that lower cladocera densities do not necessarily mean that cyanobacteria will grow rapidly or that blooms will occur. Even with very low cladocera densities, the cyanobacteria densities may be very low (**Figure 3G**). This means that although the cladocera densities may be able to predict the changing trends of the fluctuations in cyanobacteria abundance, they cannot predict the specific cyanobacteria abundance values in wild ecosystems. In addition, the predation of cladocera on cyanobacteria and the species/morphotypes response strategies of cladocera to cyanobacterial differ in different climate regions (Jeppesen et al., 2020; Amorim et al., 2020). Therefore, studies on the quantitative relationship between cladocera and cyanobacteria in different climatic regions may yield significantly different conclusions.

The unique non-linear relationship between the cladocera density and cyanobacteria density can be well explained by chaos theory. Chaos theory holds that when the external pressure crosses a certain threshold, the system state bifurcates, and the system enters a chaotic state from a stable state (May, 1976; Scheffer, 2009). After entering a chaotic state, even small differences in the initial conditions will eventually cause very large differences in the system state over time. For the case analyzed in this paper, the sites had similar water temperatures in both the GA and GC groups and even higher nutrient levels in the GC group, and in fact, these sites had the potential for cyanobacterial blooms. However, in the GC group, due to the high cladocera densities, these cladocera maintained a strong predation ability on cyanobacteria, the potential for cyanobacteria to grow rapidly was suppressed, and the amplitudes of the fluctuations in cyanobacteria densities remained within a very small range. When the cladocera density was below the threshold value, the predation effect of cladocera on cyanobacteria was not sufficient to offset the effects of other environmental conditions on the cyanobacteria density. As the cladocera density decreased, the effects of the differences in initial environmental conditions (such as TP, and water temperature) on the cyanobacteria density increased almost exponentially. The amplitudes of the fluctuations in cyanobacteria density increase sharply, which is a phenomenon that indicates that the cladocera density cannot be used to constrain and predict cyanobacteria abundances. The cyanobacteria control threshold of cladocera may be the critical point at which the cladocera-cyanobacteria predation relationship changes from a stable state to a chaotic state.

According to the above description, we cannot predict the specific values of cyanobacteria densities based on cladocera when the cladocera densities are very low. However, even in the "chaotic" state, we can still see that the maximum cyanobacterial density can reach a clear boundary, that is, the amplitudes of the fluctuations in the cyanobacteria densities can be predicted. This means that although precise predation dynamics cannot be predicted, the ranges of these predation behaviors can be described and can be predicted to remain constant as long as external conditions do not change (Scheffer, 2009). In fact, there are many non-linear relationships that are similar to the cladocera-cyanobacteria relationship, and more non-linear studies should be conducted in the future to reveal the complex mechanisms in natural aquatic ecosystems and improve the human ability to understand and respond to complex changes.

Ecological thresholds need to be verified by a large number of data and cases in order to achieve successful management applications. Although this paper investigates a large number of sample sites, this conclusion is limited to specific regions and climatic conditions. In the future, the quantitative non-linear relationship between cladocera and cyanobacteria should be verified under more climate types to obtain more general rules.

Conclusion

The purpose of this paper is to obtain a quantitative relationship between cladocera and cyanobacteria in wild ecosystems. According to the verification results of the coefficient of variation, the threshold detection path of "finding edge scattered points-piecewise regression" proposed by us is an effective method for detecting the tipping points in "L"-shaped scatters. The results of the screening analysis of groups showed that cladocera comprised the group with the best ability to control cyanobacterial abundances among zooplankton. The threshold detection results showed that the changes in cyanobacterial density with changes in cladocera density had obvious non-linear characteristics. There is a specific cladocera density (7.5 ind/L); when the cladocera density is less than this critical point, the amplitudes of the fluctuation in cyanobacteria densities will increase sharply. The analysis of TP and water temperature showed that these two important environmental conditions did not have significant disturbing effects in the analysis in this paper. The results of the analysis of the effect of body size shows that the body sizes of cladocera do play a role in controlling the cyanobacteria density, but the most critical factor for the success of cyanobacterial control is the greater density, rather than the dominance of large cladocera. Our study provides some evidence for understanding the complex changes in aquatic ecosystems, especially the occurrences and control mechanisms of cyanobacterial blooms. The threshold for cladocera being able to control cyanobacteria proposed by us may have important application value in water environment management. This threshold also provides a quantitative standard for early warnings of cyanobacteria blooms in temperate, shallow, and slow-flowing rivers.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

DL: conceptualization, data curation, formal analysis, investigation, methodology, resources, software, validation, and writing—original draft. PH: conceptualization, investigation, funding acquisition, methodology, supervision, validation, and writing—review and editing. CL and JX: writing—review and editing and investigation. LH, XG, DW, and JW: writing—review and editing. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by Biodiversity Survey and Assessment Project of the Ministry of Ecology and Environment, China (2019HJ2096001006).

Acknowledgments

We would like to thank Junxia Wang, Yajuan Zhang, Chunchen Li, Mingyao Li, Shiwei Shan, and others for their assistance in the field survey.

Conflict of interest

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

Publisher's note

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ORIGINAL RESEARCH
published: 15 July 2022
doi: 10.3389/fevo.2022.899993

Drivers of Spatiotemporal Eukaryote Plankton Distribution in a Trans-Basin Water Transfer Canal in China

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Specialty section:
This article was submitted to
Conservation and Restoration
Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 19 March 2022
Accepted: 17 June 2022
Published: 15 July 2022

Citation:
Li Y, Khan FH, Wu J, Zhang Y, Jiang Y, Chen X, Yao Y, Pan Y and Han X (2022) Drivers of Spatiotemporal Eukaryote Plankton Distribution in a Trans-Basin Water Transfer Canal in China. Front. Ecol. Evol. 10:899993. doi: 10.3389/fevo.2022.899993

Planktonic eukaryotes are important components of aquatic ecosystems, and analyses of the whole eukaryotic planktonic community composition and function have far-reaching significance for water resource management. We aimed to understand the spatiotemporal variation and drivers of eukaryotic plankton distribution in the Middle Route Project of the South-to-North Water Diversion in Henan Province, China. Specifically, we examined planktonic assemblages and water quality at five stations along the canal and another one located before the dam in March, June, September, and December 2019. High-throughput sequencing revealed that the eukaryotic plankton community was primarily composed of 53 phyla, 200 genera, and 277 species, with Cryptophyta, Ciliophora, and norank_k_Cryptophyta being the dominant phyla. Redundancy analysis of the eukaryotic community and environmental factors showed that five vital factors affecting eukaryotic plankton distribution were oxidation-reduction potential, nitrate nitrogen, pH, total phosphorus, and water flow velocity. Furthermore, the geographical distribution of eukaryotic communities was consistent with the distance decay model. Importantly, environmental selection dominantly shaped the geographical distribution of the eukaryotic community. In summary, our study elucidates the ecological response of planktonic eukaryotes by identifying the diversity and ecological distribution of planktonic eukaryotes in trans-basin diversion channels.

Keywords: eukaryotic plankton, high-throughput sequencing technology, the Middle Route Project of South-to-North Water Diversion, redundancy analysis, distance-attenuation analysis

INTRODUCTION

The Middle Route Project (MRP) of the South-to-North Water Diversion (SNWD) in China is the world's longest inter-basin water diversion project, with a total length of 1,432 km. Since water diversion started in 2014, more than 44 billion m^3 of water has been diverted to northern China through open channels, directly benefiting 140 million people and greatly alleviating the water resource shortage in northern China. Since the MRP canal, which is a concrete-bottom channel, does not intersect with other rivers, it is considered as a new closed artificial aquatic system. The community structure of canal planktonic eukaryotes is currently undergoing dynamic evolution and is dependent on the hydrological, hydrodynamic, and nutrient factors. Moreover, the rapid proliferation of biomass of specific groups occurs during specific periods and affects water quality, thereby leading to potential ecological problems (Liang et al., 2017). Therefore, safe and high-quality water in the MRP of the SNWD is a priority and should be ensured by studying the distribution of the planktonic eukaryote community in the MRP canal.

Planktonic eukaryotes primarily include phytoplankton and zooplankton; both are important links that play central roles in the energy flow and material cycle functional in aquatic ecosystems (Yang et al., 2014; Yan et al., 2020). Phytoplankton and zooplankton participate in the biogeochemical processes of aquatic ecosystems, such as the material cycles, by releasing pollutants through catabolism and synthesis. Considering that distribution characteristics and ecological functions provide crucial insights for the management and maintenance of an ecosystem (Bunse and Pinhassi, 2017), studying the community composition and distribution of planktonic eukaryotes is a high-priority area of aquatic ecosystem research.

Aquatic microorganisms are sensitive to water quality and are important indicators for biological monitoring and water quality evaluation. Studies have shown that the community structure of planktonic fungi is affected by water type, pH, temperature, electrical conductivity, and physical-chemical properties of organic matter, including nitrogen and phosphorus levels. Previous research involving cloning and sequencing data from GenBank has confirmed that variations in the nutritional status of the aquatic body altered the community composition of planktonic eukaryotes (Lefranc et al., 2005). This indicated that planktonic eukaryotes can be used to infer the nutrient status of the water body.

Planktonic eukaryote classification has traditionally been performed by optical microscopy and is influenced by multiple factors including sampling conditions, preservation techniques, and the substantial inherent variation and divergence in these organisms, especially at the species level. With the development of DNA sequencing technology (Philippe et al., 2015; Goodwin et al., 2016), high-throughput sequencing has been widely applied in biological evaluation, including the assessment of freshwater microflora and fauna (Stein et al., 2014; Visco et al., 2015; Su et al., 2017; Needham et al., 2018). Compared to traditional taxonomic methods, high-throughput sequencing technology can identify specific DNA fragments efficiently and quickly, providing more reliable and richer biological information (Keck et al., 2017; Vasselon et al., 2017; Zhang et al., 2017).

Few studies have been conducted on the MRP channel (Nong et al., 2020). Previous studies primarily focused on the water source area and the upstream tributaries (Chen et al., 2018; Han et al., 2021). Due to the unique channel structure, low nutrient levels, and continuously disturbed hydrological characteristics, the water conditions of the main MRP canal differ from those of the Danjiangkou Reservoir. Therefore, this study explored the community characteristics and succession rules of planktonic eukaryotes during trans-basin long-distance water diversion; here, we aimed to reveal the key factors driving their evolution to facilitate the prediction of water quality in the MRP of the SNWD. Specifically, we used high-throughput sequencing technology to survey the planktonic eukaryote community structure and to analyze the environmental factors affecting community succession in the MRP in Henan Province, China. These results will provide empirical support for scientific operations and management in similar aquatic conservation projects.

MATERIALS AND METHODS

Study Area

This study was conducted on the MRP of the SNWD, which starts from Taocha (TC) of Danjiangkou Reservoir (32.67°N–39.98°N, 111.71°E–116.27°E), with an average width of 54 m and an average water depth of 6.95 m. The MRP flows northward for 1,276 km through four provinces to Tuancheng Lake in Beijing, the capital of China. On average, 9.5 billion m^3 of water is diverted annually (Zhang et al., 2021). The MRP of the SNWD spans the subtropical humid climate zone and the temperate monsoon climate zone, with distinct seasonal temperature variations and annual average precipitation ranging between 542.7 and 1173.4 mm. Six sampling stations were set up along the MRP of the SNWD to collect and analyze water samples in the canal in Henan Province every 3 months (**Table 1** and **Figure 1**). In 2019, four samples were collected in March (spring), June (summer), September (autumn), and December (winter). Hydrological data were obtained from the management offices located along with the MRP.

Determination of Physical and Chemical Factors of Water Samples

The water samples in this study were collected in accordance with surface water sampling standards under sunny or cloudy weather conditions to minimize the impact of rainfall or other extreme weather on water quality. Physical and chemical variables were determined according to China Surface Water Environmental Quality Standard (GB3838-2002). water temperature (WT), pH, dissolved oxygen (DO), electrical conductivity (Cond), and oxidation-reduction potential (ORP) were measured in the field with a YSI 6920 (YSI Inc., Yellow Springs, OH,

TABLE 1 | Locations of sampling stations in the MRP of the SNWD in Henan Province.

City/Country	Station	Code	Distance from the danjiangkou reservoir (km)
Danjiangkou Reservoir	Qushou	QS	0
Xichuan Country	Taocha	TC	1
Yexian Country	Yexian	YX	190
Zhengzhou City	Zhengzhou	ZZ	320
Jiaozuo City	Jiaozuo	JZ	380
Anyang City	Anyang	AY	510

The distance in the table represents the straight-line distance from each sampling station to the Danjiangkou Reservoir.

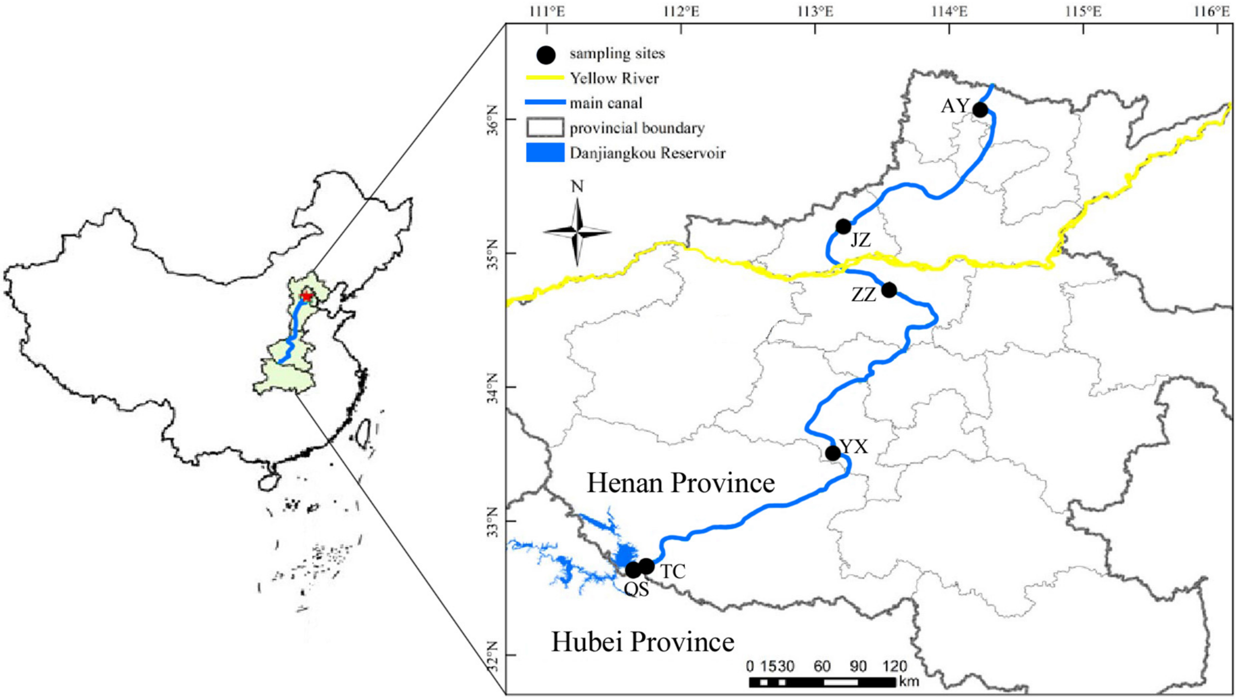

FIGURE 1 | Locations of sampling stations in the MRP of the SNWD.

United States). Two-liter water samples were collected using a sterilized column water extractor and sent to the laboratory for chemical analysis within 24 h. Permanganate index (COD_{Mn}) was determined using the potassium permanganate digestion titration method. Chlorophyll a (Chl a) concentration was spectrophotometrically measured after extraction in 90% ethanol. Total phosphorus (TP) was determined with acidified molybdate to form reduced phosphomolybdenum blue, which was spectrophotometrically measured. Total nitrogen (TN) was assayed using alkaline persulfate digestion and UV spectrophotometry, while ammonia nitrogen (NH_4^+-N) and nitrate-nitrogen (NO_3^--N) were measured by the spectrophotometric method using Nessler's reagent and thymol spectrophotometry, respectively. Total organic carbon (TOC) was determined using a TOC analyzer (Multi N/C 3100, Analytik Jena Ltd., Germany), concerning the environmental protection standard "Determination of water quality of total organic carbon combustion oxidation—non-dispersive infrared absorption method" (HJ501-2009).

DNA Extraction and Sequencing of Planktonic Eukaryote Samples

First, 1,500 ml of each water sample was filtered through 0.22-μm acetate fiber sterile microporous membrane, and each sample was filtered three times. After filtration, the filter membrane was cut into blocks and placed in a 50-ml sterile centrifugal tube for eukaryotic plankton collection. The total DNA in the sample was extracted using an Omega Water DNA Kit (Omega Bio-Tek, Norcross, GA, United States). The concentration and purity of extracted DNA were determined using a micro-ultraviolet spectrophotometer. To obtain target DNA sequences of different samples, the V4 region of 18S rDNA was obtained from the floating eukaryotes using the universal primers SSU0817F (5′-TTAGCATGGAATAATRRAATAGGA-3′) and 1196R (5′-TCTGGACCTGGTGAGTTTCC-3′). PCR was carried out using TransStart FastPfu DNA Polymerase (TransGen AP221-02; TransGen Biotech) in a 20-μl reaction volume. The reaction conditions for PCR amplification were 95°C for 3 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; 72°C for 10 min; and 10°C until stopped. The PCR mixture included 5 × FastPfu Buffer (4 μl), 2.5 mM dNTPs (2 μl), 5 μM forward primer (0.8 μl), 5 μM reverse primer (0.8 μl), FastPfu Polymerase (0.4 μl), BSA (0.2 μl), and DNA template (10 ng), with ddH_2O added to attain a final volume of 20 μl. The amplification products of different samples were homogenized to a final concentration of 10 nmol/L and then mixed in the same volume. The sequencing was performed by Shanghai Meiji Biomedical Technology Co., Ltd. (Shanghai, China).

Analysis of Planktonic Eukaryote High-Throughput Data

The extracted DNA samples were sent to Shanghai Meiji Biomedical Technology Co., Ltd., for purification and MiSeq amplification. Flash 1.2.11 was used for paired-end sequence splicing and Fastp 0.19.6 was used for quality control; chimeras were removed to obtain the optimized sequences. The optimized sequences were clustered using Uparse in the SILVA 128/18S eukaryotic database by operational taxonomic unit (out) clustering; an OTU abundance table was established for subsequent analysis, and quantitative insights for microbial ecology (QIIME) was used for bioinformatics analysis. According to the sequence similarity, the ribosomal database project (RDP) classification algorithm was used to classify OTUs with 97% similarity, and the community composition of each sample was evaluated at each classification level. In addition, mothur was used to calculate the Chao, Ace, Simpson, and Shannon index values for alpha diversity analysis. STAMP was used to compare and analyze the differences among eukaryotic community structures of different samples ($P < 0.05$).

Data Analysis

All data were processed using Excel 2019 (Microsoft Corporation, Redmond, United States). SPSS 25 (IBM SPSS Inc., Chicago, IL, United States) was used to calculate the mean and *SD* of environmental factors. Detrended correspondence analysis of MiSeq sequencing OTU data showed that the longest gradient length was less than 3; thus, redundancy analysis (RDA) was performed next. Mantel analysis was used to analyze the relationship between eukaryotic community diversity and water physicochemical parameters. Non-metric multidimensional scale analysis (NMDS) and correlation analysis were used to evaluate the community distribution of planktonic eukaryotes. Both NMDS and Mantel analyses were conducted in R 4.0.4 (R Programming Language, University of Auckland, New Zealand). Using the "vegan" package in R, variance partitioning analysis (VPA) and variance decomposition analysis were used to calculate the interpretation rate for geographical distance and environmental selection of the planktonic eukaryote community structure in the canal in Henan Province. The geographic distance based on the principal coordinates of neighbor matrices (PCNM) variables was obtained from the longitude and latitude of each sampling point calculated using the PCNM function of the "vegan" package in R. The Bray–Curtis (community similarity) distance matrix was calculated using the vegdist function of the "vegan" package in R to determine relative species abundance and environmental factors at each sampling site. ArcGIS 10.7 (ESRI, Redlands, CA, United States) was used as a spatial analysis tool to visualize the sampling stations of the canal in Henan Province.

TABLE 2 | Physico-chemical variables of the MRP of the SNWD in different seasons.

Season	Spring	Summer	Autumn	Winter
WT (°C)	8.08 ± 1.65	25.30 ± 2.29	26.93 ± 1.05	12.68 ± 1.67
pH	8.72 ± 0.16	8.73 ± 0.10	8.77 ± 0.34	8.58 ± 0.20
Cond (uS/cm)	258.67 ± 5.28	285.75 ± 5.96	254.28 ± 5.97	204.25 ± 25.41
ORP (mv)	207.00 ± 37.14	203.45 ± 29.96	134.72 ± 22.90	156.70 ± 46.05
DO (mg/L)	8.69 ± 1.03	8.51 ± 0.39	7.89 ± 0.80	9.85 ± 0.88
TN (mg/L)	1.13 ± 0.28	1.27 ± 0.47	1.18 ± 0.25	1.48 ± 0.12
TP (mg/L)	0.007 ± 0.001	0.006 ± 0.002	0.009 ± 0.003	0.003 ± 0.004
COD_{Mn} (mg/L)	2.04 ± 0.35	1.85 ± 0.30	1.89 ± 0.52	1.97 ± 0.45
TOC (mg/L)	4.15 ± 0.45	4.01 ± 1.32	4.01 ± 0.91	3.2 ± 1.70
NH_4^+-N (mg/L)	0.03 ± 0.01	0.10 ± 0.06	0.11 ± 0.04	0.04 ± 0.03
NO_3^--N (mg/L)	0.74 ± 0.24	0.84 ± 0.13	0.92 ± 0.20	1.08 ± 0.34
Chla (mg/L)	0.78 ± 0.51	1.27 ± 0.36	1.16 ± 0.76	1.54 ± 0.34
Vel (m /s)	0.57 ± 0.64	0.56 ± 0.30	0.69 ± 0.15	0.52 ± 0.66
Flow (m^3/s)	135.08 ± 30.36	129.65 ± 27.24	161.4 ± 22.03	126.12 ± 36.91

RESULTS

Analysis of Physical and Chemical Properties

During the investigation period, all variables except TN were consistent with the classes I–II water quality of the Surface Water Environmental Quality Standard (GB38382-2002). The mean and standard deviation (SD) of the environmental factors at the six sampling stations are shown in **Table 2**. WT, Cond, ORP, DO, TP, and NH_4^+-N greatly fluctuated by season throughout the year, whereas other variables showed no significant fluctuation between seasons (**Table 2**).

Community Structure Analysis of Planktonic Eukaryotes

A total of 3,504,894 optimized sequences with an average length of 378 bp were obtained from 72 samples collected from six stations in this study (**Figure 2**).

The plankton eukaryotes of the canal included 53 phyla, 158 families, 200 genera, 277 species, and 754 OTU, among which Cryptophyta, Ciliophora, and norank_k_Cryptophyta were the dominant phyla (**Figure 2**). Among the OTUs observed from QS to YX, those from YX to ZZ gradually increased, whereas those from YX to ZZ gradually decreased. Additionally, the greatest number of OTUs was detected in summer and the fewest in autumn. Of the various eukaryotes, the relative abundance of Cryptophyta was the highest in QS (41.83%) and the lowest in JZ (18.54%, **Figure 2i**). The relative abundance of unclassified_d_Eukaryota was the highest in TC (20.37%) and the lowest in JZ (18.54%). The relative abundance of Cryptophyta and unclassified_d_Eukaryota decreased with increased water transport distance. The relative abundance of Ciliophora was the lowest in QS (9.65%) and the highest in JZ (40.02%). The relative abundance of norank_k_Cryptophyta was the lowest in TC (9.72%) and the highest in ZZ (25.37%). The relative abundance of Ciliophora and norank_k_Cryptophyta gradually increased with the distance of water transfer (**Figure 2i**). The relative abundances of Ascomycota and Choanoflagellida at the AY sampling station were the highest (7.94 and 8.16%, respectively) and were significantly higher than those at the other sampling stations.

Regarding seasonal changes, the relative abundance of Cryptophyta was higher in summer (44.57%, **Figure 2ii**). The relative abundance of Ciliophora was lowest in autumn (13.57%), whereas those of Choanoflagellida and norank_k_Cryptophyta were the highest in autumn (8.31 and 31.89%, respectively). Other seasonal variations were not significant. Among them, the relative abundance of Kathablepharidae peaked in spring

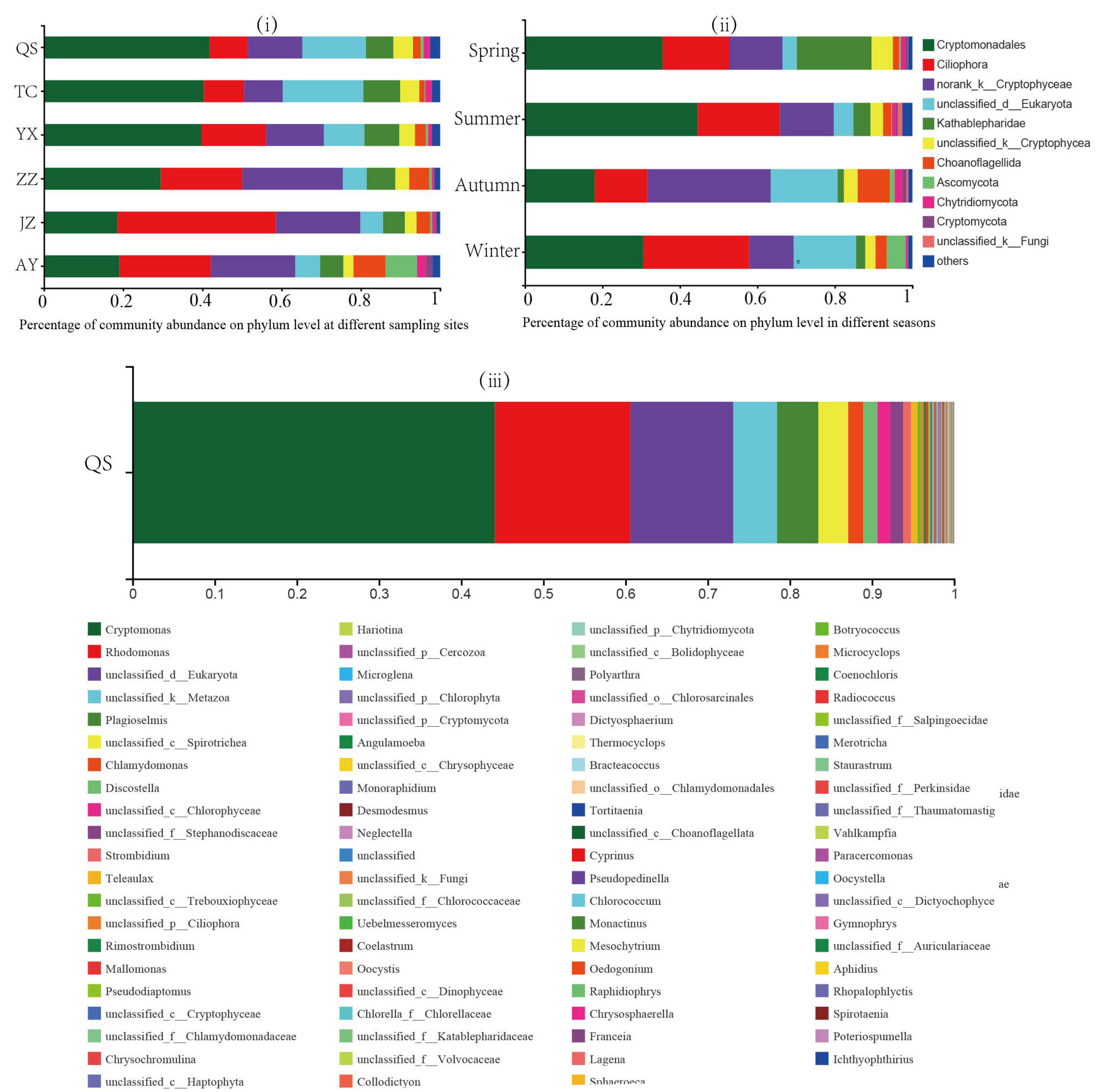

FIGURE 2 | Percent (%) of community abundance at the phylum level. Each color represents a plankton eukaryote species. Community structure of planktonic eukaryotes **(i)** at each sampling station and **(ii)** in each season, and **(iii)** community abundance at QS sampling points.

(19.25%), which was significantly higher than that in other seasons. The relative abundance of Ascomycota was 0.24% in summer and 4.82% in winter.

Among these sampling sites, we detected the percentage of community abundance at the genus level at the QS sampling site (**Figure 2iii**). Among them, Cryptomonas accounted for a large proportion (44.11%), followed by Rhodomonas, accounting for 16.50%.

Analysis of Planktonic Eukaryote Alpha Diversity

The abundance and diversity of planktonic eukaryotes of the canal in Henan Province differed among seasons (**Figure 3**). Ace and Chao indices of AY were the lowest in winter, Shannon and Simpson indices of ZZ were the lowest in autumn, and Ace, Chao, Shannon, and Simpson indices of QS and TC were the highest in summer (**Figures 3iii,iiii**). The Shannon and Simpson diversity indices of TC and QS were higher in summer than those of other stations and seasons, which indicated that compared with the changes in hydrological conditions, the eukaryotic community of TC was more significantly affected by the Danjiangkou Reservoir. As water transport distance increased, the number of species in the planktonic eukaryote community gradually decreased (**Figures 3i,ii**).

Non-metric Multidimensional Scale Analysis of Planktonic Eukaryotes in the Canal in Henan Province

Adonis analysis showed that there were significant differences among eukaryotic communities in different seasons (R^2 = 0.41, P < 0.001). The NMDS results of the planktonic eukaryote OTUs at the six sampling stations showed that there were seasonal changes in the planktonic eukaryote community of the canal in Henan Province (**Figure 4**). The planktonic eukaryote community gathered in spring, which differed from other seasons; in spring, the dispersion degree of eukaryotic communities was higher, and the community composition varied greatly among the samples.

At the spatial scale, the eukaryotic communities of AY and JZ were quite different from those of other stations. The eukaryotic communities in TC and QS had low similarity in autumn, but high similarity in other seasons. The eukaryotic communities of YX and AY had the highest similarity in spring and the lowest similarity in autumn. Overall, the community structure of planktonic eukaryotes greatly differed across both temporal and spatial scales.

Redundancy Analysis of the Planktonic Eukaryote Community and Environmental Factors

Variance inflation factors were used to analyze the collinearity of environmental factors. The environmental factors with a variance inflation factor of > 10 were removed, and the factors with relatively low collinearity were retained. Detrended correspondence analysis showed that the longest gradient length for the four axes was less than 3; therefore, the RDA linear model

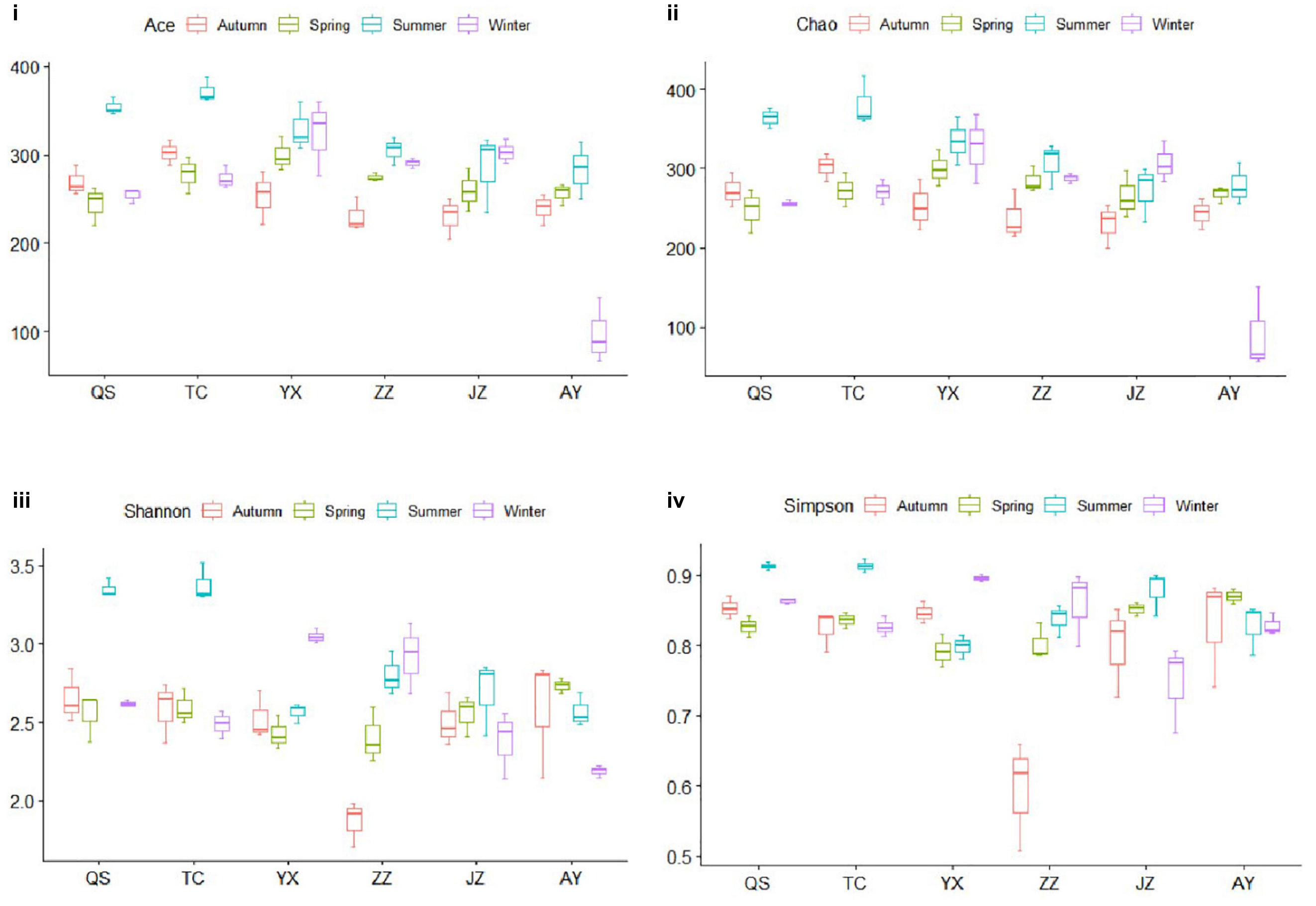

FIGURE 3 | Evaluation of alpha diversity. The Ace index **(i)** represented richness and evenness, the Chao index **(ii)** represented the number of OTUs in the samples, and the Shannon and Simpson indices represented community diversity (**iii,iv**, respectively). The boxes with a horizontal line in the figure panels represent the median value, and the upper and lower lines of each box indicate the maximum and minimum values, respectively.

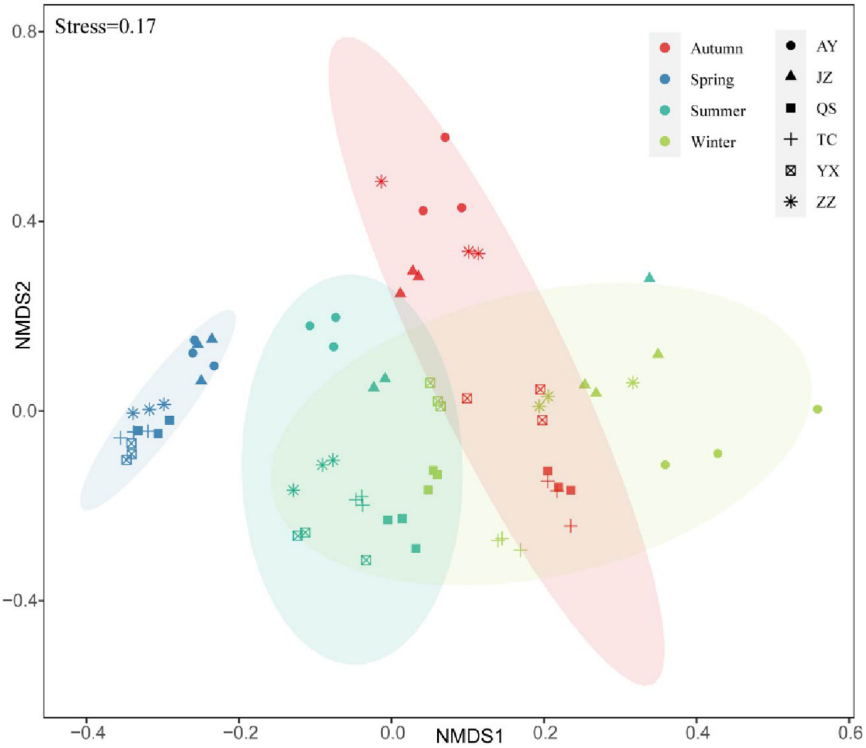

FIGURE 4 | Non-metric multidimensional scale (NMDS) analysis of the planktonic eukaryote community.

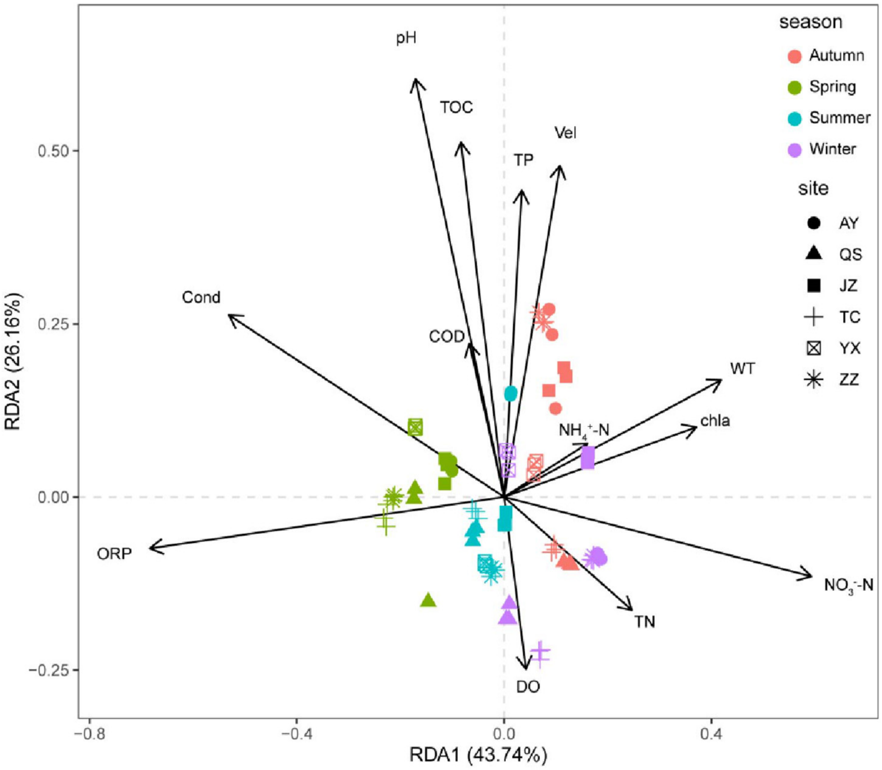

FIGURE 5 | Redundancy analysis (RDA) ranking of planktonic eukaryote community and physicochemical factors of the canal in Henan Province.

was selected. Simultaneously, we eliminated environmental factors that were not significantly related to species ($P > 0.05$). Our RDA results are shown in **Figure 5**. ORP, WT, Vel, NO_3^--N, and pH had significant effects on the eukaryotic community of the canal. The total explanatory variance of the first two RDA axes was 75.98%. ORP, Cond, and DO were the main drivers of spring and summer communities, and ORP had a higher impact on the eukaryotic community in spring and summer than in other seasons. The community in autumn and winter significantly differed from other seasons, and the community in autumn was influenced by multiple environmental factors, such as Vel. The winter community was significantly affected by DO, TP, and WT. At the spatial scale, TC and QS were significantly affected by ORP in spring and summer and DO in winter. The eukaryotic communities at other sampling stations were also affected by different environmental factors in different seasons. Overall, the driving factors of the planktonic eukaryote community in the canal had significant temporal and spatial differences.

Mantel Analysis of the Planktonic Eukaryote Community and Environmental Factors

The Mantel test was used to determine the relationship between environmental factors and species. At the phylum level, ORP, pH, Cond, TP, PCNM2, and distance were the main correlation parameters of eukaryotic community diversity and abundance (**Figure 6**). The pH, Cond, ORP, TP, PCNM2, and distance had significant effects on community abundance ($P < 0.05$). The pH, Cond, ORP, PCNM2, and distance had significant effects on Shannon diversity ($P < 0.05$), and TP had significant effects on Simpson diversity ($P < 0.05$).

Geographical Distribution Patterns of Planktonic Eukaryotes

To further explore the effects of geographical distance and environmental heterogeneity on the eukaryotic community structure of the canal, the linear relationship between the community similarity of plankton and geographical distance and the environmental difference was established and analyzed using distance decay (**Figure 7**). The results showed that eukaryotic community similarity was significantly negatively correlated with both geographic distances (**Figure 7i**, $P < 0.05$) and environmental heterogeneity (**Figure 7ii**, $P < 0.05$), which indicated that the geographical distribution of the planktonic eukaryote community followed the distance decay model, where community similarity gradually decreased with increased geographic distance and environmental heterogeneity. The results of this study also indicated that the distribution of the eukaryotic community was influenced by both dispersal limitation and environmental selection (**Figure 7**).

To clarify the relative effects of season, geographical distance, and environmental heterogeneity on planktonic eukaryote community diversity, VPA was used to calculate the explanation rate of eukaryotic community diversity (**Figure 8**). The

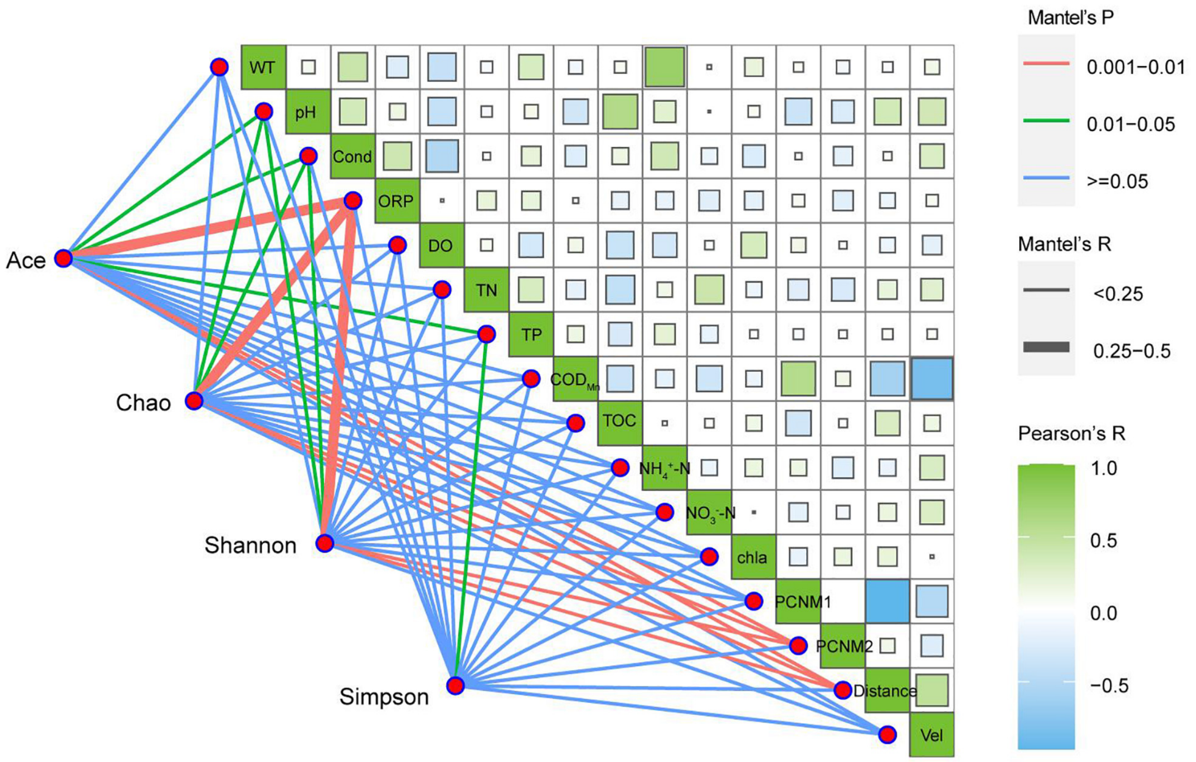

FIGURE 6 | Mantel analysis of alpha diversity in planktonic eukaryotes.

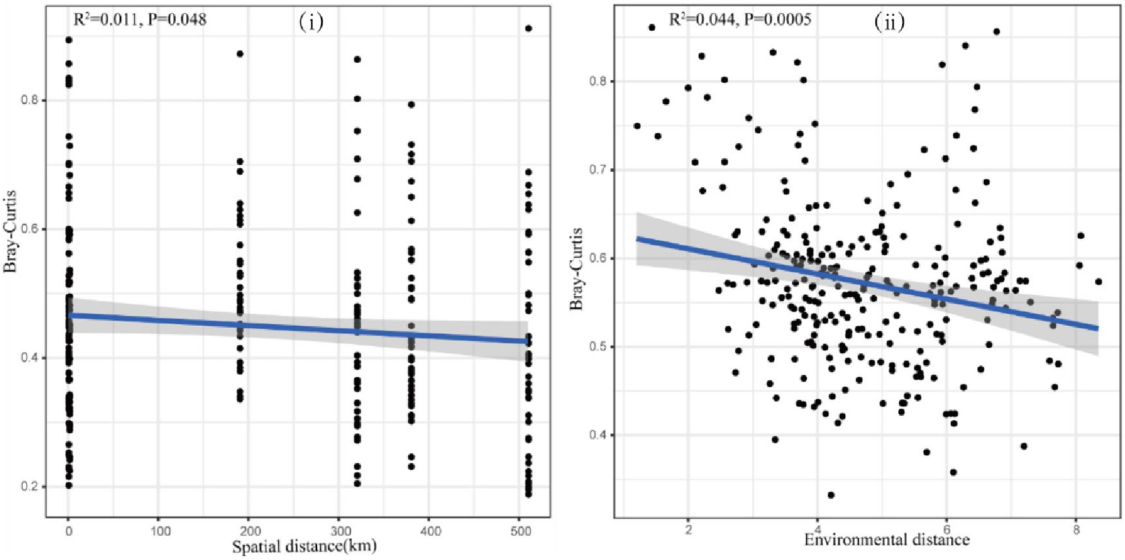

FIGURE 7 | Distance–decay relationship between **(i)** eukaryotes and geographical distance and **(ii)** environmental difference.

results showed that environmental heterogeneity, geographical distance, and seasonality accounted for 46, 8, and 13%, respectively, of planktonic eukaryote community diversity, and 21% of diversity was unexplained. These results indicated that environmental screening contributed more to eukaryotic community diversity than dispersal limitation. Environmental factors and spatial factors accounted for 1% of the eukaryotic community diversity, which was less than environmental factors and season (6%). This finding indicated that the interaction between environmental factors and seasons was greater than that between environmental factors and spatial factors in the process of community construction.

DISCUSSION

In this study, we described the spatio-temporal dynamics of planktonic eukaryotes in the MRP of the SNWD in Henan Province by performing high-throughput sequencing of the SSU rRNA.

As it contains both highly conserved regions needed for primer annealing and variable regions to allow detailed taxonomic classification, and therefore, is routinely used for molecular identification of eukaryotic microorganism species (Goodwin et al., 2016), 18S rDNA gene was chosen as a target here. In addition, the DNA can be easily amplified because of its high copy number (Guillou et al., 2013). High-throughput sequencing can provide more information related to the classification of planktonic eukaryotes than traditional identification methods, such as isolation culture identification and PCR-denaturation gradient gel electrophoresis (Kagami et al., 2012). Therefore, using high-throughput sequencing technology, community structure information of phytoplankton, zooplankton, and other eukaryotes can be obtained quickly and accurately, and the connection between different eukaryotic species can be rapidly integrated. Compared with the study of specific eukaryotes alone (such as phytoplankton and zooplankton), it is more beneficial to understand the integrity of the community structure of planktonic eukaryotes.

Community Composition of Planktonic Eukaryotes

There were 53 phyla and 277 species of planktonic eukaryotes in the Henan Province canal, which comprehensively represented the community composition structure and diversity; this included Cryptophyta, Ciliophora, and norank_k_Cryptophyta

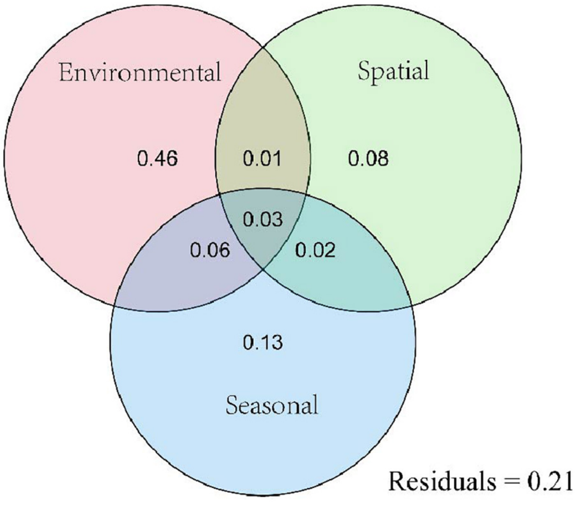

FIGURE 8 | Variance partitioning analysis (VPA) showing the effects of environmental factors, seasons, and geographical distance on eukaryotic communities.

as the dominant phyla. The eukaryotic plankton communities displayed a wide variety of growth characteristics that accounted for their adaptability to the canal environment. Medium-trophic water bodies are reportedly dominated by Cryptophyta, Bacillariophyta, and Pyrroptata, whereas eutrophic water bodies displayed abundant Chlorophyta and Cyanophyta (Tao et al., 2016). After establishing the community structure of plankton in the canal, we next determined the water quality and found it to be moderate. A previous study reported that the trophic degree increased with water transport distance (Chen et al., 2017). Our results concur with this study as we found that with increasing water transport distance along the canal, the relative abundances of Ciliophora, Choanoflagellida, and norank_k_Cryptophyta also increased. However, the relative abundances of Cryptophyta and unclassified_Eukaryota gradually decreased with increased water transport distance. Ciliophora is considered a predator of bacteria but can have a symbiotic relationship with algae (Stoecker et al., 2017; Grujcic et al., 2018; Stoecker and Lavrentyev, 2018). In addition, a previous study reported that the presence of free-living eukaryotes, such as ciliates and microalgae, changed with nutrient status (Lefranc et al., 2005). In agreement, we noticed that as water transport distance increased along the canal, the trophic degree also increased.

Additionally, the eukaryotes present in the canal were closely associated with the nutrient status of the canal. Temporally, eukaryotic communities had higher similarity in spring, whereas it was the lowest in autumn (**Figure 4**). We also observed that the canal WT was lowest in spring. Thus, we hypothesized that low temperatures likely remove some species that thrive at high temperatures, making communities more similar. This was consistent with the lower Shannon and Simpson indices of the eukaryotic community in spring. Cryptophyta was reported to have a higher population density at lower WT (Moreau et al., 2007). A previous study on the phytoplankton niche in Chaohu Lake reported that Cryptophyta displayed a wide range of ecological adaptations and could make full use of the habitat resources (Wang et al., 2010). Our results of lower WT in winter and spring, which made Cryptophyta the dominant species of the canal, are consistent with the results of these past studies. Noticeably, the diversity of samples was the lowest in autumn and the highest in summer (**Figure 4**). Furthermore, our results showed that Cryptophyta significantly and negatively correlated with water velocity ($P < 0.001$, **Figure 6**), and the relative abundance of Cryptophyta was the lowest at higher flow rates in autumn. Moreover, there was a significant negative correlation between Kathablepharidae and WT ($P < 0.001$, **Figure 6**), and the relative abundance of Kathablepharidae was the highest when WT was the lowest in spring. Thus, both WT and Vel exerted important effects on the planktonic eukaryote species in a season-dependent manner.

Effects of Environmental Factors on the Planktonic Eukaryote Community

Previous research indicates that WT is a vital environmental factor affecting the growth and development, community structure, abundance, and horizontal and vertical distribution of microorganisms in aquatic ecosystems (Shi et al., 2020). Furthermore, increased WT accelerates the growth and proliferation of the plankton population, and plankton species exhibit differences in adaptability to WT. It is reported that WT drives the change in dominant species by regulating cell activity and, thus, controls the plankton community structure (Stoecker et al., 2017). Stoecker et al. (2017) noticed that the evenness and diversity of samples in autumn were lower than those in other seasons, but WT was the highest in autumn compared to other seasons. A study evaluating the dynamics and functional roles of algal and bacterial communities in winter in two seasonal snow-covered lakes in Central Europe revealed that the algal biomass of the two lakes in winter was lower than the long-term summer average (Bullerjahn et al., 2020). This observation was in contrast with our results.

Phytoplankton biomass and spatial distribution largely depend on flow conditions. For example, a study of Zhongxin Lake in Shanghai Chongming Island reported that turbulence was negatively correlated with phytoplankton biomass, and flow velocity also showed a strong inverse correlation with Chl a concentration (Li et al., 2013). An inhibitory effect of turbulence on phytoplankton growth was also reported by another study that used ecological models and water quality data (Zhao et al., 2020). This study also found that the effect of nutrients on phytoplankton growth was the opposite of that of turbulence. Strong turbulence increases shear stress and may lead to mechanical damage or lysis of cells, which results in cell rupture or death (Zhou et al., 2016). In addition, turbulent systems enhance sediment re-suspension, leading to attenuated light penetration and decreased nutrient absorption, which may negatively affect phytoplankton survival (Li et al., 2019). In our study, the amount of water in the canal was not significantly affected by summer rainfall as the water distribution was controlled by upstream reservoirs, and, thus, the inhibition of the eukaryotic community by turbulence was weakened. Consequently, WT became the main factor affecting microbial growth in the water. Therefore, it is likely that the higher flow and velocity of the canal in autumn resulted in decreased community diversity and evenness, and turbulence had a greater effect on the eukaryotic community than WT.

Nutrient status and nutrient concentrations differently affect microbial species (Marc et al., 2014). Nitrogen and phosphorus are usually the limiting factors during the growth stage of microorganisms (Carlsson et al., 2012). It is reported that nutrient sufficiency affects the growth rate and productivity of plankton and bacterial community composition (Jong-Geol et al., 2019). Furthermore, a study revealed that the rapid absorption and utilization of nitrogen was the main cause of algal proliferation (Wu et al., 2009). Moreover, in Germany's Kielstau Catchment, the contribution of nutrient regimes was higher than that of flow regimes to both trait- and taxonomy-based temporal beta diversity and its components (Wu et al., 2022). The results of this study showed that NH_4^+-N and NO_3^--N significantly and negatively correlated with eukaryotic community diversity and abundance, which suggested that community growth consumed a large amount of nitrogen. The Cond and ORP values were determined by the type and concentration of dissolved ions and WT. The change of Cond likely the degree of eutrophication in the reservoir (Yan et al., 2021). Both Cond and ORP were significantly correlated with the eukaryotic community diversity and abundance (**Figure 6**), which indicated that the degree of eutrophication had a direct impact on the community. Additionally, pH, plankton growth, and reproduction displayed interdependence. In water bodies with low pH, plankton growth is limited and the number of individuals decreases, whereas high pH (alkaline) water bodies are more conducive to photosynthesis by plankton to form organic matter and increase plankton reproduction (Wang et al., 2016). Furthermore, the community composition of microbial eukaryotes also relates to the change in pH and DO concentration. The growth, reproduction, and other physiological processes of microorganisms, including respiration and death, consume a large amount of DO (Ouyang and Wei, 2013) leading to a significant negative correlation between eukaryotic community diversity and abundance and DO. Moreover, a positive correlation was noticed between pH and the eukaryotic community composition.

Previous research has confirmed the distance–decay relationship of bacterial communities with spatial similarity in lakes (Logares et al., 2013), reservoirs (Liu et al., 2015), and rivers (Isabwe et al., 2018). Earlier studies have shown that changes in ecological processes, such as biological diffusion and species sorting, are correlated with water dispersal time rather than river network density (Read et al., 2015), and these changes play an important role in bacterial community composition. Although dispersal limitation and environmental screening are considered important drivers that control microbial geographic distribution (Hanson et al., 2012; Zhang et al., 2018), it is not yet clear whether this effect is only due to dispersal limitation and/or environmental screening (Huszar et al., 2015). Furthermore, a study on the Guadiana River in Spain reported that environmental screening had a greater impact on the benthic diatom community than dispersal limitation (Urrea and Sabater, 2009). Moreover, a study on the Lancang River of China revealed that benthic diatoms were primarily affected by dispersal limitation (Sun et al., 2020). This study showed that the geographical distribution of eukaryotic communities in the canal followed a distance–decay pattern, that is, community similarity decreased with an increase in both geographical distance and environmental heterogeneity. The VPA results indicated that environmental screening had a greater influence on the geographical distribution of eukaryotic communities than dispersal limitation. Under the dynamic water transfer mode, the flow was stable, and the planktonic eukaryotes overcame most of the diffusion barriers, and the migration rate was higher. Therefore, the effect of dispersal limitation on the geographical distribution of eukaryotes in this canal was less than that of environmental screening.

CONCLUSION

This study investigated the community composition of the planktonic eukaryotes in the MRP of the SNWD using MiSeq sequencing technology and identified factors influencing the community composition. Our results showed that the eukaryotic communities were diverse, as there were 277 species in 53 phyla, including Cryptophyta, Ciliophora, norank_k_Cryptophyta, and Choanoflagellida. The community structure of eukaryotes and the relative abundance of dominant phyla significantly varied among different channel sections. Although the eukaryotic community was affected by environmental and spatial variables, seasonal variation was the most significant factor. The influence of environmental screening on the geographical distribution of eukaryotic communities was greater than the diffusion limitation of the MRP canal. The analysis of eukaryotic diversity indices and water quality showed that the eukaryotic community in the MRP of the SNWD was sensitive to physicochemical parameters. In addition, a large proportion of planktonic eukaryotes of the canal could not be annotated, as the public DNA database of eukaryotes are yet to register this information. Therefore, our study advocates the construction of an aquatic DNA database that will enrich species information and improve the proportion of species annotated in subsequent studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: MG-RAST (accession: mgp102285). Further inquiries about the datasets of this study should be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YL and YZ conceived the study. FK, YZ, JW, YL, and YP analyzed the data. FK and YL wrote the manuscript. YP helped to revise the manuscript. All authors collected the data, provided the important inputs on the manuscript, contributed to the article, and approved the submitted version.

FUNDING

This study was funded by the National Natural Science Foundation of China (No. 51879130) and the higher discipline innovation and talent introduction base of Henan Province (No. CXJD2019001).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.899993/full#supplementary-material

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

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ORIGINAL RESEARCH
published: 22 July 2022
doi: 10.3389/fevo.2022.914623

Longitudinal Variations in Physiochemical Conditions and Their Consequent Effect on Phytoplankton Functional Diversity Within a Subtropical System of Cascade Reservoirs

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: 07 April 2022
Accepted: 22 June 2022
Published: 22 July 2022

Citation:
Shen H, Ye L, Cai Q and Tan L (2022) Longitudinal Variations in Physiochemical Conditions and Their Consequent Effect on Phytoplankton Functional Diversity Within a Subtropical System of Cascade Reservoirs. Front. Ecol. Evol. 10:914623. doi: 10.3389/fevo.2022.914623

The social and environmental impacts of large dams are quantifiable and have been well documented, while small dams have often been presumed to be less environmentally damaging than large dams. The purpose of this study was to analyze longitudinal gradients in environmental, hydrodynamic variables and their impact on phytoplankton function, within a cascade of four reservoirs (XuanMiaoGuan, XMG; TianFuMiao, TFM; XiBeiKou, XBK; ShangJiaHe, SJH) and one reservoir bay (Huangbohe Bay, HBH), located from upstream to downstream in the Huangbo River, Hubei Province, China. Our results showed that water temperature, total nitrogen, and soluble silicate increased along the cascade reservoir system, while the concentration of dissolved oxygen and total phosphorus decreased. We identified 16 phytoplankton functional groups, and the predominant groups, including **D** (*Synedra* and *Stephanodiscus hantzschii*), **E** (*Dinobryon divergens*), **Lo** (Dinoflagellate: *Peridinium bipes* and *Peridiniopsis*), **X2** (*Chroomona*), and **Y** (*Cryptomonas*), changed longitudinally from up to down in the cascade reservoirs. The number of dominant functional groups increased along the longitudinal gradient, indicating that the function of the phytoplankton community was more stable. Functional group **D** was the dominant phytoplankton functional group among the four reservoirs, and **Lo** group was dominant except SJH. The phytoplankton functional groups in the HBH have been completely changed due to the backwater jacking of the main stream of the Yangtze River. Euphotic depth, suspended solids, and nutrients were apparently the key factors driving variations in phytoplankton functional groups among the reservoirs. Notably, the patterns we observed were not all consistent with the cascading reservoir continuum concept (CRCC) that typically characterizes large rivers. Thus, our findings contribute to the further theoretical development of the CRCC, which may not apply widely to all cascade systems.

Keywords: longitudinal heterogeneity, continuum, phytoplankton functional group, cascade reservoirs, Huangbo River

INTRODUCTION

River is the major conjunction of terrestrial and aquatic ecosystems and has important ecological functions such as water supply, flow regulation, and moderation of climate (Costanza et al., 1997; Cai et al., 2003). However, the constructed dam that fragmented the river ecosystem leads to modifications of the river's original conditions and water dynamics, resulting in changes in abiotic and biotic compartments (Zhao et al., 2017; Liu et al., 2020; Castro et al., 2021). Cascade reservoirs can maximize the utilization of water resources such as water supply, seasonal flood regulation, electricity production, and navigation (Rosenberg et al., 2000; WCD, 2000).

The cascade dams ignore the long-term environmental impacts (McCartney et al., 2000). Dams cause considerable changes in surrounding basins by disrupting the continuous gradient of the rivers (Nogueira et al., 2018; Xiao et al., 2019). Several hypotheses have been proposed to depict and interpret the longitudinal variations in physical, chemical, and biological attributes of river ecosystems. Various studies have shown that the longitudinal distribution of functional feeding groups only partially follows the Reservoir Continuum Concept (RCC) framework (Tomanova et al., 2007; Jiang et al., 2011; Jelil et al., 2021). In contrast, the serial discontinuity concept (SDC) emphasizes that non-free-flowing rivers, such as regulated streams, often have discontinuous changes in riverine geomorphology, as well as biological populations and communities (Ward and Stanford, 1983). The longitudinal connectivity has been severely disrupted by transformation of the rivers into cascade reservoirs (Miranda et al., 2008; Wu et al., 2010). Research of these aquatic systems has emphasized the isolated reservoirs, with no specific study focused on the spatial distribution along a single river basin (Miranda et al., 2008). Nevertheless, studies in such cascades considering step-like continuous systems are rare despite their obvious hydrological and suspected functional interconnectivity downstream. A subsequent addition to lotic theory, the cascading reservoir continuum concept (CRCC) shows connectivity since it deals with data concerning biotic and abiotic among the serial reservoirs (Barbosa et al., 1999; Da Silva et al., 2005). The downstream reservoirs are affected by the features of the upstream reservoirs. CRCC has provided some theoretical postulates about the effects of upstream reservoirs on the downstream ones. However, CRCC was proposed in the study of large rivers, and those concerning small cascade reservoirs are still rare.

Previous studies have considered the impact of cascade reservoirs from the aspects of water quality and biological community changes, and those concerning functional groups were rare (Rodgher et al., 2005; Perbiche-Neves and Nogueira, 2010; Nogueira et al., 2018; Wu et al., 2021). For example, the study on phytoplankton in cascade reservoirs has been carried out in Brazilian Iguacu River and Lancang-Mekong River in China (Li et al., 2013; Nogueira et al., 2018). As important primary producers in ecosystems, phytoplankton is considered to be a natural bioindicator for its complex and rapid responses to fluctuations in environmental conditions, and is well suited to study the impact of the construction of cascade reservoirs (Lewitus, 2002; Darchambeau et al., 2014; Wentzky et al., 2020). The Reynolds functional approach is more effective in evaluating the phytoplankton responses to changes in environmental conditions and has been widely used in the temperate and subtropical areas of the world (Kruk et al., 2002; Reynolds et al., 2002). But this approach in small cascade reservoirs is still not well documented, especially considering that they are not more important due to the social and environmental benefits (Wang et al., 2020; Zaidel et al., 2021). Consequently, the effects of these cascade systems on phytoplankton functional groups still require attention (Moura et al., 2013).

This study of phytoplankton ecology in reservoirs along the Huangbo River Basin (HRB) in Central China has two main objectives. First, a single river system is selected to study the impact of cascade reservoirs on phytoplankton functional groups. Moreover, our results are helpful to clarify the changing trend of phytoplankton functional groups in cascade reservoir system and enrich the CRCC theory.

MATERIALS AND METHODS

Investigation Area

Huangbo River, with a length of 162 km, is one of the first-grade branches between Three Gorges Reservoir and Gezhou Reservoir at the north shore of the Xiling Gorge of Yangtze River, located in Yichang, Hubei Province, China (30°42′–31°29′ N, 110°08′–111°34′ E; **Figure 1**). The HR drains a basin of 1931.5 km^2 and flows from north to south, entering the Yangtze River 1.5 km upstream of the Gezhou Dam. The region occupied by the HRB has a subtropical monsoon climate with a hot rainy summer. The mean annual temperature is 16.9°C, with the highest mean temperature in July and lowest in January; the annual frost-free period is 223–273 days; mean annual precipitation and runoff are approximately 1,101 mm and 28.4 $m^3 \cdot s^{-1}$, respectively (Wei et al., 2021).

The HR is a low-lying river that runs from the north to the south. It is canyon-shaped with the highest altitude at 1,962 m, lowest at 61.5 m, and a relative height of 1,895.5 m. A series of reservoirs, such as XuanMiaoGuan (XMG), TianFuMiao (TFM), XiBeiKou (XBK), and ShangJiaHe (SJH), were constructed in the HR to meet the demand for water for agricultural irrigation in eastern Yichang from 1966 to 2005. The total installed capacity of cascade hydropower stations reaches about 6.6×10^4 kW, and the total annual power generation is 2.2×10^8 kWh. After the impoundment of the Gezhouba Reservoir (GZBR) was filled to an altitude of 66 m above sea level in June 2003, the lower 10.4 km stretch of this river became Huangbohe Bay (HBH, a representative bay of GZBR), with a flow velocity of 1.8–3.6×10^{-3} $m \cdot s^{-1}$ (Bao et al., 2021). Basic habitat characteristics of the cascade reservoirs (Bay) of HRB are shown in **Table 1**.

Field Sampling

Samples were collected every 3 months during the dry (October, January) and rainy (April, July) seasons from 2011 to 2012. Collections were taken at 18 sampling stations distributed along

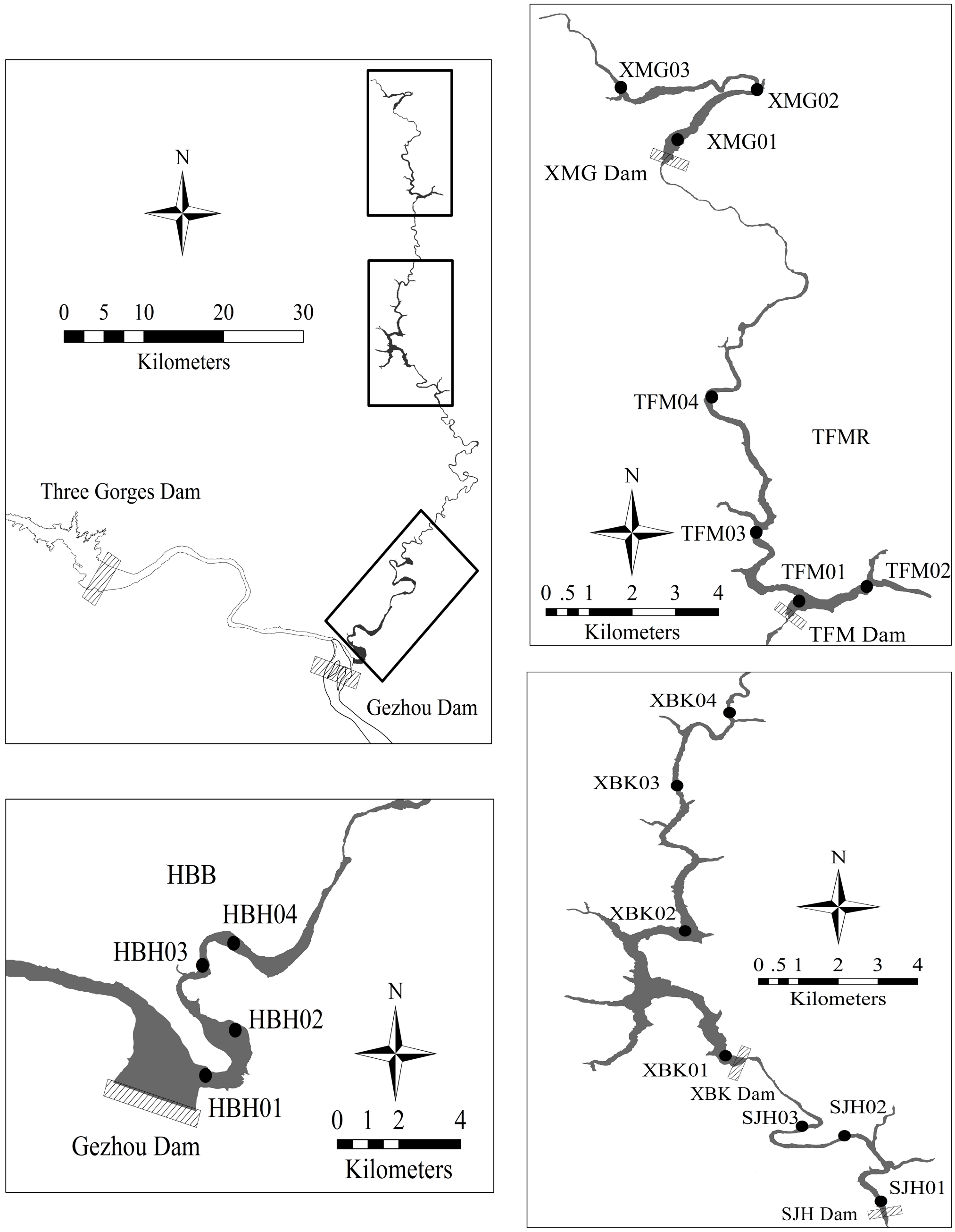

FIGURE 1 | The location of sampling sites in the cascade reservoirs of Huangbo River Basin.

different reservoirs of the main mid-lower course of the HRB. The selected stations are shown in **Figure 1**. Integrated water samples for analyses of water quality and phytoplankton community were collected at 0.5 m below the surface with a 5-L plexiglass water sampler. Excluding SJH in January due to bad weather and harsh road conditions, samples were collected at each site, resulting in a total of 69 samples. Water samples for nutrients were kept cool and shaded in acid-cleaned plastic containers, before being transported to laboratory. An Environmental Monitoring System probe (YSI 6600EDS, United States) was used to measure water temperature (WT) and dissolved oxygen (DO), pH, specific conductance (Cond), and turbidity (Turb) at 0.5 m below the water surface. Water depth was also measured by YSI 6600EDS. Water transparency was measured with Secchi Disk, and suspended solids were concentrated by filtering a known volume of water through a weighed pre-ignited glass fiber filter (Whatman type GF/C). An additional known volume of water was filtered through the GF/C for chlorophyll *a*

TABLE 1 | Basic habitat characteristics of the cascade reservoirs (Bay) of Huangbo River Basin.

	Damming year	Drainage area (km^2)	Normal water level (m)	Installed capacity (10^{7}m^3)	Mean outflow (m^3/s)	Retention time (days)	Types
XMG	2005	380	495	3.9	5.6	50.7	Class B
TFM	1978	553.6	409	6.1	8.5	39.3	Class B
XBK	1991	862	322	16.1	12.3	115.5	Class B
SJH	1978	937	243.8	1.1	14.2	7.7	Class A
HBB	1981	1931.5	66.5	14.56	10.2	>20	Class B

Class A: residence time < 20 days was a fully mixed system with river characteristics.

Class B: 20 < residence time < 300 days can be grouped into intermediate system between rivers and natural lakes has both river-like and lake-like characteristics (Straškraba and Tundisi, 1999).

(chl. *a*) determination (Xu et al., 2009). All the filters were immediately placed in a dark cooler and stored at (−20°C) until the laboratory analysis.

Chemical Analyses and Phytoplankton Assemblages

In the laboratory, the following variables were measured. Different forms of nitrogen (TN, NO_3-N, NH_4-N), phosphorus (TP, PO_4-P), and silicon (SiO_2-Si) were measured using Skalar (San++, The Netherlands; Shen et al., 2014). Total organic carbon (TOC) and dissolved organic carbon (DOC) were measured using Shimadzu (TOC-VCPH, Japan). Suspended solids (TSS) and their two fractions, volatile (VSS) and non-volatile fractions (NVSS), were measured according to a Standard Operating Procedure for Total Suspended Solids Analysis (Xu et al., 2009). The Chl. *a* concentration was determined using a spectrophotometer (Shimadzu UV-1601, Japan) by measuring the absorbance of the extract at various wavelengths (750, 663, 645, and 630 nm; Cai, 2007).

Samples for phytoplankton analysis were preserved with 5% formalin and neutral Lugol's solution, then isolated through sedimentation for 48 h and concentrated to a final volume of 30 ml, and at least 100 random transects for over 300 cells of phytoplankton were counted in 0.1 ml under an Olympus CX21 microscope at 400 × magnification (Huang et al., 1999; Cai, 2007). Taxa identification was done according to Hu and Wei (2006) and John et al. (2002). Algal biomass was calculated using the approximation of cell morphology to regular geometric shapes, assuming that the fresh weight unit is expressed in mass, where 1 $mm^3 \cdot L^{-1}$ = 1 $mg \cdot L^{-1}$ (Huang et al., 1999; Wang et al., 2011). We combined species contributing > 5% to the total biomass into functional groups using the criteria of Padisák et al. (2009) and Shen et al. (2014).

Data Analysis

We calculated the euphotic zone depth (Zeu) as 2.7 times the Secchi depth (Cole, 1994). In the absence of stratification for small reservoirs, mixing depth (Zmix) was taken equal to the average depth of the reservoir (Naselli-Flores, 2000). The ratio between the euphotic and mixing depths (Zeu: Zmix) was used as a measure of light availability (Shatwell et al., 2012). Relative water column stability (RWCS) was calculated following Padisák et al. (2003): $RWCS = (D_b - D_s)/(D_4 - D_5)$, where D_b is the density of the bottom water, D_s is the water density at surface, a depth of 1 m was artificially considered "surface water." D_4 and D_5 are the densities of water at 4 and 5°C, respectively. Phytoplankton data were log(x + 1) transformed to reduce the effects of extreme values, and the relationship between functional groups and environmental factors was analyzed using the CANOCO 4.5 software (Lepš and Šmilauer, 2003; Shen et al., 2014). A forward selection of environmental factors was applied to avoid using collinear environmental factors in the same constrained ordination model. An initial detrended corresponded analysis suggested that redundancy analysis (RDA) should be used because the length of the first axis exceeded 3 SDs. The relationships between functional groups and environmental variables were also assessed by Pearson correlations with IBM SPSS Statistics 20.0 (IBM, Chicago, IL, United States).

RESULTS

Longitudinal Patterns of Physical and Chemical Properties

Reservoirs varied widely in most of the environmental variables. Significant differences in the nutrient parameters along the cascade reservoirs were observed during the sampling periods ($p < 0.01$; **Table 2**). TN (range from 0.94 to 2.71 $mg \cdot L^{-1}$) and SiO_2-Si (1.7 to 4.5 $mg \cdot L^{-1}$) showed a progressive increase in the last two reservoirs, especially HBH (**Figure 2A**), NO_3-N (0.79–2.05 $mg \cdot L^{-1}$), TOC (1.18–1.73 $mg \cdot L^{-1}$), and DOC (1.07–1.68 $mg \cdot L^{-1}$; except a little higher values in TFM) showed a slight increase from the upper part to the mid and lower sections of the cascade reservoirs, while TP and PO_4-P in the four upper reservoirs showed a slight decrease in this longitudinal pattern (**Table 1** and **Figure 2A**). As for the highest concentrations of nutrients, the mean concentrations of NH_4-N and TP were 45 and 123.9 $\mu g \cdot L^{-1}$, respectively. We found that changes in the water quality of the first three reservoirs are relatively stable, while HBH has decreased, which may be mainly affected by the backwater jacking of the main stream of the Yangtze River.

There was no significant variation among the four abiotic variables (WT, Cond, Zmix, and RWCS). Remarkable variations existed between the other physical variables (TSS, VSS, NVSS, pH, Turb, DO, Zeu and Zeu: Zmix) along the cascade system (**Table 2**). Surface WT increased slightly down the cascade (range from 17 to 20°C). DO decreased slowly from 7.4 to 5.3 ml. L^{-1} (**Figure 2B**). The mean specific conductance of the

TABLE 2 | The summary statistics of environmental variables and results of non-parametric test between the cascade reservoirs.

	XMG	TFM	XBK	SJH	HBB	*p*
TN ($mg\cdot L^{-1}$)	0.94 ± 0.12	1.31 ± 0.21	1.32 ± 0.32	1.85 ± 0.83	2.71 ± 0.64	<0.01
NO_3-N ($mg\cdot L^{-1}$)	0.79 ± 0.1	1.11 ± 0.2	1.09 ± 0.19	1.61 ± 0.78	2.05 ± 0.42	<0.01
NH_4-N ($mg\cdot L^{-1}$)	0.06 ± 0.06	0.07 ± 0.06	0.05 ± 0.03	0.06 ± 0.04	0.45 ± 0.55	<0.01
TP ($\mu g\cdot L^{-1}$)	26.3 ± 11.1	24.3 ± 9.8	19.7 ± 6.8	19.1 ± 7.2	123.9 ± 51.6	<0.01
PO_4-P ($\mu g\cdot L^{-1}$)	13.3 ± 7.9	11.2 ± 7.0	8.5 ± 5.5	9.1 ± 4.4	105.9 ± 45.2	<0.01
SiO_2-Si ($mg\cdot L^{-1}$)	1.7 ± 0.5	1.9 ± 0.3	2.1 ± 0.4	3.3 ± 1.6	4.5 ± 2.3	<0.01
TN: TP	41 ± 15	77 ± 35	61 ± 20	104 ± 37	23 ± 5	<0.01
TOC ($mg\cdot L^{-1}$)	1.18 ± 0.25	1.45 ± 0.3	1.24 ± 0.11	1.62 ± 0.42	1.73 ± 0.29	<0.01
DOC ($mg\cdot L^{-1}$)	1.07 ± 0.25	1.34 ± 0.22	1.20 ± 0.11	1.55 ± 0.42	1.68 ± 0.27	<0.01
TSS ($mg\cdot L^{-1}$)	5.0 ± 2.3	5.4 ± 4.3	2.2 ± 1.2	4.3 ± 2.2	9.2 ± 7.3	<0.01
VSS ($mg\cdot L^{-1}$)	2.7 ± 1.3	2.6 ± 1.5	1.5 ± 1.1	2.5 ± 1.0	2.5 ± 1.1	0.02
NVSS ($mg\cdot L^{-1}$)	2.4 ± 1.2	2.8 ± 3.0	0.8 ± 0.5	1.8 ± 1.4	6.7 ± 6.5	<0.01
Water tempature (°C)	17.0 ± 6.5	18.1 ± 6.0	19.1 ± 6.1	19.8 ± 4.2	20.0 ± 6.8	0.78
Cond ($mS\cdot cm^{-1}$)	0.387 ± 0.03	0.402 ± 0.05	0.392 ± 0.02	0.410 ± 0.01	0.380 ± 0.06	0.18
pH	8.7 ± 0.8	8.8 ± 1.1	9.3 ± 1.1	8.0 ± 1.2	8.1 ± 1.0	<0.01
Turb (NTU)	4.4 ± 3.8	4.5 ± 3.8	1.3 ± 1.6	2.9 ± 2.0	11.1 ± 11.1	<0.01
DO ($ml\cdot L^{-1}$)	7.4 ± 1.6	7.0 ± 1.2	6.5 ± 0.7	6.4 ± 0.5	5.3 ± 1.4	<0.01
Zeu (m)	5.1 ± 2.1	6.5 ± 3.3	10.2 ± 3.3	5.8 ± 3.1	3.4 ± 2.1	<0.01
Zmix (m)	15.9 ± 16.5	13.2 ± 13.4	16.8 ± 16.6	5.1 ± 6.6	6.8 ± 1.6	0.15
Zeu: Zmix	1.1 ± 1.1	1.2 ± 0.9	1.5 ± 1.4	4.3 ± 4.3	0.53 ± 0.32	0.03
RWCS	138 ± 125	94 ± 92	114 ± 135	133 ± 100	72 ± 107	0.24
Chl *a* ($\mu g\cdot L^{-1}$)	12.4 ± 10.3	7.5 ± 6.6	4.0 ± 1.6	7.7 ± 4.3	6.3 ± 5.7	<0.01
Density ($10^6 cells\cdot L^{-1}$)	7.4 ± 5.3	6.5 ± 9.2	1.1 ± 0.7	2.2 ± 1.4	1.3 ± 0.9	<0.01
Biomass ($mg\cdot L^{-1}$)	4.5 ± 4.5	2.7 ± 2.7	0.7 ± 0.9	1.1 ± 0.5	0.6 ± 0.4	<0.01

Results are the mean values and standard deviation (standard deviation for the entire data) for each reservoir during the period.
VSS, volatile suspended solids; NVSS, non-volatile suspended solids; Cond, specific conductance; Zeu, the euphotic zone depth; Zmix, mixing depth.

reservoirs was about 0.4 $mS\cdot cm^{-1}$. The highest RWCS was 409 in XBK in July, while the lowest (−2) in TFM in January. The mean suspended solids (TSS, VSS, and NVSS were 2.2, 1.5, and 0.8 $mg\cdot L^{-1}$, respectively) and Turb (1.3 NTU) were lower in the XBK. The highest value of Zeu (10.2 m) was recorded in the XBKR, for the longest retention time (115.5 days) in the cascade reservoirs (**Table 1**). Higher Zeu were always present in front of the dam in the four reservoirs (**Figure 2C**). The reservoirs (except HBH) showed stratification in April and July of 2012, with high light availability (Zeu: Zmix > 1), and the lowest value of Zmix was 1 m.

According to the results of principal component analysis (PCA; **Figure 3**), the first PCA axis explained 53.33% of the variance in the environmental variables, and the second axis explained 24.20% of the variance. The PCA results showed that all samples in each reservoir were separated independently into five groups by the phytoplankton density, DO, RWCS, pH, Zeu, WT, bottom WT, TN, SiO_2-Si, and TSS. Furthermore, distinct spatial pattern was detected in the PCA ordination diagram (**Figure 3B**).

Composition and Abundance of Phytoplankton

The 124 algal species identified in the cascading reservoirs are members of the following seven major taxonomic categories: Bacillariophyta (55), Chlorophyta (37), Cyanophyta (10 taxa), Cryptophyta (7), Pyrrophyta (10), Chrysophyta (3), and Euglenophyta (2). In the subtropical cascade reservoirs, 80 species were sorted into seven major taxonomic categories and belonged to 16 functional groups using the functional approach (>5% of the total biomass) described by Reynolds (1984) and Reynolds et al. (2002). The 43 descriptor species (>10% of the total biomass) were members of 13 functional groups (**Table 3**). The typical traits of these functional groups are exhibited in **Table 3**. Phytoplankton biomass was characteristically low and varied widely among reservoirs (**Figure 4**). Biomass ranged from 0.05 to 16.2 $mg\cdot L^{-1}$, with higher biomass in the upstream (XMG and TFM) and lower in the downstream (XBK, SJH, and HBH). Phytoplankton functional groups occurred in the cascade reservoirs, with one or more species contributing to their compositions (**Figure 4A**). Groups **D**, **E**, **Lo**, **X2,** and **Y** were classified as dominant phytoplankton functional groups (**Figure 4**).

The diatom of group **D** (*Synedra acus* and *S. hantzschii*) was the most important in quantity, sharing this importance with the dinoflagellates *P. bipes* and *Peridiniopsis* (*Peridiniopsis* sp., *Peridiniopsis kevei*, *Peridiniopsis penardiforme*, *Peridiniopsis cunningtonii*, *Peridiniopsis niei,* and *Ceratium hirundinella*) in group **Lo** of phytoplankton community in the XMG. Biomass was dominated by groups **Lo** (46.3%) and **D** (23.9%) upstream of the reservoir (XMG03). Group **D** also dominated in the TFM, XBK, and SJH with over 20% of the contribution. The dominant and co-dominant groups in the riverine zone of XBK

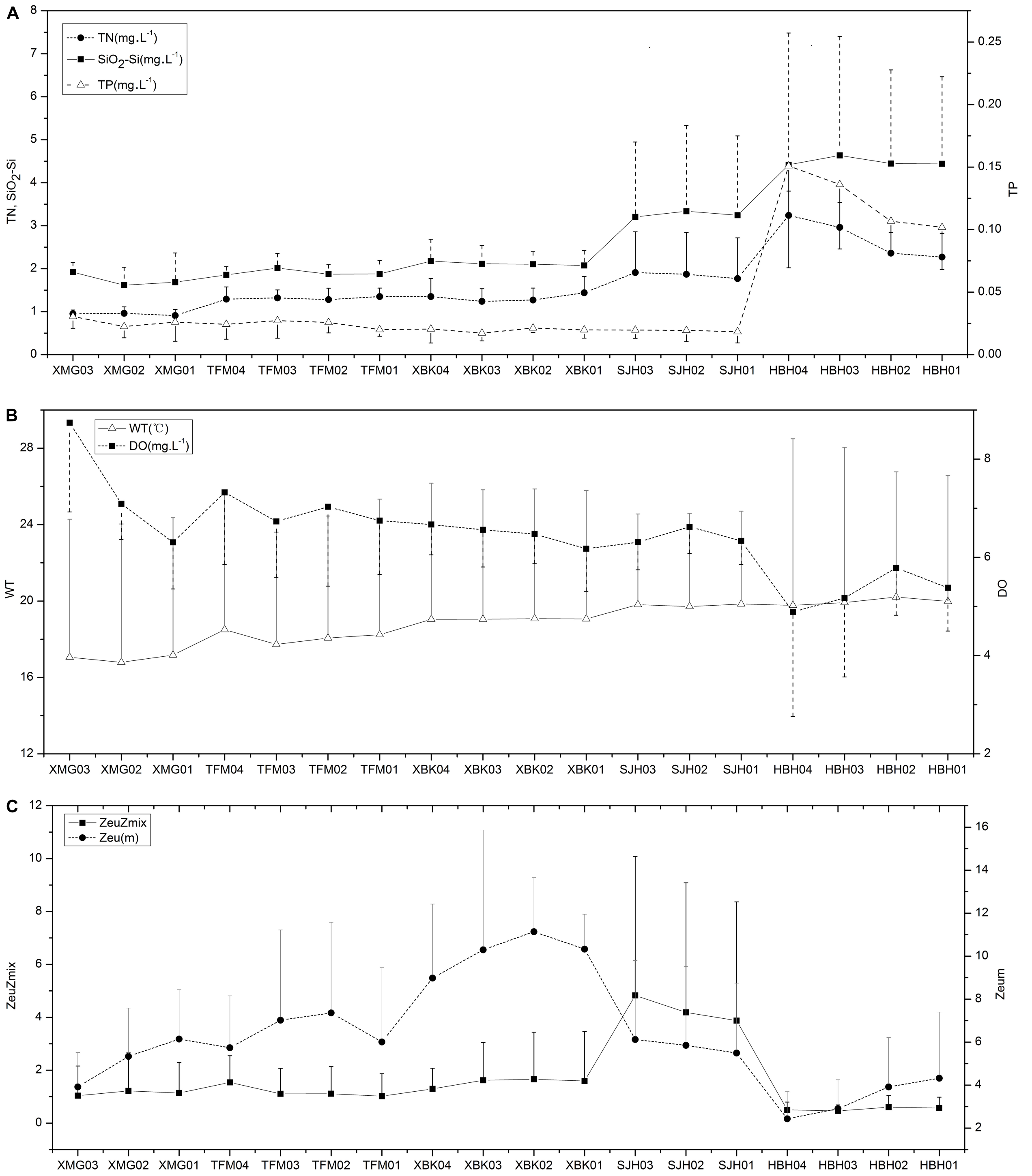

FIGURE 2 | Spatial variation of environmental variables in the cascade reservoirs **(A)** TN, SiO2-Si and TP; **(B)** WT and DO; **(C)** Zeu, Zmix and Zeu: Zmix.

were groups **D** (34.1%), **Lo** (16.9%), and **X2** (14.5%), formed by small *Chroomonas* (*Chroomonas acuta* and *Chroomonas caudate*) and Chlorophyta (*Pyramidomonas delicatula*, *Chlamydomonas reinhardtii*). The biomass of groups **X2** and **Lo** decreased while **D** increased downstream of the XBK reservoir; the dominance of **X2** and **Lo** groups was supplanted by the **D** group. In SJH, groups **Lo** and **D** dominated (28.4%, 20% of biomass) though the dominance of group **D** declined. Group **E** (*D. divergens*) and the crytophyceans in group **Y** (*Cryptomonas ovata*, *Cryptomonas erosa*, *Cryptomonas* sp.,

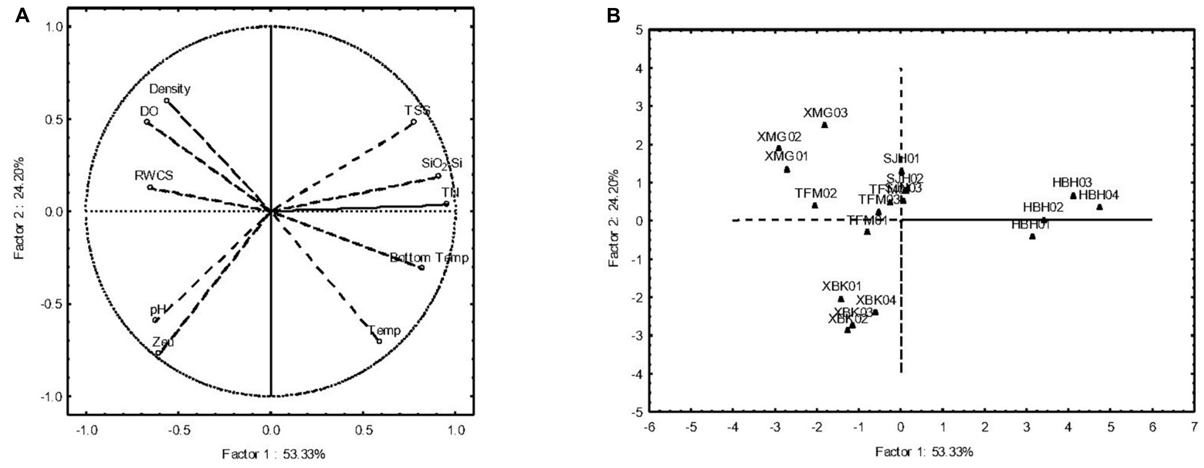

FIGURE 3 | Ordination diagram of PCA for environmental variables and phytoplankton community characteristics **(A)** and samples **(B)**.

TABLE 3 | Trait of phytoplankton functional groups detected in the cascade reservoirs quoted from Reynolds et al. (2002) and Padisák et al. (2009).

Functional group	Habitat template	Typical representatives	Tolerances	Sensitivities
B	Mesotrophic small- and mediumsized lakes with species sensitive to the onset of stratification	*Cyclotella* sp.; *Cyclotella ocellate; Cyclotella bodanica; Cyclotella stelligera; Cyclotella hubeiana; Stephanodiscus minutulus*	Light deficiency	pH rise, Si depletion
C	Mixed, eutrophic small-medium lakes	*Asterionella formosa*	Light, C deficiencies	Si exhaustion stratification
D	Shallow turbid waters including rivers.	*Synedra acus; Stephanodiscus hantzschii*	Flushing	Nutrient depletion
E	Usually small, shallow, base poor lakes or heterotrophic ponds	*Dinobryon divergens*	Low nutrients (resort to mixotrophy)	CO_2 deficiency
G	Nutrient-rich conditions in stagnating water columns; small eutrophic lakes and very stable phases in larger river-fed basins and storage reservoirs	*Pandorina morum*	High light	Nutrient deficiency
J	Shallow, mixed, highly enriched systems (including many low-gradient rivers)	*Tetraedron minimum; Scenedesmus bijugatus; Coelastrum microporum*	?	Settling into low light
Lo	Deep and shallow, oligo-eutrophic, medium to large lakes	*Peridinium bipes; Peridiniopsis penardiforme; Peridiniopsis* sp.; *Peridiniopsis cunningtonii; Peridiniopsis kevei; Peridiniopsis niei; Ceratium hirundinella; Peridiniopsis penardii var. robusta*	Segregated nutrients	Prolonged or deep mixing
MP	Frequently stirred up, inorganically turbid shallow lakes	*Oscillatoria* sp.; *Cocconeis placentula; Surirella* sp.	High turbidity, low light	Stratification nutrient depletion
P	Eutrophic epilimnia at higher trophic states	*Aulacoseira sulcate; Staurastrum gracil; Aulacoseira* sp.	Mild light and C deficiency	Stratification Si depletion
TB	Highly lotic environments (streams and rivulets)	*Aulacoseira varians; Cymbella pusilla; Cymbella ventricosa; Achnanthes linearis; Frustulia vulgaris*		
W1	Ponds, even temporary, rich in organic matter from husbandry or sewages	*Phacus* sp.	High BOD	Grazing
X2	Shallow, meso-eutrophic environments	*Chroomonas acuta; Chroomonas caudata; Pyramidomonas delicatula; Chlamydomonas reinhardtii*	Stratification	Mixing, filter feeding
Y	Wide range of habitats, which reflect the ability of its representative species to live in almost all lentic ecosystems when grazing pressure is low	*Cryptomonas ovata; Cryptomonas erosa; Rhodomonas lacustris; Cryptomonas coerulea; Cryptomonas* sp.	Low light	Phagotrophs

Cryptomonas coerulea, and *Rhodomonas lacustris*) were the high biomass contributors. In HBH, **Lo**, **X2** (*C. acuta*, *C. caudate*, *P. delicatula*, *C. reinhardtii*) and **Y** supplanted **D** group became dominant biomass contributors. Despite low biomass contributions, groups **MP** (*Oscillatori*a sp., *Cocconeis placentula,* and *Surirella* sp.) and **P** (*Aulacoseira sulcata*, *Aulacoseira*

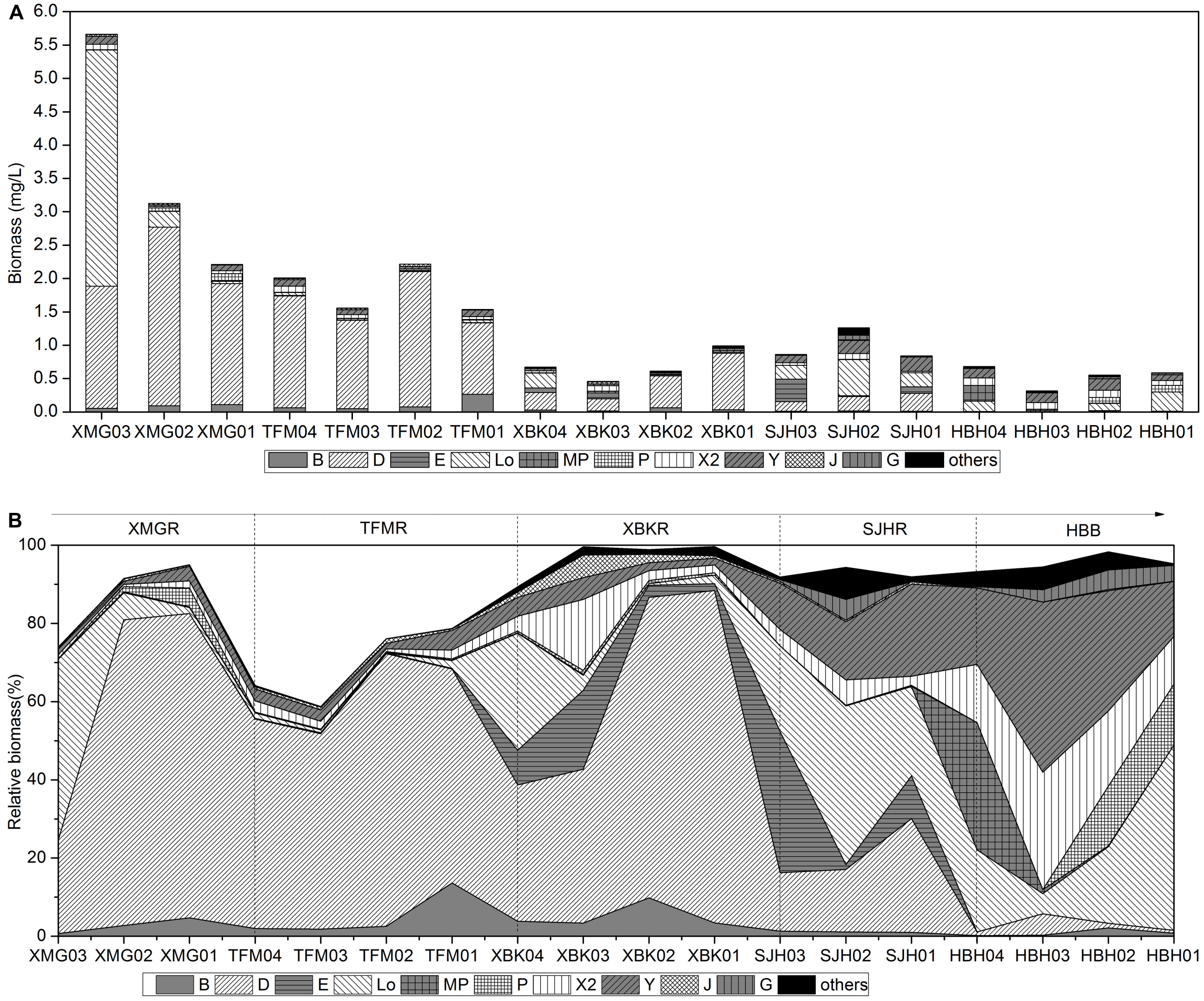

FIGURE 4 | Longitudinal variation of the biomass **(A)** and relative biomass (%) **(B)** of the main phytoplankton functional groups in the cascade reservoirs.

sp., and *Staurastrum gracile*) were also important as they were present in the upper reach (**MP**) and the river mouth region (**Figure 4A**).

Relationships Between Phytoplankton Functional Groups and Environmental Variables

Redundancy analysis was the appropriate method for linear ordination (gradient lengths of the first two axes are 2.73 and 2.88, respectively). The results of the RDA ordination for phytoplankton functional groups and environmental variables on axes 1 and 2 are shown in **Figure 5A**. The Monte Carlo test revealed that the first canonical axis and all canonical axes were significant (F = 52.32, p = 0.002; F = 9.94, p = 0.002; 499 random permutation).

The correlations for the first (0.84) and second (0.70) axes were high (**Table 4**), indicating a strong relationship between phytoplankton and selected environmental factors. All canonical axes cumulatively explained 99.43% of the total variation in RDA, and the first two redundancy axes jointly accounted for 97% of the species–environmental variables relation (axis 1: 78.0%; axis 2: 21.0%; **Table 4**). Nine significant variables of environmental data were screened by forward selection, DO (F = 28.98, p = 0.001), TN (F = 5.76, p = 0.005), TSS (F = 4.98, p = 0.007), NO_3-N (F = 5.44, p = 0.012), VSS (F = 4.82, p = 0.013), Cond (F = 4.48, p = 0.012), Zeu (F = 3.84, p = 0.022), SiO_2-Si (F = 3.54, p = 0.033), and RWCS (F = 3.37, p = 0.029). These variables accounted for 58.5% variance of species data for the first two axes (axis 1: 47.0%; axis 2: 11.5%). The first RDA axis was mainly positively correlated to DO (0.66) and negatively to TN (−0.50), NO_3-N (−0.50), and SiO_2-Si (−0.38). Axis 2 was positively correlated with VSS (0.26; **Figure 5A**).

The spatiotemporal dynamic changes of phytoplankton functional groups in cascade reservoirs of the Huangbo River were well presented in the RDA diagram (**Figure 5B**).

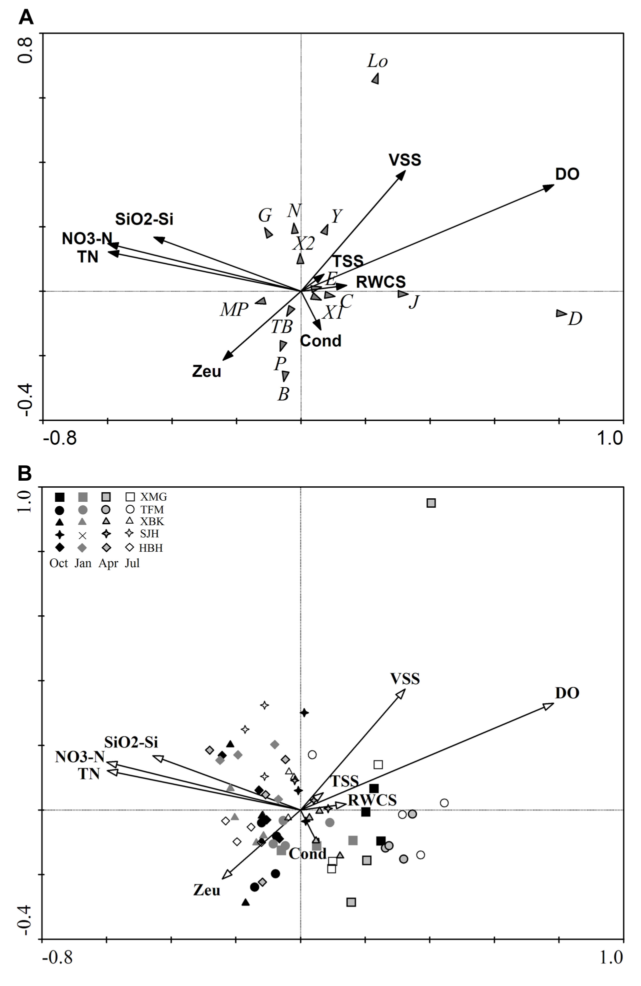

FIGURE 5 | Ordination diagram of RDA for environmental variables and phytoplankton functional groups **(A)** and samples **(B)**.

The first RDA axis illustrates differences between upstream and downstream regions. Upstream reservoirs showed high concentrations of DO while the downstream regions were characterized by high nitrogen nutrient levels (TN, NO_3-N, and SiO_2-Si). Pearson correlations found significant correlations ($p < 0.05$) between the biomass of main functional groups and most of the examined physicochemical factors (WT, DO, Zeu, nutrients, suspended solids, and hydrodynamic conditions; **Table 5**). The biomass of groups **X2** and **Y** was negatively correlated with Zeu and TN:TP ratio and positively correlated with WT, TOC, DOC, and VSS. Group **Y** was also positively correlated with TP, PO_4-P, and TSS and negatively correlated with pH and Zeu: Zmix. In contrast, the biomass of group **D** was positively correlated with DO and VSS and negatively correlated with nutrients (N, P, Si, and C). The significant environmental variables for group **Lo** were DO and VSS; for groups **MP** and **P,** significant environmental variables were WT, Turb, SiO_2-Si, TSS, and NVSS; for **E**, significant environmental variables were Zeu, Zeu: Zmix, and TN:TP ratios. Total phytoplankton biomass was significantly related to DO, Zeu, Zmix, TN, NO_3-N, SiO_2-Si, TSS, and VSS.

Significant differences in major environmental factors existed between different locations by RDA, which are not displayed in this study. In XMG, pH and DO were the principal environmental factors related to phytoplankton community and accounted for 73.4% variance of species data in the first two axes ($F = 12.4$, $p = 0.002$). In TFM, however, the central environmental factors changed into Zeu: Zmix, TN, RWCS, and Turb and explain 86% of the species data ($F = 17.3$, $p = 0.002$); SiO2-Si, NO_3-N, and Cond replaced the previous factors as the important factors in XBK with a probability are 59% ($F = 5.8$, $p = 0.008$). Phytoplankton growth is limited by Cond, pH, and Zeu: Zmix in SJH, where the phytoplankton could also be explained as 64% ($F = 4.5$, $p = 0.002$); No remarkable factor was selected in the RDA to explain phytoplankton distribution in HBH ($p > 0.05$).

DISCUSSION

Longitudinal Variations of Physicochemical Variables

It is reported that the capture of nutrients by reservoirs with low retention time along the basin would increase the nutrient level in the downstream of the cascade. In the Tietê River, Brazil, the uppermost reservoir in a series of nine impoundments captured most of the nutrients released from São Paulo, Brazil (Barbosa et al., 1999), and decreased linearly with descent down the cascade. In the Iguaçu River, Brazil, Da Silva et al. (2005) observed that nutrients decreased in a downstream direction while in Tennessee River, United States, nutrients increased (Miranda et al., 2008), but nutrient ratios changed, reflecting nutrient-specific gradients along the cascade reservoirs. The values of TP also showed a decreased gradient, while TN and SiO_2-Si increased along the longitudinal cascade reservoir system in the Huangbo River, China. The nutrient levels in the cascade reservoirs may be recorded as a result of specific watershed differences in morphometric features, retention time, hydrodynamic conditions, external loads, and retention by dams (Barbosa et al., 1999; Rodgher et al., 2005). Like nutrients, other water quality characteristics are affected by impoundments and exhibit gradients along cascade reservoirs. The values of DO displayed descending gradients in Tennessee River (Miranda et al., 2008) and Huangbo River; however, surface WT revealed an ascending gradient variation along the longitudinal cascading system in Tietê River (Barbosa et al., 1999), Iguaçu River (Da Silva et al., 2005), and Huangbo River.

The discontinuity also presented in the system, and there was a sharp decline in PO_4-P, TOC, DOC, and suspended solids in XBK, with the same trends for chl. *a*, density, and biomass of phytoplankton. In addition, the light condition and RWCS were also interrupted by dams. In general, the proposed concept of CRCC was partly verified in the reservoirs in Huangbo River unlike the established gradients in Tietê River, Tennessee River, and Iguaçu River (Barbosa et al., 1999; Da Silva et al., 2005; Miranda et al., 2008). In contrast, more attention from limnologists was paid to longitudinal patterns within reservoirs

TABLE 4 | Redundancy analysis results for phytoplankton functional groups and environmental variables.

	Eigen values	Species-environment correlations	Cumulative percentage variance		Sum of all canonical eigenvalues
			Species data	Species-environment relation	
Axis 1	0.470	0.838	47.0	78.0	
Axis 2	0.115	0.699	58.5	97.0	
Axis 3	0.009	0.598	59.4	98.6	
Axis 4	0.005	0.626	59.9	99.4	
					0.602

TABLE 5 | Significant Pearson correlations between the biomass of the 10 main phytoplankton functional groups and the biomass of total phytoplankton, and 20 environmental variables (n = 69).

	WT	Cond	pH	DO	Turb	Zeu	Zmix	Zeu: Zmix	TN	NO_3-N	TP	PO_4-P	TN: TP	SiO_2-Si	TOC	DOC	TSS	VSS	NVSS	RWCS
B			–0.24*				0.43**													
D				0.56**					–0.45**	–0.46**	–0.26*	–0.26*		–0.35**		–0.28*		0.21*		
E						0.22*		0.62**					0.43**							
J	0.41**	–0.29**		0.29**					–0.29**	–0.28**		0.03*						0.25*		0.47**
G			–0.34**															021*		
Lo				0.45**														0.32**		
MP	0.21*	–0.27*		–0.3**	0.43**				0.20*	0.23*				0.46**			0.41**		0.46**	
P	0.29**	–0.41**			0.43**	–0.24*								0.33**			0.37**		0.39**	0.40**
X2	0.21*					–0.24*							–0.31**		0.25*	0.22*		0.21*		
Y	0.24*		–0.46**			–0.39**		–0.21*			0.22*	0.22*	–0.36**		0.35**	0.32**	0.21*	0.14**		
Chl. *a*	0.41**	–0.40**		0.27*		–0.42**	–0.22*						–0.25*		0.31**	0.25*	0.33**	0.46**	0.25*	0.27*
Density	0.32**	–0.35**		0.52**		–0.40**			–0.29**	–0.29**			–0.26*		0.21*		0.36**	0.54**	0.27*	0.34**
Biomass				0.74**		–0.34**	–0.22*		–0.38**	–0.39**				–0.23*			0.24*	0.53**		

$^*p < 0.05$; $^{**}p < 0.01$.

than among reservoirs, leading to informational deficiencies in the literature (Miranda et al., 2008). As a consequence, we could not predict which limnological characteristic parameter will show continuous longitudinal gradients along the cascade reservoirs. Longitudinal gradients may reset and restart multiple times as the river travels through a different basin (Barbosa et al., 1999; Miranda et al., 2008). Thus, the exact patterns in Huangbo River may not apply directly to other cascade systems.

The Impact of Physiochemical Conditions on Phytoplankton

In the cascade reservoirs, physicochemical conditions were successfully described by the predominant coexistent phytoplankton species. We found that groups **D**, **E**, **Lo**, **X2,** and **Y** occurred in the cascade reservoir system, however, with relatively different contributions. **D** group was more important in the former three reservoirs, while **E**, **Lo,** and **Y** groups became more representative in XBK, SJH, and HBH. The relative biomass of **D** group decreased in SJH and HBH, while the **Lo**, **X2,** and **Y** groups increased along the longitudinal cascade reservoirs from upstream to downstream. In view of the above description, we concluded that the longitudinal distribution characteristics of phytoplankton in the cascade reservoirs of the Huangbo River could be summarized as groups **D** and **Lo** in XMG, only **D** in TFM, and **D**, **Lo**, **E** groups in XBK, while in SJH, the dominant groups were **Lo**, **D**, **E,** and **Y**, based on biomass changes of functional groups. Due to the influence of jacking from the mainstream of GZB, the phytoplankton in HBH has changed greatly compared with the four reservoirs and **Y**, **Lo,** and **X2** groups dominant in HBH, which was consistent with the study of Xiangxi bay (Wang et al., 2011).

D group was represented by lanceolate, pinnate diatoms (*Synedra*), and centric diatom (*S. hantzschii*), which were also the main contributors of the group on this occasion. **D** group was more important in the upper three reservoirs and exhibited a higher biomass over the reservoirs, commonly found in shallow, turbid waters, with species sensitive to nutrient consumption (Reynolds et al., 2002; Shen et al., 2014). Although the group typically occurs in spring (Yin et al., 2011), it was observed in the other seasons in our study. In fact, diatoms can become dominant in the flooding and mixed waters because dissolved silicate is replenished from deeper water (Stević et al., 2013; Yi et al., 2020). As a C-strategist, this small-celled and fast-growing species is tolerant of mixing and low light (Reynolds, 2006). In this study, group **D** was well adapted to high VSS though strongly negatively correlated with nutrients (C, N, P, and Si) and light conditions. As reported in the lower Salado River (Argentina), group **D** was also directly correlated with Cond in the region (Devercelli and Farrell, 2013).

Group **E**, with the representative species of *D. divergens*, can tolerate low nutrients (resort to mixotrophy) but is sensitive to CO_2 deficiency (Reynolds et al., 2002). This group is wildly distributed in the northern hemisphere and reported in moderately to very nutrient-rich ponds and lakes (John et al., 2002; Hu and Wei, 2006). However, the correlation analysis showed its significant relation with light availability and TN:TP ratio. Many different species of *Dinobryon* are distributed widely in different nutritional water, and most of them were recorded once and predominantly occurred in clear-water lakes and ponds with low nutrient contents, low temperature, and high transparency (Feng, 2008).

Groups **Lo** and **Y** became more representative in SJH and HBH. The **Lo** group mainly comprises dinoflagellates species in the reservoirs. The representative species of group **Lo** were *P. niei*, *P. bipes*, *C. hirundinella,* and *Peridiniopsis penardii* var. *robusta*. This group was usually observed in mesotrophic lakes, tolerated segregated nutrients, and sensitive to prolonged or deep mixing as emphasized by Reynolds et al. (2002). According to former research, most of the dinoflagellates were capable of using their slower swimming velocities to perform diel vertical migration where nutrient-depleted conditions occurred in aquatic ecosystems (Xu et al., 2010a; Shen et al., 2014). High temperature resulted in group **Lo** in a Mediterranean reservoir (Becker et al., 2010) and Lake Sakadaš along the Danube (Stević et al., 2013). In this study, group **Lo** had no significant correlation to WT and nutrients. Concerning *P. niei*, high abundances have been found in Danjiangkou Reservoir and Three Gorges Reservoir, leading to bloom in some periods (Xu et al., 2010b; Shen et al., 2014). This species can be a dominant species in winter and spring for a short time, and is widely distributed in waters whose trophic status varies from oligotrophic to eutrophic (Amorim and Moura, 2022).

The **Y** group was characterized by *C. ovata* and *C. erosa*, occurred in the system, and dominated in the downstream throughout the year. These two species have similar ecological requirements, which is well adapted to live in small, low light, and enriched lakes (Reynolds et al., 2002). The relatively high surface to volume ratio of cryptomonads facilitates their rapid absorption of nutrients and fast growth (Bovo-Scomparin and Train, 2008). The group Y was dominated by wide suitable habitat during the mixing period, where there was low grazing pressure and a quiet water body (Becker et al., 2010). In this study, group **Y** was characterized by low Zeu and TN:TP ratio, high concentration of TP, PO_4-P, TOC, and DOC. Thus, in HBH, lowest eutrophic depth and TN:TP ratio and the highest nutrients contribute to the high biomass. The results obtained from RDA analysis also showed a virtual correspondence to Rychtecký and Znachor, 2011 in the Římov Reservoir, Czech Republic, and a young canyon reservoir in Southwest China (Liao et al., 2020).

The habitat template for group **X2** is shallow, meso-eutrophic environment with species-tolerant stratification but sensitive to mixing regime and filter feeding (Padisák et al., 2009). The representative species of the group are *C. acuta* and *C. caudata*, existing in all waters along the Huangbo River. This group presented a high biomass only in the downstream area, but never became dominant in the cascade reservoirs except in HBH. These small unicellular flagellates are generally considered as "C" strategists with high surface/volume ratio (Bovo-Scomparin and Train, 2008), high intrinsic growth rates, and high metabolic activity and low light requirement in common (Grime, 1977; Reynolds, 1988). However, they differ in sedimentation velocity and silica requirement (Mieleitner and Reichert, 2008). **X2** group was positively correlated with VSS, nutrients (TN, NO_3-N, and SiO_2-Si), and negatively correlated with Zeu and Cond, which was not consistent with observations in the Middle Paraná River (Devercelli, 2006; Devercelli and Farrell, 2013). The present habitat for **X2** is well consistent with what RDA indicated and also with shallow eutrophic lakes of Huaihe River, China (Yi et al., 2020).

It is widely recognized that spatial heterogeneity plays a functional role in aquatic ecosystems (Dutilleul and Legendre, 1993; Becker et al., 2010), and phytoplankton communities are regulated mainly by hydrology, WT, the availability of light, nutrients, and mixing conditions (Costa et al., 2009; Rigosi and Rueda, 2012; Shen et al., 2014). In some reservoirs, although both internal and external variables determine the structure of the phytoplankton community, physical variables and hydrodynamics generally predominate (Rangel et al., 2012; Xu et al., 2021). Phytoplankton community structure was driven by light and nutrient, which have illuminated by Reynolds (2006). The results also highlighted the hydrodynamic conditions, light availability, mixing regime and nutrients as the main factors related to the phytoplankton functional groups in the cascade reservoirs. In terms of importance, nutrients seemed to play a more important role in the four reservoirs in this study. The results verified by the correlations between the biomass and environmental factors. The importance of environmental factors showed spatial differences in terms of influence. The major environmental factors shape phytoplankton in different waters for the main functional groups. Heterogeneity in the distribution of phytoplankton was clearly demonstrated as in Mangueira Lake (Crossetti et al., 2013). Reservoirs were traditionally considered as an independent system, separated from the surrounding watersheds and other parts of the river basin (Miranda et al., 2008). Despite the shortcomings of the paradigm, our research was restricted to five reservoirs in phytoplankton assemblages and physicochemical variables, and obvious longitudinal gradients were identified in the cascade reservoir system of the Huangbo River. In this study, the effects of the cascading dams on phytoplankton assemblages were obvious and more complex than those in a single reservoir (Amorim and Moura, 2022). The operations of cascade reservoirs can cause eutrophication of downstream reservoirs, longitudinal connectivity loss in RCC, and cascading and accumulation effects on phytoplankton communities.

CONCLUSION

Instead of seeing each part of the reservoir as independent, we regarded them as an interdependent system. For the spatial pattern, the biomass of functional groups was interfered by the strong influence of the dams, especially the series of dams in the study region. The number of dominant functional groups increased along the longitudinal gradient, indicating that the function of the phytoplankton community was more stable. Functional group **D** was the dominant phytoplankton functional group among the four reservoirs, and **Lo** group was dominant except SJH. Among the main environmental variables, the depth of euphotic layer, nutrients were the limiting factors of algae growth, and the effects of nutrients were apparently more important. Furthermore, the study confirmed that CRCC existed in the cascade reservoirs of Huangbo River, which were not completely consistent with it in large rivers. The phytoplankton functional groups in the HBH have been completely changed due to the backwater jacking of the main stream of the Yangtze River.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

HS and QC conceived the ideas and designed the study. HS and LT sampled the phytoplankton. HS, LY, LT, and QC analyzed the data and led wrote the manuscript. All authors contributed, and approved it for publication.

FUNDING

This study was funded by the National Key R&D Program of China (2018YFD0900806), the Shandong Provincial Natural Science Foundation (ZR2016CL05), the Technology Projects (A) Category, Chinese Academy of Sciences (XDA23040500), and the State Key Laboratory FEBL Research (2019FBZ01).

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

frontiers | Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 25 July 2022
DOI 10.3389/fevo.2022.940254

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

RECEIVED 10 May 2022
ACCEPTED 04 July 2022
PUBLISHED 25 July 2022

CITATION
Xiang H, Li K, Cao L, Zhang Z and Yang H (2022) Global patterns and drivers of coniferous leaf-litter decomposition in streams and rivers. *Front. Ecol. Evol.* 10:940254. doi: 10.3389/fevo.2022.940254

Global patterns and drivers of coniferous leaf-litter decomposition in streams and rivers

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Many streams and rivers are heterotrophic ecosystems that are highly dependent on cross-ecosystem subsidies such as leaf litter (LL). Terrestrial LL can be consumed by macroinvertebrates and microbes to fuel the detrital-based food webs in freshwaters. To date, our knowledge of LL decomposition in freshwaters is largely based on broadleaved LL, while the patterns and drivers of coniferous leaf-litter (CLL) decomposition in streams and rivers remain poorly understood. Here, we present a global investigation of CLL decomposition in streams and rivers by collecting data from 35 publications. We compared LL breakdown rates in this study with other global-scale studies (including conifers and broadleaved species), between evergreen and deciduous conifers, and between native and invasive conifers. We also investigated the climatic, geographic (latitude and altitude), stream physicochemical characteristics, and experimental factors (e.g., mesh size and experimental duration) in influencing CLL decomposition. We found that the following: (1) LL breakdown rates in this study were 18.5–28.8 and 4.9–16.8% slower than those in other global-scale studies when expressed as per day and per degree day, respectively. Conifer LL in coarse mesh bags, for evergreen and invasive conifers, decomposed 13.6, 10.3, and 10.8% faster than in fine mesh bags, for deciduous and native conifers, respectively; (2) CLL traits, stream physicochemical characteristics, and experimental factors explained higher variations in CLL decomposition than climatic and geographic factors; (3) CLL nutritional quality (N and P), water temperature, and experimental duration were better predictors of CLL decomposition than other predictors in categories of LL traits, stream physicochemical characteristics, and experimental factors, respectively; and (4) total and microbial-mediated CLL breakdown rates showed linear relationships with latitude, altitude, mean annual temperature, and mean annual precipitation. Our results imply that the replacement of native forests by conifer plantation would impose great impacts on adjacent freshwaters by retarding the LL processing rate. Moreover, future climate warming which is very likely to happen in mid- and high-latitude areas according to the IPCC 6th report would accelerate LL decomposition, with a potential consequence of food depletion for detritivores in freshwaters during hot summers.

KEYWORDS

leaf-litter trait, ecosystem functioning, water temperature, leaf-litter breakdown rate, latitude, freshwater, cross-ecosystem subsidy

Introduction

Leaf-litter (LL) decomposition is one fundamental ecological functioning that links nutrients cycling, food web structure, and energy transfer within and across ecosystems (Anderson and Sedell, 1979; Wallace et al., 1997; Manning et al., 2021). The input of terrestrial LL to freshwaters imposes great impacts on individuals, populations, and communities of macroinvertebrates and microbes (Graça, 2001; Hayer et al., 2022), which can translate the C stored in LL into coarse particulate organic matter and CO_2 (Marks, 2019). An increasing number of studies advanced our understanding of LL decomposition in freshwater ecosystems. Many biotic (e.g., LL traits, shredders, and fungi) and abiotic (e.g., water temperature, dissolved oxygen (DO), and latitude) factors have been identified as key drivers of LL decomposition in freshwaters at local, regional, and global scales (Woodward et al., 2012; Tiegs et al., 2019; Xiang et al., 2022). However, most previous studies focused on broadleaved species and ignored the decomposition of coniferous leaf litter (CLL) in freshwaters. For example, Follstad Shah et al. (2017) investigated the temperature sensitivity of LL decomposition in streams at a global scale in which CLL represents only 2.8% of all the cases. On the contrary, the decomposition of CLL in terrestrial ecosystems has been intensively investigated, especially for *Pinus*, which is the most well-studied genus than any other genus (Berg, 2014).

More studies should be conducted on investigating CLL decomposition in freshwaters, considering that many conifers are globally distributed species; conifers are common and dominant species in many mid- and high-latitude areas (e.g., temperate forests), and coniferous plantation is among one of the biggest forest changes that have invaded in many regions (Richardson and Rejmánek, 2004; Essl et al., 2010; Ferreira et al., 2016). Coniferous LL differs from broadleaf species in many ways such as nutritional quality and LL input time, and the shift between conifers and broadleaf species may have great impacts on communities of macroinvertebrates and microbes (Riipinen et al., 2010; Kanasashi and Hattori, 2011; Ferreira et al., 2017). The lack of information on CLL decomposition in freshwaters is, therefore, unrealistic and may lead to biased results when investigating general patterns of LL decomposition in freshwaters on a global scale. In addition, CLL naturally differs from broadleaved LL in ways of leaf morphology, nutrient concentration (e.g., N and P), chemical (e.g., lignin) and physical (e.g., toughness) characteristics, and, especially, the nutritional quality, which is lower than broadleaved LL (Martínez et al., 2013; Balibrea et al., 2020). Leaf-litter traits refer to characteristics that have correlations with LL decomposability or palatability, which include nutrient concentrations and chemical and physical characteristics in this study. Lower quality (e.g., higher lignin) of LL may influence the responses of LL decomposition to other factors such as water temperature and macroinvertebrate communities (Leroy and Marks, 2006; Fernandes et al., 2012). Therefore, the global patterns and drivers of LL decomposition found in previous large-scale studies may not be applicable for CLL.

To the best of our knowledge, there is no global-scale study of CLL decomposition in freshwaters. However, experiences gained from previous studies may help us predict some general patterns. Leaf-litter traits such as lignin and N have long been acknowledged as driving predictors of LL decomposition in freshwaters (Boyero et al., 2016; Kennedy and El-Sabaawi, 2017; Zhang et al., 2019). Coniferous LL is a low-quality food resource for microbes and detritivores due to the high concentration of lignin and low concentration of N (Richardson et al., 2004). Therefore, we should expect slower LL breakdown rates of conifers than broadleaf species in freshwaters (Graça and Pereira, 1995; Albariño and Balseiro, 2002). However, there are no consistent results regarding which traits are the most important predictors of LL decomposition. Leaf-litter decomposition also has been shown to change along the latitudinal gradient: total, macroinvertebrate-mediated, and microbial-mediated LL decomposition decreased when approaching the poles (Follstad Shah et al., 2017; Boyero et al., 2021) or no significant change of total LL decomposition with increasing absolute latitude (Irons III et al., 1994). These latitudinal patterns of LL decomposition in streams and rivers are usually associated with changes in mean annual temperature (MAT), microbes (e.g., fungi), and macroinvertebrates: lower MAT toward the poles, the highest abundance and richness of fungi at mid-latitude areas, and increasing abundance and richness of shredders toward the poles (Boyero et al., 2011a; Seena et al., 2019). Conifers are mainly distributed in mid- and high-latitude areas (Essl et al., 2010) where the abundance and richness of macroinvertebrates and microbes are relatively high and MAT is low (Seena et al., 2019). Therefore, the latitudinal trend of CLL decomposition may differ from the reported global patterns that included a low proportion of conifers and had a wider distribution range of sampling sites.

Stream physicochemical characteristics such as water temperature and water nutrient concentrations are essential abiotic factors driving LL decomposition in streams (Boyero et al., 2011b; Woodward et al., 2012). A growing number of studies addressed the importance of water temperature in driving LL decomposition in freshwaters (Bernabé et al., 2018; Fenoy et al., 2022). However, most studies suggested accelerated total, microbial-mediated, and macroinvertebrate-mediated LL breakdown rates under warmer temperature conditions (e.g., 1–4°C higher) due to benefited individuals and communities of microbes and detritivores (e.g., stimulated activities and higher density) (Boyero et al., 2011b; Ferreira et al., 2015b; Griffiths and Tiegs, 2016). Some others found no or negative effects of increased water temperature on LL decomposition through the inhibition effects on communities of macroinvertebrates and microbes (Bernabé et al., 2018; Peréz et al., 2021). However, the higher water temperature may reduce the negative effects of LL quality on LL decomposition (Fernandes et al., 2012). Therefore, the observed relationships between water temperature and broadleaved LL breakdown rates may be different from that of conifers due to the lower quality (e.g., higher lignin). Water nutrient concentrations are also closely correlated with LL decomposition in freshwaters. Nutrient concentrations (e.g., NO- 3 and PO_4^{3-}) in freshwaters worldwide have been increasing and will continue to increase due to intensive anthropogenic activities, such as growing fertilizer and sewage input (Vilmin et al., 2018; Beusen et al., 2022). The responses of LL decomposition to increasing water nutrient concentrations (e.g., NO- 3 and PO_4^{3-}) may be hump shaped (Woodward et al., 2012) or positive, and this effect was stronger for nutrient-poor LL (e.g., low N) than for nutrient-rich LL (Ferreira et al., 2015a). In addition, nutrient enrichment significantly decreased macroinvertebrate abundance in freshwaters, and this effect was stronger in tropical than in temperate areas (Nessel et al., 2021). Because CLL has lower quality than broadleaved LL, conifers are mainly distributed in temperate regions. Therefore, the relationships between CLL decomposition and water nutrient concentrations may differ from that of broadleaf species.

Here, we collected data on CLL breakdown rates in streams and rivers with associated climatic (MAP, mean annual precipitation; MDTR, mean diurnal temperature range, and MAT), geographic (altitude and latitude), experimental (mesh size, duration, and initial LL mass), and stream physicochemical (e.g., water depth, NO- 3, and PO_4^{3-}) factors from 35 publications (Supplementary Datasets and Appendix). We aimed to (1) compare the mean values of CLL breakdown rates with that of LL (including conifers and broadleaf species) reported in other global-scale studies and test the effect of mesh size (coarse vs. fine), leaf habit (evergreen vs. deciduous), and invasion status (native vs. exotic) on CLL decomposition; (2) select the driving intrinsic (CLL traits), environmental (climatic and geographic), and experimental factors on CLL breakdown rates; and (3) investigate the responses of CLL breakdown rates to the gradients of MAT, MAP, latitude, and altitude. We predicted that (1) CLL breakdown rates would be lower than other reported LL breakdown rates; (2) LL traits such as N and lignin would have greater impacts on CLL decomposition than other factors; and (3) CLL breakdown rates would increase with increasing MAT and MAP toward the poles but decrease with increasing altitude.

Materials and methods

Dataset

To evaluate the current state of knowledge on CLL decomposition in streams and rivers on a global scale, we built a database by searching online databases (ISI Web of Science and China National Knowledge Infrastructure for papers published in English and Chinese, respectively, prior to January 2022) with the following search terms: "(litter OR leaf) AND (conifer OR needle) AND (stream OR river) AND (breakdown OR decomposition OR decay)." The selected publications were based on several criteria: (1) only studies that were conducted in natural lotic ecosystems (i.e., streams and rivers) or experimental channels adjacent to streams and rivers were included to avoid experiments that were conducted in lentic ecosystems (e.g., lakes and ponds) or mesocosms; (2) the study should have measured LL breakdown rates of at least one conifer species; and (3) studies that measured the breakdown rates of mixed LL species in one leaf-litter bag were excluded due to the LL-mixing effects on LL decomposition (Leroy and Marks, 2006). In total, we screened 541 independent observations (324 and 217 observations in coarse and fine mesh bags, respectively) from 35 publications (Figure 1, Supplementary Datasets). The database included 541 and 428 cases for LL breakdown rates expressed as per day and per degree day, respectively, spanning 105° of latitude and large MAP (310–2,250 mm), MAT (0.36–19.8°C), and altitudinal gradients (1–3,600 m a.s.l.) in five continents.

Leaf-litter breakdown rates expressed as k_d (per day) and k_{dd} (per degree day) were collected from each publication. When k_d and/or k_{dd} were not provided in the publication, the following breakdown rate coefficient was used: $W_t = W_I^{*}e^{-kt}$, where W_I and W_t are the initial and final dry LL mass, respectively, and t is the incubation time (day) (Bärlocher, 2020), and k_{dd} was calculated by replacing the time (t) in days with the total water temperature accumulated over the experimental period. The total water temperature accumulated over the experimental period was calculated with mean water temperature multiplied by experimental duration in this study.

FIGURE 1
Global distribution of the 54 sites of coniferous leaf-litter decomposition in streams and rivers. There were 541 and 428 records of coniferous leaf-litter breakdown rates expressed as per day and per degree day, respectively. The colors of circles represent the number of records of leaf-litter breakdown rates (including per day and per degree day) at each site.

Leaf-litter breakdown rates of the replications at each sampling trial/date were collected if they were provided in the original publication. If not, the mean values of k_{d} or k_{dd} at each sampling date were recorded. For studies that reported LL breakdown rates in both coarse (>1 mm) and fine (≤ 1 mm) mesh bags, they represent the total (k_{tot}) and microbial-mediated LL breakdown rates (k_{micro}), respectively, and macroinvertebrate-mediated LL breakdown rates (k_{inver}) were calculated with Wt as the differences in mass remaining in coarse and fine mesh bags (Hladyz et al., 2010).

Factors that have potential impacts on LL breakdown rates were extracted from the publications. First, climatic and geographic factors including MAT, MAP, MDTR, latitude, longitude, and altitude were recorded. If geographic data (i.e., latitude, longitude, and altitude) were missing in the original study, they were determined by using Google Earth (version 7.1.8.3036). An open climatic dataset (CRU TS v4.04, between 1901 and 2019), which is based on Google Earth on a 0.5° latitude by 0.5° longitude grid (Osborn and Jones, 2014; Harris et al., 2020), was used to obtain the climatic data (MAT, MDTR, and MAP) if they were not provided in the original studies. Second, we collected LL traits including nutritional (e.g., N and P), chemical (e.g., tannin and cellulose), and physical quality (e.g., toughness). We manually extracted LL traits data that were available only in figures. Leaf-litter nutrient concentrations that were recorded as mg/g were transformed into percentages for ease of comparison. Because some studies did not calculate stoichiometry ratios of LL traits, a third LL trait was calculated when two out of the three stoichiometry-related traits were present; for example, the lignin/N ratio was calculated if concentrations of N and lignin were available. Third, experimental factors including a mesh size of LL bags, experimental duration (day), and initial CLL mass (g) were recorded. A mesh size larger than 1 mm that allowed the access of most macroinvertebrates was classified as a coarse mesh size representing total LL breakdown rates, while a mesh size smaller than 1 mm that excluded most macroinvertebrates was classified as a fine mesh size representing microbial-mediated LL breakdown rates (Follstad Shah et al., 2017). Finally, stream physicochemical characteristics that include physical characteristics (e.g., depth, width, and velocity) and stream water quality (e.g., pH, NO- 3, and PO_4^{3-}) were extracted.

Other information extracted from the publications includes site names of decomposition experiment locations (continent) and plant classification information (species and family name). Plant functional types (PFTs) were also classified as leaf habit (evergreen vs. deciduous) and invasion status (exotic vs. native). The invasion status was determined by checking whether the sampling site is in the species' natural distribution range (http://temperate.theferns.info/) when this information was not provided in the original publication. Data collected from islands that are >1,000 km away from continents (Azores) were not assigned to any continent.

Data analysis

First, a one-way analysis of variance (ANOVA) was conducted to compare the mean values of LL breakdown rates (both k_d and k_{dd}) in this study with other global-scale studies (Boyero et al., 2016; Follstad Shah et al., 2017; Zhang et al., 2019; LeRoy et al., 2020). These studies were selected based on several criteria: (1) field experiments or meta-analysis included worldwide data; (2) data of LL breakdown rates should be obtained from natural senesced LL of various species; and (3) the decomposition experiments should be conducted in lotic ecosystems (e.g., streams and rivers). We conducted the analyses with three different datasets: (1) all data combined; (2) data for coarse mesh bags; and (3) data for fine mesh bags. Tukey's honestly significant difference (HSD) was used for the *post-hoc* multiple comparison in the ANOVA analysis. Second, t-tests were used to investigate whether the CLL breakdown rates differ between coarse and fine mesh bags. The effects of leaf habit and invasion status on CLL breakdown rates were also checked with t-tests. As there were only three studies (37 cases) that had values of macroinvertebrate-mediated LL breakdown rates, we did not conduct any analysis for this variable.

Third, stepwise multiple regression analyses were conducted to investigate the associations of climatic and geographic factors, LL traits, experimental factors, and stream physicochemical characteristics with CLL breakdown rates. We selected factors that were significantly correlated with CLL breakdown rates (Supplementary Table S1). For the selected predictors, any potential collinear predictors were identified and removed ($R \geq 0.70$) before running the analyses. If the collinearity was close to the threshold, we further calculated the variance inflation factors (VIFs) to ensure that they would not inflate the variance of models. The predictors were kept when the VIFs were below 4. Toughness and phenol-related traits were excluded in the stepwise multiple regression analyses due to the limited species (<5) that had measurements of these LL traits. The stepwise multiple regression analyses were conducted in two steps: (1) we conducted the analysis for the four categories of predictors individually (model A) and (2) we included all the factors screened in model A (model B). In addition, the included predictors in models A and B should have observations >10 times the number of variables.

Finally, linear models were used to explore the responses of CLL breakdown rates to important predictors selected in the stepwise multiple regression analyses. As some climatic and geographic factors were excluded in the regression analyses (e.g., latitude), we were interested in the latitudinal and altitudinal trends of CLL decomposition in lotic ecosystems and how climate changes (temperature and precipitation) would affect CLL breakdown rates. In this case, all climatic and geographic factors, LL traits including N and P, mean water temperature, and PO_4^{3-} were included in the linear models. These linear models were conducted with three different datasets: all data pooled together and data from coarse and fine mesh bags. This kind of data treatment was aimed to test whether there are differences between the responses of total and microbial-mediated CLL breakdown rates to these predictors. Before conducting all analyses, outliers, normality, and homogeneity of variance were examined, and data were log-transformed to meet normality and homogeneity assumptions. Consequently, CLL breakdown rates, C/P, lignin/N, experimental duration, initial litter mass, velocity, discharge, NO- 3, NH+ 4, PO_4^{3-}, and conductivity were log-transformed. All statistical analyses were carried out using IBM SPSS Statistic 24.0.

Results

Comparison of leaf-litter breakdown rates in this study with other global studies

We found consistently lower LL breakdown rates of conifers in this study than in other global-scale studies regardless of whether it was expressed as per day or per degree day or whether it was in coarse or fine mesh bags (Figure 2). Mean CLL breakdown rates in this study were -2.37 ± 0.02 ($n = 543$, mean ± SE, data were log-transformed) and -3.20 ± 0.02 ($n = 430$, mean ± SE), which were 18.5–28.8 and 4.9–16.8% lower than the reported LL breakdown rates in other global-scale studies when expressed as per day and per degree day, respectively. This trend was similar when data were analyzed separately in coarse and fine mesh bags, except that the mean breakdown rates (per degree day) in coarse mesh bags did not differ from one study (Boyero et al., 2016).

Predictors of CLL breakdown rates

We did find that CLL breakdown rates differed between mesh sizes, leaf habits, and invasion status (Figure 3).

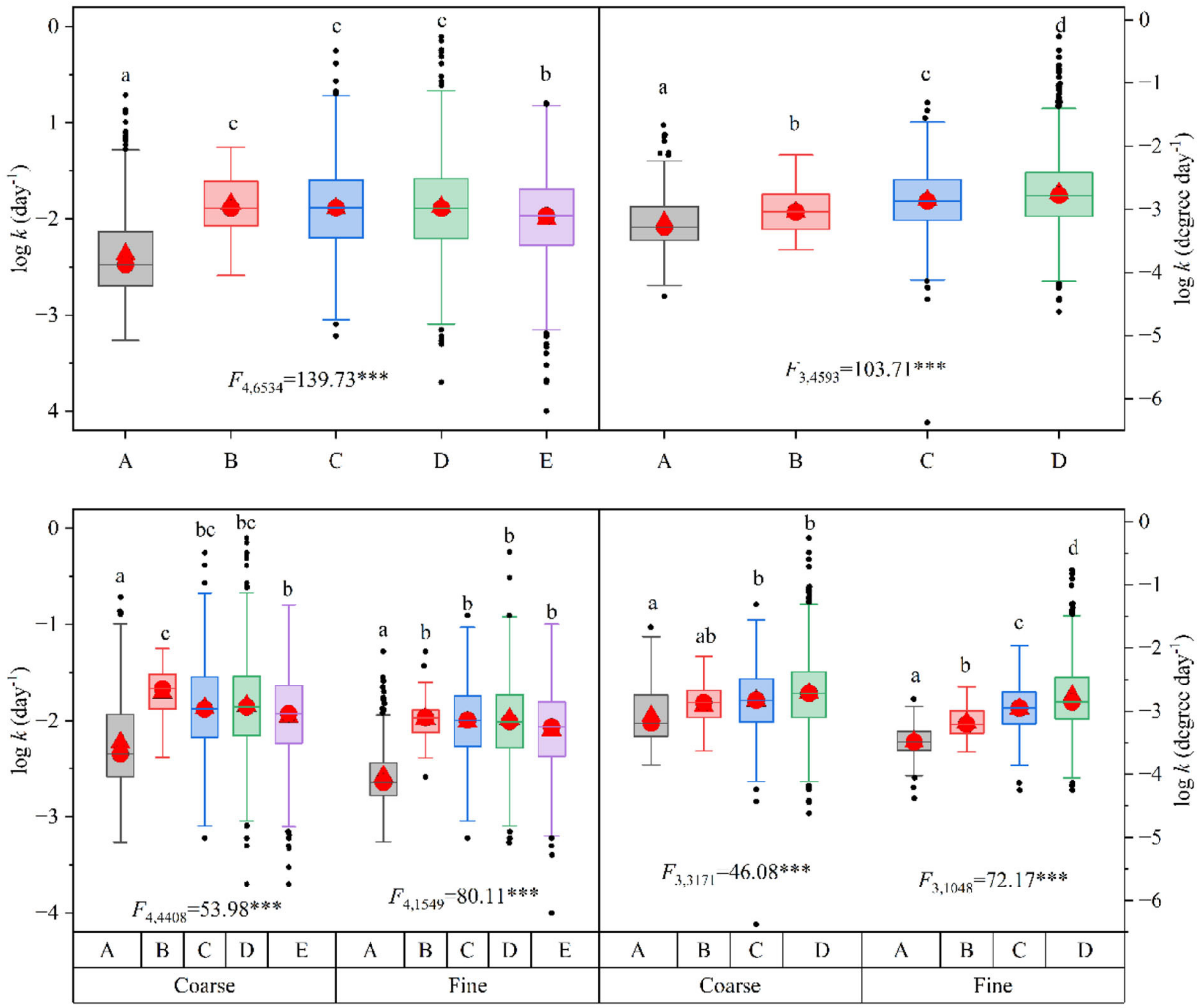

FIGURE 2
Comparisons of leaf-litter breakdown rates (per day, left; per degree day, right) in this study (A) with other global-scale studies [B, Boyero et al., 2016; C, Follstad Shah et al., 2017; D, LeRoy et al., 2020; E, Zhang et al., 2019]. Box plots summarize the distribution of all values. Box plots indicate the interquartile ranges (box part), 25th and 75th percentiles (lower and upper error bars), median values (red circles in the box), mean values (red triangles in the box), and outliers (black dots). Lowercase letters above each box indicate a significant difference among studies after one-way ANOVA and *post-hoc* Tukey's *t*-test (parameters with the same letter are not significantly different between studies, $P > 0.05$) was used. ***$P < 0.001$.

Coniferous LL decomposed 13.6 and 11.6% faster in coarse than in fine mesh bags when expressed as per day and per degree day, respectively (Figures 3A,D). Unexpectedly, deciduous CLL (*Larix*) had 11.3% slower breakdown rate (per day) than evergreen species (Figure 3B), and this trend was the same even though data were divided into coarse and fine mesh bags (Supplementary Figures S1A,C). However, the deciduous CLL breakdown rate in fine mesh bags was 4.8% faster than evergreen species when expressed as per degree day (Supplementary Figure S1C) and did not differ in coarse mesh bags nor when data were pooled. Exotic CLL breakdown rates (per day) were 10.6 and 8.7% higher than native species when data were combined (Figure 3C) and in coarse mesh bags (Supplementary Figure S1B), respectively. By contrast, exotic conifers had 3.4% faster breakdown rates, while they had 3.2% slower breakdown rates (per degree day) when data were combined (Figure 3F) and in fine mesh bags (Supplementary Figure S1D), respectively.

Altitude, MAP, and MDTR were the best-fit climatic and geographic predictors for CLL breakdown rates (per day), which together explained 22.1% of the variation (Table 1), whereas MAT and MDTR only explained 2.8% of the variations in CLL breakdown rates when expressed as per degree day. Nitrogen was the best-fit LL trait predictor, which explained 39.3% of the variations in CLL breakdown rates (per day).

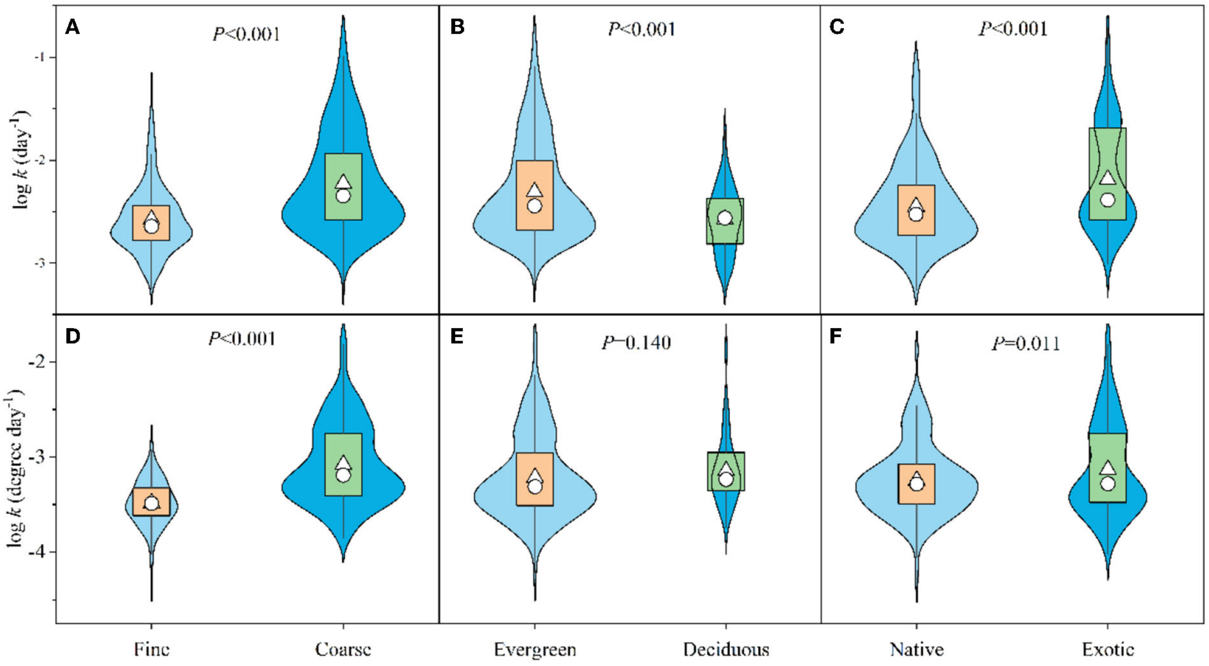

FIGURE 3
Comparisons of leaf-litter breakdown rates (per day, upper; per degree day, lower) between coarse and fine mesh bags **(A,D)**, evergreen and deciduous species **(B,E)**, and native and exotic species **(C,F)**. Box plots summarize the distribution of all values. Box plots indicate the interquartile ranges (box part), 25th and 75th percentiles (lower and upper error bars), median values (circles in the box), and mean values (triangles in the box).

When expressed as per degree day, P and N/P became the best-fit LL trait predictors, which together explained 70.4% of the variation in CLL decomposition. The best-fit experimental predictors for CLL breakdown rates were mesh size and experimental duration regardless of whether it was expressed as per day or per degree day. Mean water temperature was the best-fit physicochemical characteristic for the prediction of CLL breakdown rates (per day). By contrast, 81.8% of the variation in CLL breakdown rates (per degree day) was explained by discharge, pH, and minimum water temperature. When the selected four categories of predictors were pooled together, mean water temperature, mesh size, duration, and N concentration of LL were responsible for 23.6% of the variation in CLL breakdown rates (per day), whereas P, experimental duration, and minimum water temperature together explained 91.2% of the variation in CLL breakdown rates (per degree day). However, the best-fit predictors (in each category and with the four categories of predictors pooled) changed when CLL breakdown rates were analyzed individually in coarse and fine mesh bags (Table 1). In addition, when analyzed individually, most of the four groups of predictors showed linear relationships with CLL breakdown rates (per day and per degree day) regardless of whether data were pooled, in coarse or in fine mesh bags (Supplementary Table S1).

Relationships between CLL breakdown rates and driving predictors

Coniferous LL breakdown rates (per day) increased with increasing N, P, mean water temperature, PO_4^{3-}, MAT, and MAP, but decreased with increasing MDTR and altitude (Figure 4). There were no latitudinal trends of CLL breakdown rates regardless of whether it was expressed as per day or per degree day when all data were pooled (Figure 4E). However, we found a decreasing trend of CLL breakdown rates toward the poles in coarse mesh bags. On the contrary, CLL breakdown rates increased toward the poles in fine mesh bags (Supplementary Figure S2A). CLL breakdown rates (per day) decreased with increasing altitude when data were pooled (Figure 4I), in coarse and in fine mesh bags (Supplementary Figure S2C). However, CLL breakdown rates (per degree day) increased with increasing altitude in coarse mesh bags (Supplementary Figure S2D). Coniferous LL breakdown rates increased but decreased with increasing MAT and MAP when expressed as per day and per degree day, respectively (Figures 4F,G). However, there were no linear relationships between MAP and CLL breakdown rates (Supplementary Figures S2I,J) and between MAT and CLL breakdown rates (per degree day) in coarse mesh bags (Supplementary Figure S2F). The relationships between MDTR

10.3389/fevo.2022.940254

TABLE 1 Results of multiple regressions examining global-scale variations in coniferous leaf-litter breakdown rates (k_{d}, per day; k_{dd}, per degree day).

Dataset	*k*	Factors	Variables	Regression	*F*	R^2	Excluded variables
All	k_{d}	Environmental factors	Alt (15.0), MAP (4.1), MDTR (3.0)	$\log k = -3.178 – 2.27 \times 10^{-4}\mathrm{Alt} + 0.080\mathrm{MDTR} + 2.35 \times 10^{-4}\mathrm{MAP}$	$F_{3,536} = 50.734^{***}$	0.221	MAT
		Leaf-litter traits	N (39.3)	$\log k = -2.221 – 0.408\mathrm{N}$	$F_{1,138} = 89.298^{***}$	0.393	C, P, C:N, C:P
		Experimental factors	Mesh size (9.9), duration (33.2)	$\log k = -1.911 + 0.038\log(\text{mesh size}) - 0.368\log\mathrm{Duration}$	$F_{2,469} = 177.882^{***}$	0.431	Litter mass
		Physicochemical characteristics	$\mathrm{T_{mean}}$ (9.7)	$\log k = -2.834 + 0.039\mathrm{T_{mean}}$	$F_{1,140} = 15.011^{***}$	0.097	Depth, pH, alkalinity, PO3-4
		All	$\mathrm{T_{mean}}$ (2.3), mesh size (4.2), duration (15.5), N (1.6)	$\log k = -2.388 – 0.132\log\mathrm{Duration} + 0.016\ \mathrm{T_{mean}} + 0.027\text{Mesh size} - 0.135\mathrm{N}$	$F_{4,256} = 19.792^{***}$	0.236	Alt, MAP, duration
	k_{dd}	Environmental factors	MAT (1.4), MDTR (1.4)	$\log k = -3.342 – 0.014\mathrm{MAT} + 0.034\mathrm{MDTR}$	$F_{2,424} = 6.141^{**}$	0.028	MAP
		Leaf-litter traits	N:P (1.6), P (68.8)	$\log k = -3.687 + 7.058\mathrm{P} + 0.005(\mathrm{N{:}P})$	$F_{2,115} = 136.629^{***}$	0.704	n/a
		Experimental factors	Mesh size (21.3), duration (2.9)	$\log k = -3.245 + 0.054\text{Mesh size} - 0.136\log\mathrm{Duration}$	$F_{2,362} = 57.670^{***}$	0.242	Litter mass
		Physicochemical characteristics	Discharge (69.3), pH (11.1), $\mathrm{T_{min}}$ (1.4)	$\log k = 0.966 + 0.498\log\mathrm{Discharge} - 0.694\mathrm{pH} - 0.048\mathrm{T_{min}}$	$F_{3,89} = 133.768^{***}$	0.818	Width, conductivity, $\mathrm{T_{max}}$, $\mathrm{T_{range}}$
		All	P (77.3), duration (5.7), $\mathrm{T_{min}}$ (8.2)	$\log k = -2.330 + 5.422\mathrm{P} - 0.375\log\mathrm{Duration} - 0.085\mathrm{T_{min}}$	$F_{3,56} = 193.115^{***}$	0.912	MAT, MDTR, pH, discharge, N:P
Coarse	k_{d}	Environmental factors	AbsLat (7.8), Alt (4.5)	$\log k = -1.074 – 0.022\mathrm{AbsLat} - 2.37 \times 10^{-4}\mathrm{Alt}$	$F_{2,320} = 22.289^{***}$	0.122	MDTR
		Leaf-litter traits	P (74.7), N:P (2.4)	$\log k = -2.308 + 5.716\mathrm{P} - 0.006(\mathrm{N{:}P})$	$F_{2,87} = 146.248^{***}$	0.771	N
		Experimental factors	Duration (12.5), mesh size (20.5)	$\log k = -2.173 – 0.306\log\mathrm{Duration} + 0.06\text{Mesh size}$	$F_{2,253} = 62.238^{***}$	0.330	Litter mass
		Physicochemical characteristics	PO3-4 (18.5), conductivity (5.0)	$\log k = -2.894 + 0.167\log\text{PO3-4} + 0.219\ \mathrm{Conductivity}$	$F_{2,108} = 16.630^{***}$	0.235	Depth, pH, NO- 3, $\mathrm{T_{mean}}$
		All	PO3-4 (31.3)	$\log k = -3.052 + 0.433\log\text{PO3-4}$	$F_{1,34} = 15.469^{***}$	0.504	AbsLat, alt, P, N:P, duration, conductivity
	k_{dd}	Environmental factors	Alt (2.2)	$\log k\text{=-}3.171 + 1.46 \times 10^{-4}\mathrm{Alt}$	$F_{1,300} = 6.723^{**}$	0.022	n/a
		Leaf-litter traits	P (72.2)	$\log k = -3.435 + 6.032\mathrm{P}$	$F_{1,88} = 228.315^{***}$	0.722	N:P
		Experimental factors	Duration (7.2), mesh size (4.0)	$\log k = -3.089 – 0.177\log\mathrm{Duration} + 0.041\text{Mesh size}$	$F_{2,234} = 14.845^{***}$	0.113	Litter mass
		Physicochemical characteristics	Discharge (69.3), conductivity (5.4)	$\log k = -2.720 + 0.556\log\mathrm{Discharge} - 0.762\mathrm{Conductivity}$	$F_{2,90} = 132.916^{***}$	0.747	Width, $\mathrm{T_{max}}$, $\mathrm{T_{min}}$, $\mathrm{T_{range}}$
		All	P (77.3), duration (5.7), discharge (5.5)	$\log k = -3.739 + 3.378\mathrm{P} - 0.309\log\mathrm{Duration} + 0.439\log\mathrm{Discharge}$	$F_{3,56} = 143.990^{***}$	0.885	Alt, conductivity
Fine	k_{d}	Environmental factors	MAP (23.3), alt (5.3), MDTR (9.1)	$\log k = -3.474 – 2.72 \times 10^{-4}\mathrm{MAP} - 1.73 \times 10^{-4}\mathrm{Alt} + 0.084\mathrm{MDTR}$	$F_{3,213} = 42.909^{***}$	0.377	AbsLat, MAT
		Leaf-litter traits	Lignin (56.5), C (9.8)	$\log k = -3.275 – 0.02\mathrm{Lignin} + 0.032\mathrm{C}$	$F_{2,51} = 52.208^{***}$	0.374	N
		Experimental factors	Mesh size (10.2), duration (51.1)	$\log k = -2.174 + 0.563\text{Mesh size} - 0.349\log\mathrm{Duration}$	$F_{2,213} = 168.860^{***}$	0.613	n/a

(Continued)

TABLE 1 Continued

Dataset k	Factors	Variables	Regression	F	R^2	Excluded variables
	Physicochemical characteristics	T_{mean} (19.0)	$\log k = -2.868 + 0.036T_{mean}$	$F_{1,74} = 35.971$***	0.190	Width, T_{min}, T_{range}, conductivity
	All	Duration (31.6), MDTR (9.2), lignin (3.0), mesh size (10.5)	$\log k = -2.065–0.393\log\text{Duration} + 0.069\text{MDTR} + 0.059\text{Lignin}-5.895\text{Mesh size}$	$F_{4,90} = 26.714$***	0.543	MAP, alt, T_{mean}
k_{dd}	Environmental factors	MAP (10.2)	$\log k = -3.265–1.52 \times 10^{-4}\text{MAP}$	$F_{1,126} = 14.335$***	0.102	MAT, AbsLat
	Leaf-litter traits	Cellulose (19.5)	$\log k = -2.457–0.049\text{Cellulose}$	$F_{1,67} = 16.216$***	0.195	Lignin
	Experimental factors	Duration (3.0)	$\log k = -3.318–0.092\log\text{Duration}$	$F_{1,126} = 3.921$*	0.030	n/a
	Physicochemical characteristics	T_{range} (13.6)	$\log k = -3.01–0.038T_{range}$	$F_{1,67} = 10.531$**	0.136	NO- 3, alkalinity, T_{max}
	All	Duration (29.6), T_{range} (19.9), cellulose (18.0)	$\log k = -1.055–0.443\log\text{Duration}–0.047\text{Cellulose}–0.046T_{range}$	$F_{3,65} = 45.064$***	0.675	MAP

Five different datasets were included: (1) environmental factors (MAP, mean annual precipitation; MAT, mean annual temperature; absolute latitude, AbsLat; altitude, Alt; and MDTR, mean diurnal temperature range); (2) leaf-litter traits; (3) experimental factors; (4) stream physicochemical characteristics, and (5) the predictors selected in the above four models. Data in parentheses indicate the contribution of the exact predictor to coniferous leaf-litter decomposition. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

and CLL breakdown rates (per day and per degree day) when all data were pooled were contrary to the relationships between MAT and CLL breakdown rates (Figure 4H). However, CLL breakdown rates (per day) increased but decreased with increasing MDTR in coarse and fine mesh bags, respectively (Supplementary Figure S2G).

Discussion

Slower leaf-litter breakdown rates in this study than in other studies

Our result supports the first hypothesis that LL breakdown rates in this study were significantly lower than that in other global-scale studies (Boyero et al., 2016; Follstad Shah et al., 2017; Zhang et al., 2019; LeRoy et al., 2020). This result advocates many other studies that found lower CLL breakdown rates than broadleaved LL in streams at local, regional, and global scales (Graça and Pereira, 1995; Albariño and Balseiro, 2002; Zhang et al., 2019). Although, some studies claimed that there were no differences in LL breakdown rates between conifers and broadleaved species at local (Ferreira et al., 2017) and global scales (Ferreira et al., 2016). The slower CLL breakdown rate was not surprising because CLL usually has a poorer nutritional quality (e.g., lower N) than other species (Richardson et al., 2004). Microbes and macroinvertebrates prefer a high nutritional quality over less nutritious LL, which resulted in a lower breakdown rate of poor nutritious LL (Gessner and Chauvet, 1994; Balibrea et al., 2020; Oliveira et al., 2022). Another possible explanation may be associated with temperature because most sampling sites in this study were in mid- and high-latitude areas and had no site in tropical areas. Consequently, MAT and water temperature in this study were lower than those mentioned in other large-scale studies, which may contribute to the reduced LL breakdown rates (Boyero et al., 2011b; Follstad Shah et al., 2017). Given that conifer plantation is one of the main types of forest changes that occurred worldwide (Essl et al., 2010; Ferreira et al., 2016; Kawamura et al., 2021), our results, therefore, have implication on predicting the ecological consequences of the replacement of native species by conifer plants (e.g., *Pinus*) in freshwater ecosystems such as LL decomposition.

Relationships between LL traits and CLL decomposition in streams and rivers

As expected, LL traits explained a relatively high proportion of the variation in CLL decomposition. This result is in accordance with many other local and global-scale studies that revealed the predominant role of LL traits in determining

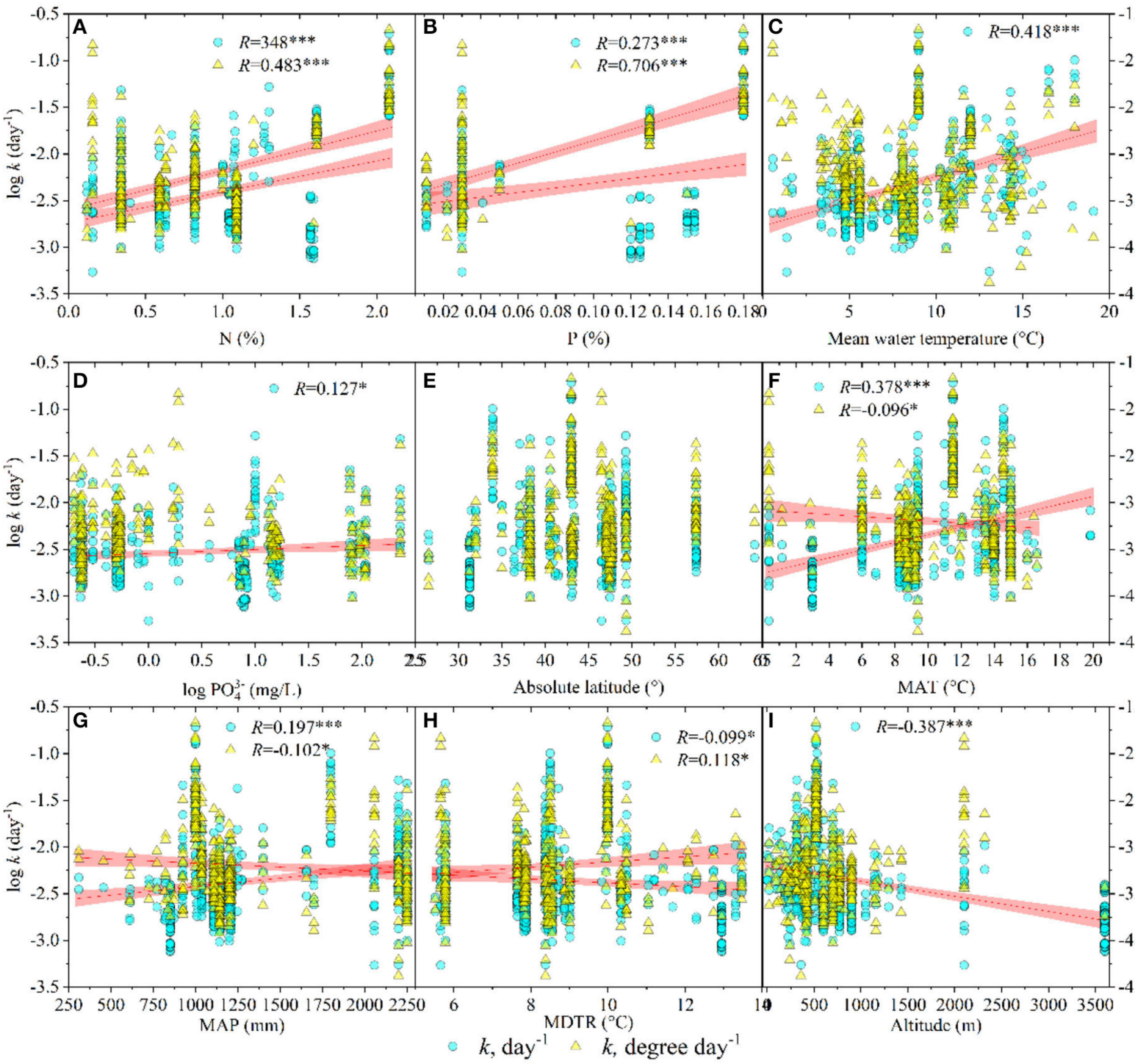

FIGURE 4
(A–I) Relationships between coniferous leaf-litter breakdown rates (per day, circle; per degree day, triangle) and selected important leaf-litter traits (N and P), stream physicochemical characteristics (mean water temperature and PO_4^{3-}), and geographic and climatic (latitude; MAT, mean annual temperature; MAP, mean annual precipitation; MDTR, mean diurnal temperature range; and altitude) predictors. *R* values with significant correlations are shown in the figure. ***$P < 0.001$. Linear fitted lines and 95% confidence intervals are shown.

LL decomposition in streams and rivers (Leroy and Marks, 2006; Goncalves et al., 2017; Zhang et al., 2019). Also, we found that the controlling LL traits differed depending on whether it was expressed as per day or per degree day and whether in coarse or fine mesh bags. This result adds evidence that there was no consistent conclusion regarding which LL trait is the most important predictor of LL decomposition in freshwaters. Furthermore, our result underscored that LL nutritional quality (N and P) mainly drives the decomposition of CLL in streams and rivers, and the result agrees with the findings for many broadleaved LL and CLL (Kennedy and El-Sabaawi, 2017; Jabiol et al., 2019). Nitrogen and P concentrations are important indicators of LL decomposability. Many researchers found positive effects of N content on LL decomposition in freshwaters through enhanced biomass, diversity, and activity of microbes (e.g., fungi) and macroinvertebrates (Garcia et al., 2012; Jackrel and Wootton, 2015; Pereira and Ferreira, 2021). By contrast, secondary compounds such as lignin can hinder LL decomposition by reducing the abundance and richness of microbes and macroinvertebrates (Gessner and Chauvet, 1994; Leite-Rossi et al., 2016; Balibrea et al., 2020). However, we only found a negative relationship between lignin and CLL breakdown rates in fine mesh bags. Taken together, our results may indicate that microbial-mediated and total CLL decomposition were driven by lignin and the nutritional quality of LL, respectively.

Relationships between stream physicochemical characteristics and CLL decomposition

Stream physicochemical characteristics affected CLL decomposition, but the best-fit predictors are context dependent. Specifically, we found stronger impacts of mean water temperature on CLL decomposition than other physicochemical characteristics in fine mesh bags and when all data were pooled. An increasing number of studies addressed the importance of water temperature in driving LL decomposition in freshwaters at local, regional, and global scales (Boyero et al., 2011b; Bernabé et al., 2018; Xiang et al., 2019). As the positive relationships between CLL decomposition and mean water temperature appeared in both coarse and fine mesh bags, the *R* value was much higher in coarse than in fine mesh bags. We may speculate that the accelerated CLL decomposition at higher water temperature may be attributed to both macroinvertebrate- and microbial-mediated ways. Elevated water temperature can stimulate microbial-mediated LL decomposition in various ways, including stimulating activities, higher biomass, and higher richness of microbes (Ferreira and Chauvet, 2011; Canhoto et al., 2016). Similarly, macroinvertebrates decomposed LL faster under warmer conditions due to higher metabolic rate, stimulated activities, and higher density (Esther et al., 2015; Griffiths and Tiegs, 2016). Although some researchers found negative effects of warmer water temperature on macroinvertebrates and/or microbes (Boyero et al., 2011b; Martínez et al., 2014; Domingos et al., 2015), these negative effects may be weak or were suppressed by the positive effects of warming on LL decomposition. Moreover, we found significant effects of temperature range, minimum water temperature, and maximum water temperature on CLL decomposition. This result highlights that, along with mean water temperature, temperature oscillation should also be considered in future studies (Dang et al., 2009).

By contrast, PO_4^{3-} concentration was the best-fit physicochemical predictor, which showed a positive relationship with CLL decomposition in coarse mesh bags. This result adds evidence to mounting research that suggest accelerated LL decomposition by higher water nutrient concentrations (Woodward et al., 2012; Ferreira et al., 2015a; Manning et al., 2021). Phosphorus is a fundamental and usually limited nutrient in freshwater ecosystems such as streams and rivers (Elser et al., 2007; Dodds and Smith, 2016). Previous studies found that the abundance, density, biomass, and richness of macroinvertebrates may be positively affected by higher nutrient concentrations (Gulis et al., 2006; Bergfur et al., 2007; Pereira et al., 2016) to enhance LL decomposition. We may anticipate faster macroinvertebrate-mediated CLL breakdown rates in future, considering that P concentration in streams and rivers worldwide has been and will continue to increase (Vilmin et al., 2018; Harrison et al., 2019; Beusen et al., 2022). Taken together, our results imply that macroinvertebrate-mediated and microbial-mediated CLL decomposition were differently influenced by stream physicochemical characteristics, and future changes in water temperature and nutrient concentrations should be especially concerned.

Relationship between climatic and geographic factors and CLL decomposition in streams

Although we found that CLL breakdown rates had linear relationships with gradients of climatic and geographic factors, they explained relatively smaller variations in CLL decomposition in streams and rivers than other predictors (e.g., LL traits). This result agrees with previous studies that found that LL decomposition changed along the gradients of latitude (Irons III et al., 1994; Tiegs et al., 2019; Boyero et al., 2021), altitude (Fabre and Chauvet, 1998; Jinggut and Yule, 2015; Martínez et al., 2016), and temperature (Boyero et al., 2011b). Specifically, we found that CLL breakdown rates increased in fine mesh bags but decreased in coarse mesh bags toward the poles. The result for fine mesh bags is consistent with other large-scale studies, while the result for coarse mesh bags agrees (Tiegs et al., 2019) or disagrees with others (Irons III et al., 1994; Follstad Shah et al., 2017). Our result implies that macroinvertebrate-mediated CLL decomposition was reduced toward the poles and overrode the stimulated microbial-mediated CLL decomposition, which resulted in decreased total CLL breakdown rates. Along the latitudinal gradient, we found increased chemical LL quality (e.g., lower lignin and lignin/N), decreased MAT and mean water temperature, and lower water nutrient concentrations (e.g., NO-3 and PO_4^{3-}). These changes may have positive (enhanced LL quality) or negative effects (e.g., lower water temperature) on CLL decomposition in streams as evidenced in this study and many others (Woodward et al., 2012; Ferreira et al., 2015b). Therefore, we may speculate that the accelerated microbial-mediated CLL breakdown rates toward the poles may be attributed to the increased chemical LL quality, and this effect may have overridden the negative effects of decreased temperature and water nutrient concentrations on microbes. On the contrary, decreased MAT, mean water temperature, and water nutrient concentrations may have resulted in slower macroinvertebrate-mediated and hence total CLL breakdown rates toward high-latitude areas and suppressed the positive effects of enhanced CLL quality on decomposition.

In addition, we found consistently slower CLL breakdown rates in this study than the reported LL breakdown rates in tropical areas in other studies. This is interesting because previous studies found higher LL breakdown rates in tropical than in high-latitude areas or no differences between the two regions (Irons III et al., 1994; Zhang et al., 2019). This pattern may be true for broadleaved species because many previously published papers focused on broadleaved LL and ignored coniferous species. In fact, conifers are the dominant plants in many mid- and high-latitude areas such as temperate, boreal, and Mediterranean forests (Essl et al., 2010). However, the decomposition of CLL in streams is largely ignored by researchers, and the proportion of conifers was usually low in previous studies (Ferreira et al., 2015a; LeRoy et al., 2020). The low proportion of conifers in global-scale studies may have unintentionally induced higher LL breakdown rates in mid- and high-latitude areas. Therefore, we argue that the inclusion of a proper proportion of conifers may give us a better understanding of the latitudinal trend of LL decomposition in streams and rivers at a global scale, especially for macroinvertebrate-mediated LL decomposition and for temperature-normalized LL breakdown rates.

We also found that the third hypothesis was supported as MAT and MAP positively affected CLL decomposition in both coarse and fine mesh bags in streams, a result following previous studies (Boyero et al., 2011b; Follstad Shah et al., 2017). If the stimulated CLL breakdown rates were induced by the changes in CLL quality associated with increasing MAT and MAP, we should have expected slower CLL breakdown rates. Because CLL quality decreased with increasing MAT and MAP as indicated by higher lignin and lignin/N, and lower LL quality can inhibit LL decomposition through the negative effects on microbes and macroinvertebrates (Hladyz et al., 2009; Kennedy and El-Sabaawi, 2017). Similarly, faster CLL breakdown rates may not be correlated with the changes in detritivore communities associated with changing MAT and MAP. Because abundance and richness of shredders are higher in mid- and high-latitude areas (low MAT and MAP) than in low latitude areas (Boyero et al., 2011a). Alternatively, higher CLL breakdown rates caused by higher MAT may be attributed to the changed microbial communities (e.g., higher diversity) and higher water nutrient concentrations, all of which have shown to boost LL decomposition in streams (Boyero et al., 2011b; Woodward et al., 2012; Fenoy et al., 2022). As we did not have enough cases that included data from both coarse and fine mesh bags to calculate macroinvertebrate-mediated CLL breakdown rates, the relationship between CLL decomposition and MAT was stronger in fine than in coarse mesh bags. We are not sure whether macroinvertebrate-mediated CLL decomposition was stimulated by increasing MAT or not. Our result suggests that future climate change such as warming and higher MAP, which is likely to occur in mid- and high-latitude areas where conifer plants are dominant species (Essl et al., 2010), may lead to faster microbial-mediated and total CLL decomposition in streams.

Moreover, we found negative relationships between CLL breakdown rates and altitude in both coarse and fine mesh bags, while the relationship was positive in coarse mesh bags when expressed as per degree day. This decreasing (or increasing when expressed as per degree day) trend of LL decomposition along altitudinal gradient was also evidenced in other studies (Jinggut and Yule, 2015; Martínez et al., 2016). Because nutritional quality (N and P)increased while decreased with increasing altitude in fine and coarse mesh bags, respectively, the decreased CLL decomposition with increasing altitude in fine mesh bags, therefore, was not controlled by the improved CLL nutritional quality, nor by higher water nutrient concentrations (e.g., NO-3, PO_4^{3-}). Rather, the decreased temperature (MAT and mean water temperature) should be responsible for the declined microbial-mediated CLL decomposition (Taylor and Chauvet, 2014). However, both the decreased nutritional quality of CLL and lower temperature may contribute to the decelerated total CLL decomposition.

Relationship between experimental factors and CLL decomposition in streams

As expected, we found that mesh size is positively correlated with CLL decomposition in both coarse and fine mesh bags, a result in line with other studies (Zhang et al., 2019). The mesh size of LL bags can be divided into coarse and fine mesh groups to represent total and microbial-mediated LL breakdown rates, respectively. However, this classification is somehow arbitrary and there was no consistent agreement on the standard. Some researchers suggested mesh size larger than 1 mm as coarse mesh (Follstad Shah et al., 2017; Zhang et al., 2019), while others claimed 0.5 mm (Ferreira and Guérold, 2017; Kennedy and El-Sabaawi, 2017). In addition, some researchers used three or more than three categories other than the two categories of mesh size because micro-, meio-, macroinvertebrates, and macroconsumers (e.g., crabs) can contribute to LL decomposition (Moulton et al., 2010; Handa et al., 2014; Wang et al., 2020). Therefore, we suggest that more studies should be conducted on the relationship between mesh size and LL decomposition. The commonly applied 1 or 0.5 mm to differ between coarse and fine mesh bags representing total and microbial-mediated LL breakdown rates may not be able to exclude small size invertebrates (e.g., small body midge) in fine mesh bags. The standard should be adjusted to a smaller size or more size categories may be used. Moreover, CLL breakdown rates decreased with a longer experimental duration, which agrees with many other studies (Ferreira et al., 2015b; Balibrea et al., 2020). Once fallen into freshwaters, LL is processed into three different stages, namely, leaching, conditioning, and fragmentation (Gessner et al., 1999). However, the duration of these stages of CLL was longer than other species (Sakai et al., 2016; Balibrea et al., 2020). Half of the CLL breakdown rates in this study were measured within 70 days, which translates to an average of 77.5% of remaining LL mass. Therefore, we suggested that future studies of CLL decomposition in freshwaters should have a longer duration than that for broadleaved LL.

Other predictors of CLL decomposition in streams

Coniferous LL breakdown rates were higher in coarse than in fine mesh bags regardless of whether it was expressed as per day or per degree day, which is in accordance with other studies (Whiles and Wallace, 1997; Balibrea et al., 2020). This result suggests that both macroinvertebrates and microbes contributed to the decomposition of CLL. Based on the limited studies that can calculate macroinvertebrate-mediated CLL breakdown rates (37 cases), macroinvertebrates and microbes were responsible for 49.7% and 40.5% of total CLL decomposition, respectively. These values differ from the result in a global study that reported LL breakdown rates in lotic ecosystems at a global scale (unpublished data) and that the contribution of microbes was lower, while macroinvertebrates were higher than that study. Our result also differs from another study that reported higher and lower contributions of macroinvertebrates and microbes on LL decomposition based on biomass, respectively (Hieber and Gessner, 2002). The relatively high contribution of macroinvertebrates to CLL decomposition may be attributed to the high abundance and richness of detritivores in mid- and high-latitude areas (Boyero et al., 2011a) where conifers are abundant. Even though CLL is a low-quality food resource for detritivores in streams (Richardson et al., 2004), CLL can remain longer than broadleaved LL in streams and becomes the major food resource in summer when broadleaved LL are scarce or depleted (Hisabae et al., 2011; Sakai et al., 2016). In addition, CLL can also be utilized as case-building materials by invertebrates due to their refractory nature (Whiles and Wallace, 1997; Richardson et al., 2004; Márquez et al., 2017). These factors may partially explain the relatively high contribution of macroinvertebrates to CLL decomposition in streams.

Coniferous LL decomposition was affected by leaf habit that evergreen CLL decomposed faster than deciduous CLL (*Larix*). This result is unexpected because evergreen LL usually has a lower quality than broadleaved LL, which may result in slower LL breakdown rates (Kominoski et al., 2011; Ramos et al., 2021). Our result also differs from one early global-scale study that found no difference in broadleaved LL decomposition between evergreen and deciduous species (Zhang et al., 2019). As there was no consistent result regarding whether evergreen CLL had lower quality than deciduous CLL in this study, the slower deciduous CLL breakdown rates were probably because deciduous CLL was decomposed at higher altitude areas where the MAT was lower than the evergreen CLL decomposition sites. Because water temperature is usually positively correlated with LL decomposition in streams. Moreover, we found higher exotic than native CLL breakdown rates in coarse, but not in fine mesh bags, suggesting that only macroinvertebrate-mediated CLL decomposition was influenced by invasion status. This result is in line with another global-scale study (Kennedy and El-Sabaawi, 2017). Exotic CLL had higher nutritional (N) but lower chemical (lignin) quality than native CLL in coarse mesh bags. By contrast, exotic CLL showed both lower nutritional and chemical quality than native CLL in fine mesh bags. Therefore, the differences in CLL quality between native and exotic species may not be the reason why exotic CLL decomposed faster than native species in coarse mesh bags. However, exotic CLL decomposed in streams with higher MAT than native species, which may be responsible for the faster LL breakdown rates (Ferreira et al., 2015b). Our results imply that the environmental conditions (e.g., MAT) may modulate and override the effects of CLL quality on the processing rate in streams and rivers.

Conclusions

In conclusion, we found first that CLL breakdown rates were significantly lower than other species probably due to the lower quality (e.g., lower N and higher lignin) and the lower water temperature. Second, CLL decomposed faster in coarse than in fine mesh bags, and CLL breakdown rates increased with increasing mesh size. This result highlights the importance of macroinvertebrates in driving CLL decomposition, and the commonly used 1 mm or 0.5 mm mesh size to differing total and microbial-mediated LL breakdown rates may have resulted in higher values of the latter. Third, LL traits and stream physicochemical characteristics explained higher variations in CLL decomposition than climatic and geographic factors. Nutritional quality (N and P) of LL, water temperature, and water nutrient concentration (PO_4^{3-}) were identified as better predictors of CLL decomposition than other factors. Finally, CLL decomposition was stimulated by higher MAT and MAP. These observed global patterns of CLL decomposition have implications to help predict ecological responses of CLL breakdown rates and their responsive mechanisms to future climate change and the subsequent consequences on macroinvertebrate and microbial communities and global nutrient cycling in freshwater ecosystems.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

HX and ZZ conceived the ideas and designed methodology. HX collected the data and led the writing of the manuscript. KL, LC, and HY analyzed the data. All authors contributed critically to the drafts and gave final approval for publication.

Funding

This work was supported by the fellowship of the China Postdoctoral Science Foundation (2021M692728), the National Natural Science Foundation of China (32101310 and 32071587), the China Scholarship Council (202006625001), the Science Foundation of Science and Technology of Education Department of Jilin Province (JJKH20211293KJ), the Natural Science Foundation of Heilongjiang Province of China (YQ2021C031), the Postdoctoral Scientific Research Developmental Fund of Heilongjiang Province (LBH-Q20166), and the Basal Research Fund for Undergraduate Universities of Heilongjiang Province (2019-KYYWF-0598).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.940254/full#supplementary-material

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frontiers | Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 25 July 2022
DOI 10.3389/fevo.2022.929944

Genetic structure of wild rice *Zizania latifolia* in an expansive heterogeneous landscape along a latitudinal gradient

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Global aquatic habitats are undergoing rapid degradation and fragmentation as a result of climate change and changes in land use. Understanding the genetic variability and adaptive potential of aquatic plant species is thus important for conservation purposes. In this study, we investigated the genetic diversity and structure of the extant natural populations of *Zizania latifolia* from five river basins in China based on 46 microsatellite markers. We tested isolation by environment (IBE), isolation by resistance (IBR), and isolation by distance (IBD) patterns using a reciprocal causal model (RCM). Furthermore, we elucidated the impact of the environment on *Z. latifolia* genetic diversity using generalized linear models (GLMs) and spatially explicit mixed models. Low genetic diversity ($H_E = 0.125–0.433$) and high genetic differentiation ($F_{ST} = 0.641$, $\varnothing_{pt} = 0.654$) were found. Higher historical gene flow ($M_H = 0.212–2.354$) than contemporary gene flow ($M_C = 0.0112–0.0247$) and significant bottlenecks in almost all populations were identified, highlighting the negative impact of wetland fragmentation. The IBE model was exclusively supported for all populations and in three river basins. The IBD and IBR models were supported in one river basin each. The maximum temperature of the warmest month and precipitation seasonality were the plausible environmental parameters responsible for the observed pattern of genetic diversity. Local adaptation signatures were found, with nine loci identified as outliers, four of which were gene-linked and associated with environmental variables. Based on these findings, IBE is more important than IBD and IBR in shaping the genetic structure of *Z. latifolia*.

KEYWORDS

genetic structure, isolation by environment, landscape heterogeneity, latitudinal gradient, microsatellite markers, *Zizania latifolia*

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

RECEIVED 27 April 2022
ACCEPTED 30 June 2022
PUBLISHED 25 July 2022

CITATION
Wagutu GK, Fan X, Fu W, Tengwer MC, Li W and Chen Y (2022) Genetic structure of wild rice *Zizania latifolia* in an expansive heterogeneous landscape along a latitudinal gradient. *Front. Ecol. Evol.* 10:929944. doi: 10.3389/fevo.2022.929944

Introduction

Migration and adaptation are two main strategies that plants use to resist unfavorable climate alterations. The knowledge about genetic structure and dispersal modes are of important implications for the fate of plants after migration. The higher genetic homogeneity among populations, due to frequent gene exchange, may indicate that individuals are less dependent on local conditions and may be able to survive in different areas. On the other hand, some species show obvious genetic differentiation and develop local adaptations due to selective pressures associated with particular local habitats (Bischoff et al., 2006). In this case, plant fitness will be greatly impacted when environmental changes occur rapidly. Additionally, the adverse effects of climate change on plant diversity will be exacerbated by habitat fragmentation from land-use change and excessive exploitation. Aquatic habitats are often discontinuous and sandwiched between terrestrial habitats. Furthermore, given that most aquatic plants persist as meta-populations, their long-term survival depends on continuous gene flow among populations (Barrett et al., 1993; Santamaría, 2002). It is conceivable that the survival of aquatic plants will face more severe tests with increasing climate warming and habitat fragmentation. Therefore, investigating the dispersal and adaptability of aquatic plants will help predict their response to future climatic changes and formulate reasonable management strategies.

It is important to examine the factors affecting genetic patterns, especially when the natural environments are changing at an alarming rate. In discontinuous habitats, the gene flow is usually not enough to offset the alleles lost through genetic drift and inbreeding associated with habitat fragmentation. This situation leads to "isolation by distance," (IBD) where geographical distance limits gene flow (Wright, 1943). Migration involves the physical dispersal of propagules and successful establishment. However, significant genetic differentiation has occasionally been found among adjacent populations with different climates or soil conditions (Zhao et al., 2013). Due to long-term growth in special habitats, native plants have developed specific genotypes to adapt to local environments, which results in unsuccessful colonization for migrant plants. Thus, the IBD model sometimes underestimates the effects of environmental variables, and researchers propose the "isolation by environment" (IBE) model, which considers the contribution of environmental heterogeneity in shaping the distributions of spatial genetic variation (Wang and Bradburd, 2014). Widespread species are often distributed in habitats with distinct climatic conditions. Selective pressures can lead to strong differentiation of allele frequencies in some loci which will deviate from the equilibrium model and are considered to be potentially adaptive. Outlier loci detection and environmental association tests allow for the identification of such alleles, providing further evidence for IBE. The development of landscape genetics and map theory has made it easier to obtain landscape features (topographic and environmental characteristics) of habitats which are used to evaluate dispersal difficulties through different habitats. Based on this, McRae (2006) described isolation by resistance (IBR) as where resistance reflects the difficulty exerted by landscape features on gene flow.

Although these models (IBD, IBE, and IBR) often work together in forming genetic differentiation of species, especially for widespread species, most empirical research on population genetics has focused on geographical distance and topographical features as the main drivers, ignoring the contribution of environmental factors (Orsini et al., 2013).

To identify the most probable models for observed genetic structure, Cushman and Landguth (2010) introduced an analytical framework, the reciprocal causal model (RCM), for competing the three hypotheses simultaneously. The framework is based on Mantel tests (Mantel, 1967), where partial Mantels are first performed, and the resultant values are used to build the RCM. This approach reduces the simple Mantels' spurious correlations and false-positive errors. For the identification of the contribution of specific environmental variables to genetic diversity, especially in a spatial auto-correlation scenario, Rousset and Ferdy (2014) proposed an improved spatially explicit generalized linear mixed modeling (spatial GLMMs) method, implemented in R package *spaMM*. This allows for the accurate comparison of a null model (spatial as the random effect) vs. a full model (spatial and environmental variable as random effects), unlike the conventional methods, such as *glmmPQL* that do not fit a null model (Rousset and Ferdy, 2014).

For widespread species, genetic differentiation is prevalent and local adaptation is probable. While IBD is expected in such populations, the role of unique environment niches could be overlooked. The inclusion of environmental factors can improve ecological studies of different organisms and ecosystems while monitoring their biological implications has application in the development of environmental-conscious policies (Wu et al., 2022). Ecological niche models (ENMs) have been used to explore the role of ecology on the current and future distribution of aquatic species (Nzei et al., 2021; Ngarega et al., 2022). Traditional ENMs assumed that populations of a species are genetically uniform and share the same niche, ignoring the possibility of local adaptation, especially for widespread species (Wiens et al., 2009). Evidence for environment-associated gene-linked loci in a widespread species could be an indication of local adaptation. When an IBE pattern is also found, genetically-informed ENMs (gENMs) can be used to supplement this evidence through the investigation of niche differentiation (divergence or conservatism) (Ikeda et al., 2017; Bothwell et al., 2021).

Zizania latifolia (Griseb.) Turcz. Ex Stapf, a perennial aquatic grass commonly known as the Chinese wild rice, belongs to the family Poaceae, tribe Oryzeae. It is a partially clonal species that is an important ecological and genetic resource in

China (Liu et al., 1999, 2007; Yu et al., 2006; Zhou et al., 2007; Shen et al., 2011; Peng et al., 2013; Wang et al., 2013). Natural populations of *Z. latifolia* are distributed in the East of China along a wide stretch of latitudinal zones (21°-50°N). This region spans five major eco-geographic regions with varying biotic and abiotic factors that could influence species gene flow and local adaptation (Wu et al., 2003). To date, the genetic differentiation of wild rice across China has been attributed to IBD (Chen et al., 2017a; Zhao et al., 2018, 2019). This is despite the fact that the distribution of wetlands in China is characterized by heterogeneous landscapes and environments, besides being expansive and patchy. Therefore, such a wetland distribution pattern presents an opportunity to study the landscape genetics of a widespread species.

Based on the distribution of *Z. latifolia* in China, we hypothesized that IBE would best explain its genetic structure and that genetic variability is influenced by the environment. The objective was to test three gene flow models, IBE, IBD, and IBR using RCM, and the contribution of environmental variables to genetic diversity patterns using spatial GLMMs. This study would aid in delineating the impact of human- and climate-change-induced habitat degradation on the genetic structure of riparian plants at different spatial and environmental gradients. Furthermore, the environmental gradient provides a perfect model for space-for-time substitution in assessing the long-term aquatic ecosystem response to the changing environment.

Materials and methods

Study design

Twenty-eight natural populations of *Z. latifolia*, covering above 30 latitudes (N 20°21′ - 50°54′), were collected across China from five river basins: Heilongjiang River Basin, Liaohe River Basin, Huanghe River Basin, Yangtze River Basin, and Pearl River Basin (Table 1) in the autumn of 2015. For each population, young and healthy leaves of 20–23 individuals were collected at intervals of at least 10 m to avoid sampling the same clone. The leaves were dried with silica gel and taken back to the laboratory for DNA extraction.

Total genomic DNA was extracted from 0.5 g of dried leaves using a modified cetyltrimethylammonium bromide (CTAB) protocol with a 3 × CTAB buffer (Doyle and Doyle, 1987). After screening, 46 simple-sequence repeat (SSR) markers with polymorphic and clear bands were used in this study, including 26 genomic SSR (gSSR) (Richards et al., 2007; Quan et al., 2009; Wang et al., 2015; Wagutu et al., 2020a) and 20 expressed sequence tag SSR (EST-SRR) developed from *Z. latifolia* transcriptome data available at NCBI (SRX796501) following our previous approach (Wagutu et al., 2020b) (Supplementary Table 1). PCR amplification was performed following the protocol by Quan et al. (2009), and PCR products were separated on a 6% denaturing polyacrylamide gel. Fragments were visualized by silver staining and alleles were scored in reference to a 25 bp DNA ladder (Promega, Madison, WI, USA).

GenoDive 2.0 (Meirmans and Van Tienderen, 2004) was used to identify the clonal structure. To identify multi-locus genotypes, we tested the commonly used thresholds for microsatellites (0–4%) (Lo et al., 2010; Gross et al., 2012; Meirmans, 2020). No significant difference ($P < 0.05$) was found between the diversity indices (N/G; where N is the population sample size, G is the number of clones for each population) for the thresholds tested. Additionally, considering the facts (1) we sampled each individual at a 10 m radius to avoid collecting clone mates, (2) we excluded individuals with missing data, and (3) that a non-zero threshold could lead to the assignment of individuals with different genotypes into the same clone (James and McDougall, 2014), threshold 0 was used. At threshold zero, samples were assigned to their respective clones. The number of genotypes (*G*) was calculated, and repeating genotypes were excluded from further analysis. FreeNA software (Chapuis and Estoup, 2007) was used to estimate the frequency of putative null alleles. R package *genepop* v1.1.7 (Rousset et al., 2020) was used to calculate the inbreeding coefficient (F_{IS}), deviation from Hardy-Weinberg equilibrium, and linkage disequilibrium.

Outlier loci test

Different approaches for outlier detection have their advantages and disadvantages. To minimize the false positive, three approaches were used to detect outlier loci among all the 28 populations: (i) hierarchical Bayesian method implemented in BAYESCAN (http://cmpg.unibe.ch/software/bayescan/) with the parameters set to 10 pilot runs of 5,000 iterations each and a burn-in of 50,000 iterations. Outliers were identified based on 99% posterior probabilities. (ii) LOSITAN (Antao et al., 2008) was used to implement the method developed by Beaumont and Nichols (1996), where F_{ST} distribution was evaluated based on the observed and expected values at a 99% confidence interval. Here, an initial run with 60,000 simulations was conducted, followed by a computation of F_{ST} distribution based on putative neutral loci derived from the simulations. (iii) A coalescent simulation was implemented in DETSEL 1.0 (Vitalis et al., 2003) with the following parameters: mutation rate (infinite allele model, IAM) 0.005, 0.001, and 0.0001; ancestral population size $N_a = 500$, 1,000, and 10,000; population size before split $N_0 = 100$ and 500; time since an assumed bottleneck $t_0 = 50$, 100, and 1,000 generations; and time since population split $t = 100$ generations. Loci outside the 99% confidence level and present in at least five combinations in the pairwise comparisons among populations were considered outliers.

TABLE 1 Geographical information and summary of genetic diversity measures for 28 *Zizania latifolia* populations based on 46 simple-sequence repeat (SSR) loci/23 neutral loci/9 outlier loci.

Population	Location	N	G	A	N_e	H_O	H_E	F_{IS}	F_{ST}
BS	Baihilazi, Heilongjiang Province	22	21	1.652/1.565/1.667	1.45/1.338/1.406	0.338/0.246/0.328	0.228/0.185/0.196	−0.418/−0.26/−0.599	
XXT	Xiaoxintun, Heilongjiang Province	21	19	1.456/1.261/1.667	1.353/1.131/1.597	0.244/0.11/0.333	0.169/0.081/0.197	−0.406/−0.277/−0.765	
HDY	Hadayan, Heilongjiang Province	20	16	1.413/1.391/1.667	1.261/1.207/1.418	0.191/0.122/0.326	0.136/0.109/0.214	−0.378/−0.108/−0.528	
KEB	Kuerbin River, Heilongjiang Province	22	19	1.369/1.391/1.444	1.229/1.221/1.304	0.189/0.188/0.287	0.129/0.128/0.158	−0.366/−0.353/−0.787	
LQQ	Lanqitun, Heilongjiang Province	20	19	1.695/1.565/1.778	1.457/1.458/1.406	0.283/0.256/0.304	0.247/0.247/0.216	−0.14/−0.055/−0.302	
HW	Hongwei, Heilongjiang Province	20	14	1.717/1.522/1.667	1.534/1.344/1.643	0.263/0.199/0.413	0.236/0.181/0.291	−0.113/−0.061/−0.426	
YLZ	Yihaoyuliangzi, Heilongjiang Province	20	18	1.695/1.435/1.778	1.432/1.277/1.521	0.229/0.179/0.333	0.18/0.13/0.243	−0.205/−0.303/−0.199	
Mean	**Cluster I**	**20.7**	**18**	**1.571/1.447/1.667**	**1.389/1.282/1.471**	**0.249/0.186/0.332**	**0.19/0.151/0.216**	**−0.29/−0.203/−0.515**	**0.559/0.623/0.650**
JH	Jinhua, Liaoning Province	20	19	1.869/1.609/2.444	1.517/1.415/1.883	0.297/0.277/0.427	0.233/0.204/0.319	−0.264/−0.308/−0.41	
HR	Huanren, Liaoning Province	20	17	1.5/1.304/1.778	1.343/1.23/1.643	0.228/0.192/0.373	0.164/0.128/0.255	−0.389/−0.434/−0.525	
ZD	Zhangdang, Liaoning Province	20	19	1.478/1.348/1.778	1.34/1.225/1.681	0.239/0.181/0.415	0.171/0.13/0.289	−0.368/−0.328/−0.473	
DG	Donggang, Liaoning Province	20	20	1.63/1.478/2	1.33/1.269/1.599	0.246/0.202/0.406	0.177/0.151/0.28	−0.337/−0.294/−0.477	
LZX	Liaozhong, Liaoning Province	20	20	2.565/2.391/3.111	1.903/1.661/2.473	0.389/0.343/0.483	0.336/0.305/0.366	−0.16/−0.129/−0.404	
Mean	**Cluster II**	**20**	**19**	**1.809/1.626/2.222**	**1.487/1.36/1.856**	**0.28/0.239/0.421**	**0.217/0.184/0.302**	**−0.304/−0.298/−0.458**	**0.485/0.481/0.30**
HXD	Huanxiangdian, Shandong Province	20	19	1.76/1.609/2.111	1.472/1.421/1.714	0.329/0.256/0.515	0.245/0.212/0.331	−0.314/−0.218/−0.575	
DP	Dongpinghu, Shandong Province	20	15	2.347/2.261/2.778	1.807/1.869/2.034	0.369/0.394/0.452	0.317/0.338/0.335	−0.156/−0.163/−0.4	
LQ	Luqiao, Shandong Province	20	19	2.413/2.391/3	1.816/1.785/2.401	0.367/0.398/0.456	0.321/0.352/0.368	−0.125/−0.13/−0.332	

(Continued)

TABLE 1 Continued

Population	Location	N	G	A	N_e	H_O	H_E	F_{IS}	F_{ST}
MK	Mankou, Shandong Province	20	20	2.413/2.522/2.889	1.777/1.917/1.999	0.37/0.42/0.433	0.319/0.365/0.344	−0.16/−0.16/−0.338	
Mean	**Cluster III**	**20**	**18.3**	**2.234/2.196/2.694**	**1.719/1.748/2.037**	**0.359/0.367/0.464**	**0.301/0.317/0.344**	**−0.189/−0.168/−0.411**	**0.236/0.235/0.106**
CH	Changhu Lake, Hubei Province	21	21	2.956/2.435/4.111	2.257/1.938/2.71	0.429/0.391/0.534	0.379/0.352/0.433	−0.153/−0.108/−0.326	
DT	Dongting Lake, Hunan Province	21	21	2.63/2.522/3	2.03/1.852/2.364	0.393/0.371/0.46	0.368/0.361/0.368	−0.073/−0.014/−0.348	
HH	Honghu Lake, Hubei Province	20	16	2.586/2.174/3.444	1.994/1.702/2.422	0.389/0.37/0.396	0.346/0.331/0.339	−0.137/−0.117/−0.218	
LZ	Liangzi Lake, Hubei Province	21	21	2.543/2.304/3.444	1.869/1.695/2.204	0.365/0.348/0.344	0.329/0.315/0.308	−0.127/−0.114/−0.193	
LG	Longgan Lake, Hubei Province	21	21	1.478/1.522/1.556	1.293/1.284/1.451	0.25/0.24/0.381	0.161/0.16/0.244	−0.444/−0.417/−0.528	
SJ	Shengjin Lake, Anhui Province	21	21	2.673/2.261/3.333	1.989/1.851/2.339	0.391/0.379/0.487	0.358/0.347/0.367	−0.1/−0.086/−0.416	
BD	Baidang Lake, Anhui Province	22	22	2.456/2.348/2.667	1.813/1.708/1.906	0.398/0.399/0.434	0.344/0.343/0.334	−0.157/−0.137/−0.37	
Mean	**Cluster IV**	**21**	**20.4**	**2.475/2.224/3.079**	**1.893/1.719/2.199**	**0.374/0.357/0.434**	**0.327/0.315/0.342**	**−0.171/−0.142/−0.343**	**0.289/0.394/0.473**
NM	Nama, Guangxi Province	21	19	1.478/1.391/1.667	1.316/1.251/1.515	0.245/0.199/0.392	0.165/0.134/0.256	−0.424/−0.432/−0.522	
FC	Fangchenggang, Guangxi Province	21	19	1.5/1.391/1.667	1.371/1.263/1.607	0.276/0.229/0.404	0.187/0.139/0.288	−0.471/−0.606/−0.418	
BL	Beiliu City, Guangxi Province	23	22	1.456/1.435/1.667	1.311/1.308/1.432	0.245/0.215/0.394	0.157/0.143/0.239	−0.508/−0.455/−0.603	
WC	Wuchuan City, Guangdong Province	21	20	1.586/1.478/1.667	1.404/1.3/1.412	0.271/0.215/0.372	0.194/0.154/0.229	−0.389/−0.361/−0.558	
DC	Dongcheng, Guangdong Province	20	19	1.5/1.304/1.667	1.302/1.229/1.429	0.236/0.197/0.339	0.159/0.125/0.212	−0.399/−0.5/−0.59	
Mean	**Cluster V**	**21.2**	**19.8**	**1.504/1.4/1.667**	**1.341/1.27/1.479**	**0.255/0.211/0.38**	**0.173/0.139/0.245**	**−0.439/−0.471/−0.538**	**0.194/0.105/0.145**
Overall		**20.6**	**19.1**	**1.922/1.772/2.266**	**1.57/1.47/1.804**	**0.302/0.268/0.401**	**0.241/0.22/0.286**	**−0.245/−0.205/−0.44**	**0.641/0.637/0.599**

N, number of individual plants; G, multi-locus genotypes; N_a, observed alleles number; A, effective allele number; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; F_{ST}, genetic differentiation.

Following the analysis method described by Richter-Boix et al. (2011), the locus positive in more than one test was considered a robust outlier candidate. Furthermore, the loci not significant in all approaches were considered to be neutral. We obtained three data sets, including all loci (46), loci under selection (9), and neutral loci (23). The genetic variation and population divergence were calculated based on all three data sets. Simulated demographical process and gene flow were estimated for the neutral loci only.

Environmental association analysis

Simple-sequence repeat markers that were detected as outliers in more than one of the three methods used (Bayescan, Lositan, and Detsel) were used to perform genetic-environment association using the SAM approach implemented in samβada (Joost et al., 2007; Stucki et al., 2017). The SAM method uses a logistic regression model to identify the association between marker alleles (individuals coded as either present/absent for each of the marker alleles) and environmental variables across the sampling locations. The best model was selected based on significant G and Wald tests after Bonferroni correction at a 99% confidence level (Joost et al., 2007). To supplement the SAM method, we investigated the evidence for the environmental contribution to allele frequency distribution, where outlier SSR markers were evaluated for the environmental association as described by Bradbury et al. (2013). In brief, genotype data, excluding rare and fixed alleles of frequency 0.05 and 0.95, respectively, were coded into Hapmap format for loading to Tassel 5 software (Bradbury et al., 2007). The standardized 20 environmental variables and their first principal component (PC1 accounting for 99.32% variation) were used. Genetic distance calculated from the non-outlier gSSR and EST-SSR in GenAlEx 6.5 (Peakall and Smouse, 2012) was used as a covariant to control for the effect of genetic structure on association. A mixed linear model (MLM) was fit, and a significant association was considered at $P < 0.01$ after false discovery rate (FDR) correction. Loci that were significantly associated with environmental variables were considered to be under selection. Their putative identity and role were determined through BLASTx analysis in NCBI and annotated using B_{LAST}2G_O v.6.0.1 (Conesa and Götz, 2008).

Genetic variation

Genetic diversity across the loci for each population and for each locus was estimated in terms of observed and expected heterozygosity (H_O and H_E), the effective number of alleles (N_e), the total number of alleles (A, allelic diversity), and the number of private alleles (S_P and S_R: alleles that only occurred in one population and one region) for all the 46 loci using GenAlEx.

Genetic structure

Population genetic divergence was calculated by the estimation of F_{ST} using 999 permutations with *genepop*. STRUCTURE program (Pritchard et al., 2000) was used to perform Bayesian clustering analysis. Ten independent runs for each number of K clusters from 1 to 15 were performed. A total of 20,000 iterations burn-in period followed by 100,000 Markov Chain Monte Carlo (MCMC) iterations were assumed for each run with correlated allele frequencies and admixture origin assumptions. To determine the value of K, the output was interpreted with Structure Harvester (Evanno et al., 2005; Earl and vonHoldt, 2012). However, Evanno's *delta K* method has been reported to suffer philosophical and statistical errors (Verity and Nichols, 2016). Therefore, it was supplemented with the thermodynamic integration (TI) method (Verity and Nichols, 2016). Here, the *rmaverick* R package was used to estimate the true value of K by running 20 runs for $K = 1–15$ with a burn-in period of 10,000 iterations followed by 100,000 MCMC iterations under the admixture model. The value of K was estimated as described by Verity and Nichols (2016). To confirm the results of Bayesian clustering, principal coordinate analysis (PCoA) and neighbor-joining (NJ) trees were implemented to identify the substructure and the clustering pattern of the individuals using GenAlEx and MEGA-X (Kumar et al., 2018), respectively. Additionally, the analysis of molecular variance (AMOVA) was performed to determine the genetic variation among and within populations using ARLEQUIN (Schneider et al., 2000).

Genetic discontinuity

Taking into account our sampling strategy that could mask sub-structuring as a result of geographical distances between the river basins, the spatial Bayesian clustering implemented in the *geneland* R package (Guillot et al., 2011), was performed under the correlated allele frequency model with spatial uncertainty in the spatial locations fixed at 1 km and using the option to filter for the presence of null alleles. The value of K was allowed to vary from 1 to 15 for 20 independent runs each consisting of 500,000 MCMC iterations, a thinning of 100, the maximum number of nuclei in the Poisson-Voronoi tessellations fixed at 536, maximum rate of the Poisson process fixed at 150, and burn-in of 100,000 in the postprocessing. MCMC coverage was assessed by comparing the number of populations across replicate runs, with a mean posterior density as the criterion for choosing the best run. BARRIER 2.2 (Manni et al., 2004), which is based on Monmonier's maximum difference algorithm, was used to assess the genetic barriers between populations with 1,000 F_{ST} matrices generated using the *diveRsity* R package (Keenan et al., 2013).

Migration and bottleneck analysis

To detect recent demographic bottlenecks, BOTTLENECK v.1.2.02 (Piry et al., 1999) was used to perform the analysis under three models: stepwise mutation model (SMM), IAM, and two-phase mutation model (TPM) with 1,000 iterations using a two-tailed Wilcoxon's signed-rank test. Furthermore, the allelic frequency distribution mode was tested and the mode shift away from the L-shaped distribution indicated recent bottlenecks.

Historical gene flow (M_H) was estimated using MIGRATE (Beerli et al., 2019). The analysis was performed under the Brownian motion model using Bayesian inference. Defaults settings, as recommended by Beerli (2009), were used where a single long chain was run with 20 sampling increments, 5,000 recorded steps in the chain, a burn-in of 10,000, and heating of 4 parallel chains were set to estimate Θ, and M. Gene flow (M_H) was calculated as $\Theta \times M/4$, where Θ is the mutation-scaled effective population size, and M is the mutation-scaled migration rate. Contemporary gene flow (M_C) was assessed using BAYESASS (Wilson and Rannala, 2003). The analysis was performed using 5,000,000 iterations with the chain sampled after 2,000 iterations. A burn-in of 1,000,000 was used and the delta values were adjusted to ensure a 40–60% acceptance rate.

Geographic and environmental influence on the genetic structure

To compare simultaneously the three gene flow models (IBD, IBR, and IBE), we used the RCM, which is based on partial Mantels, and could eliminate the simple Mantels' spurious correlations (Cushman et al., 2006). For the analysis, we obtained some parameter matrices, including genetic distance, geographic distance, environment distance, and resistance distance.

Genetic distance between populations was calculated as pairwise F_{ST} based on the three data sets (all loci, adaptive loci, and neutral loci) using *genepop*. Geographic distance was based on Euclidean distance and was calculated using GenAlEx.

Nineteen environmental variables were extracted for the studied sites from BioClim's 30s resolution dataset (Busby, 1991), and soil type data from http://www.isric.org/ with GIS details using ArcMap 10.5 (Esri, Redlands, CA, USA). To reduce climatic variables, principal component analysis (PCA) was performed based on 21 variables (19 bioclimatic variables, elevation, and soil type) using PAST ver. 4.01 (Hammer et al., 2001). The first principal component (PC1) represented 99.32% of the variation and was highly correlated ($r > 0.9$) with eight temperature-related variables (bio_1, bio_2, bio_4, bio_6, bio_7, bio_9, bio_10, and bio_11). The PC1 was thus used to calculate the environmental distance matrix with the *vegan* package in R (Oksanen et al., 2018).

Zizania latifolia is wind-pollinated, and its seeds are edible for native waterfowl and migratory birds, which could lead to pollen and seed dispersal among populations. Additionally, water connectivity facilitates the dispersal of *Z. latifolia* propagules, while wind helps in pollen and seed dispersal. Wind and hydrological connectivity were used to calculate the resistance distance. Data for bird movements are unavailable and their influence on the genetic structure was not estimated in this study.

To obtain wind resistance distance, the R package *rWind* (Fernández-López and Schliep, 2019) was used to extract wind direction and speed data from September 20 to October 20, 2014, considering the sample collection time (Autumn of 2015) and flowering of *Z. latifolia*. We calculated the conductance distance based on the wind speed and direction for the average of the 30 days and calculated the cost distance between populations.

Hydrological connectivity was calculated on a raster water layer (Yamazaki et al., 2017) that was re-classed in Arcmap 10.5 such that pixels with permanent water bodies and major rivers were assigned a value of 0, pixels with small streams and canals were assigned a value of 1, while dry land pixels were assigned a value of 2. Pairwise least-cost distance between populations was then calculated using the *gdistance* R package.

Based on the four distance matrices, the RCM method was used to compare the different gene flow models. First, partial Mantel was performed between genetic (Gen) and geographic distances to test IBD and to partial out IBR (Gen∼IBD|IBR); second, partial Mantel was performed between the Gen and resistance matrices to test IBR and to partial out the IBD (Gen∼IBR|IBD). The difference between the contribution of geographical distance excluding the resistance and the resistance excluding the geographical distance is the score showing support for IBD when compared to IBR (Cushman et al., 2013a). If the score is positive, IBD is supported, and a zero or negative score supports IBR. The vice versa is also true. The full matrix of the partial Mantel test differences between pairs of alternative hypotheses was computed. A hypothesis was regarded as fully supported, independent of all alternatives if all the values in its column were positive and all the values in the row were negative (Cushman et al., 2013a,b). Correlation values and significance values for the partial Mantel model combinations were calculated through 9,999 corrected permutations using the *vegan* R package. The RCM analysis was performed for the entire data set and for each ecoregion, which corresponds to the clusters identified by the different methods.

We further evaluated the relationship between genetic diversity estimators and geographic and environmental variables through generalized linear models (GLMs) using the PAST software, and spatially explicit mixed modeling (Morente-López et al., 2018) using the *spaMM* R package. First, GLMs were used to explore the contribution of each environmental variable to genetic diversity estimators (H_{O}, H_{E}, and A). Second, spatial GLMMs were developed using genetic, geographic (coordinates), and environmental data. Here, we used genetic diversity estimators (H_{O}, H_{E}, and A) as response variables, each of the 21 environmental variables as fixed effects, and geographical coordinates as random effects. We transformed environmental variables as required, including using their squared values to account for non-linearity. Full models (e.g., $[A \sim \text{bio\_1} + (1|\text{lat+long})]$) and null models (e.g., $[A \sim 1 + (1|\text{lat+long})]$) were tested for associated likelihood ratio to obtain the P-value.

Genetically informed ecological niche modeling

With the realization that IBE was the supported model in the entire study site and 3 of the 5 river basins, and that our sampling strategy could have increased the bias toward IBE and against IBD, we supplemented the RCM analysis with genetically informed ecological niche models (gENMs). Besides our 28 geo-referenced collections, we obtained 131 occurrence records from the Global Biodiversity Information Facility (GBIF, http://www.gbif.org/). The GBIF occurrence records were assigned to one of the five genetic clusters identified by spatial analysis in *geneland*. The *geneland* probability surface maps for each cluster were georeferenced in QGIS (QGIS Development Team, 2009) and GBIF occurrence records were overlaid on the maps. Occurrences within ≥70% posterior probability contours were assigned to the respective clusters since the range was non-overlapping for any of the cluster populations. To model for the current distribution of the genetic populations, 19 bioclimatic variables (similar to those used in EAA) were used. First, we tested and removed highly correlated variables (Pearson correlation coefficient, $r > 0.8$) and those retained were verified for variance inflation factor (VIF) <5. To test for climate niche differentiation, we performed PCA on standardized climatic variables followed by permutational multivariate analysis of variance (perMANOVA) in the *vegan* R package (Bothwell et al., 2021). Furthermore, the ANOVA followed by Turkey's pairwise test of significant difference among groups was performed to explore the unique contribution of each bioclimatic variable to genetic group variations.

Following the approach by Bothwell et al. (2021), we developed ENMs using Maxent 3.4.4 (Phillips et al., 2006), since it performs better with presence-only data. One model was generated using all occurrence records for *Z. latifolia* and assuming no genetic structure, and the other five models were generated for each of the genetic groups (gENMs). Model performance was evaluated using the area under the receiver operating characteristic curves (AUC) and partial receiver operating characteristic curves (pROC) (Peterson et al., 2008).

Widely distributed species persist in varied environmental and geographical spaces resulting in restricted gene flow, local adaptation, and niche divergence. We tested for niche overlap and divergence among the 5 gENMs to determine if each group occupies distinct niches in the geographical space or they exhibit niche conservatism. For this, we performed a niche overlap test using Schoener's D statistic and implemented niche identity tests in ENMTools 1.4.4 (Warren et al., 2008, 2021) using 99 pseudo-replicated ENMs from pooled, randomized occurrence records for each genetic cluster pair.

Results

SSR genotyping

From 578 individuals used in this study, 536 multi-locus genotypes (MLGs) were identified, and no genotype was shared among populations. In nine populations, every individual had a unique genotype (Table 1). The individuals with clonal genotypes were excluded from further analysis. We detected 371 alleles across the 46 SSR markers, with an average of 8.1 alleles per locus. Among these loci, four gSSR markers showed a large number of alleles per locus with 28 (ZL4 and ZL5), 29 (ZL3), and 30 (ZL1) alleles per locus; five EST-SSR markers (EZ73, EZ75, EZ89, EZ121, and EZ131) only manifested 2 alleles per locus; 40 SSR markers showed private alleles unique to each of the 5 regions (Supplementary Table 2).

Null alleles were present in 87 of 1,288 population-loci combinations, which is slightly above the expected 64 by chance at a 5% significance level. The null alleles were not associated with any loci or population and there was no significant bias in F_{ST} estimation on the data compared to the estimate based on ENA correction (t-test, $P = 0.873$). The entire data set deviation from Hardy– Weinberg equilibrium (HWE) was detected at $P = 0.05$, which is expected for the natural population at a broad geographical scale (Garnier-Géré and Chikhi, 2013). Exact tests for LD indicated a significant deviation for 62 ($P < 0.05$) of 680 possible comparisons. This was slightly higher than that expected by chance (34 at $P < 0.05$). However, the significant tests involved different locus pairs indicating that loci were unlinked and statistically independent of each other.

Neutrality tests, associations between adaptive loci, and environmental variables

Among the 46 SSR loci amplified, a total of 19, 7, and 7 were detected as outlier loci by BAYESCAN, LOSITAN, and DETSEL, respectively (Figure 1A). Of these loci, 9 that

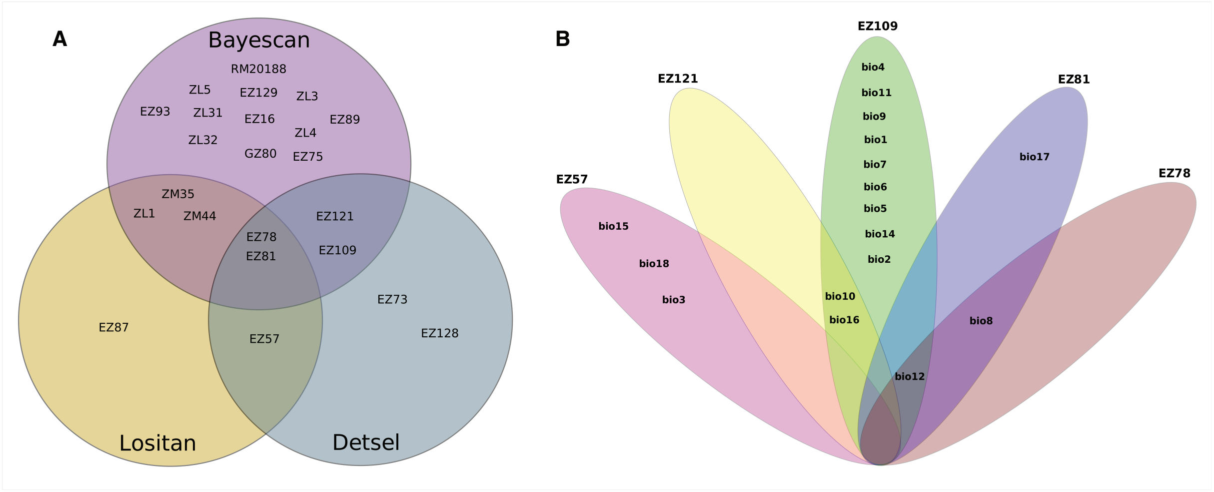


FIGURE 1
Summary of outlier loci and allele-environmental variables association. **(A)** Loci identified by each of the software, between two software and three software, respectively; **(B)** Environmental variables significantly associated with either of the loci and/or shared among and between loci.

were detected by two outlier tests were considered adaptive loci. The SAM analysis found that five of the nine loci tested were significantly associated with 17 environmental variables (Figure 1A). Three of the loci (EZ78, EZ81, and EZ121) showed the highest significant association with five temperature-related environmental variables (Figure 1B). The other two loci (EZ57 and EZ109) were associated with 15 variables both precipitation- and temperature-related (Figure 1B). Similar results were found for Tassel analysis, where 7 of the 9 loci tested were significantly associated with 15 environmental variables and PC1. Among these loci, three loci (EZ78, EZ81, and EZ121) were significantly associated with 8, 14, and 15 variables, respectively, as well as with PC1. EZ57 and EZ109 were significantly associated with one variable each. Besides PC1, among the significantly associated variables include; bio_1, bio_2, bio_3, bio_4, bio_5, bio_6, bio_7, bio_8, bio_9, bio_11, bio_12, bio_13, bio_14, bio_15, bio_17, and bio_19, eleven of which are related to temperature, while five are related to precipitation. Four loci were associated with functional genes based on the top BLAST hit and their putative roles include DNA-binding transcription factor activity, protein phosphorylation, RNA splicing, and cell differentiation, respectively (Table 2).

Genetic variation of populations

The genetic diversity of each population was evaluated based on all 46 SSR loci, 23 neutral loci, and 9 outlier loci. For all populations, the outlier loci and neutral loci showed the highest and lowest values of genetic variation (A, N_e, and H_E) (Table 1). For the different regions, the genetic diversity increased gradually from north to south, reaching the highest value in the middle and lower reaches of the Yangtze River, and then began to decrease. The lowest and the second-lowest genetic diversity occurred in the southernmost and northernmost regions (Pearl River Basin and Heilongjiang River Basin), respectively. For each basin, the genetic variations varied greatly within the regions of the Heilongjiang River Basin and Liaohe River Basin. In the bottleneck analysis, 13, 17, and 23 populations were out of mutation-drift equilibrium under SMM, TPM, and IAM models, respectively. All populations from the Pearl River Basin showed significant bottlenecks for the 3 models (Supplementary Table 3). Wright's fixation index based on all, neutral, and adaptive loci showed that all populations displayed a heterozygote excess (Table 1).

Genetic structure and discontinuity

Obvious genetic structure was found between populations with $F_{ST} = 0.641$, 0.637, and 0.599 based on all loci, neutral loci, and loci under selection, respectively. Based on all loci and neutral loci, the three approaches (PCoA, NJ tree, and STRUCTURE) consistently divided the 28 populations into five clusters and populations from the same region clustered together, which showed the clear genetic divergence among the five regions (Figures 2, 3, and Supplementary Figure 1). Outlier loci showed six clusters, with the population from the expansive Yangtze River basin clustering into two groups (Figure 3). Similarly, AMOVA results based on all loci, neutral, and adaptive loci showed high population divergence ($Ø_{pt} =$ 0.6324, 0.6546, 0.5925) (Table 3 and Supplementary Tables 4, 5). Based on neutral loci that were used to assess the landscape models, variation among populations was 65.46%, while 34.54%

TABLE 2 Putative function of adaptive loci that were outliers and associated with climatic variables.

Putative gene	Role	*E*-value	% Identity	Variable	Marker
PREDICTED: *Oryza brachyantha* homeobox protein knotted-1-like 12 (LOC102715865), transcript variant X2, mRNA	DNA-binding transcription factor activity	0	84.28	bio_8 bio_12	EZ78
PREDICTED: *Oryza sativa* Japonica Group PTI1-like tyrosine-protein kinase At3g15890 (LOC4328540), mRNA	Protein phosphorylation	0	93.37	bio_8 bio_12 bio_17	EZ81
PREDICTED: *Brachypodium distachyon* serine/arginine-rich splicing factor RS2Z33 (LOC100826232), mRNA	RNA splicing, mRNA splicing, via spliceosome, spliceosomal complex assembly	4.00E-36	91.67	bio_10 bio_16	EZ121
PREDICTED: *Oryza sativa* Japonica Group BEL1-like homeodomain protein 1 (LOC4349837), transcript variant X1, mRNA	biological processes; response to external stimuli, cell differentiation, biosynthesis	4.00E-91	79.28	bio_1 bio_2 bio_3 bio_4 bio_5 bio_6 bio_7 bio_9 bio_10 bio_11 bio_12 bio_14 bio_16	EZ109

of the variation was within the populations, both statistically significant ($P = 0.001$). Inter-cluster AMOVA showed that 47.13% variation was within the clusters, 21.28% between the clusters, and 31.59% within populations (Table 4). AMOVA between populations in each cluster showed that differentiation was the highest in the north-most region ($Ø_{pt} = 0.5887$, $P = 0.000$) and decreased toward the south, reaching the lowest in the south-most region ($Ø_{pt} = 0.1047$, $P = 0.000$). Hierarchical F_{ST} analysis using neutral loci showed a similar differentiation pattern (Table 1).

Based on the 23 neutral loci, *geneland* identified five genetic clusters and populations from similar latitudes clustered together (Supplementary Figure 2). The results suggested that genetic discontinuities existed between any adjacent regions. Similar results were shown by BARRIER. Additionally, BARRIER further displayed the genetic discontinuities existing within the Heilongjiang River Basin, like barriers between populations BS and XXT, XXT and HDY, and LQQ and HW as well as around population ZD (Supplementary Figure 3).

Historical and contemporary gene flow

MIGRATE analysis based on neutral loci detected moderate historical gene flow between populations in each river basin, with the highest in Pearl River Basin ranging from 0.546 to 2.354, and the least in Heilongjiang River Basin, ranging from 0.212 to 1.136. Gene flow among the five clusters was also moderate, but higher than among the populations within clusters, ranging from 0.211 to 14.46 (Supplementary Tables 6, 7). Conversely, BAYESASS analysis revealed low contemporary gene flow in all clusters with the highest in Pearl River Basin ranging from 0.012 to 0.258 and the least in the Yangtze River Basin, ranging from 0.0112 to 0.0247. Among the clusters, contemporary gene flow

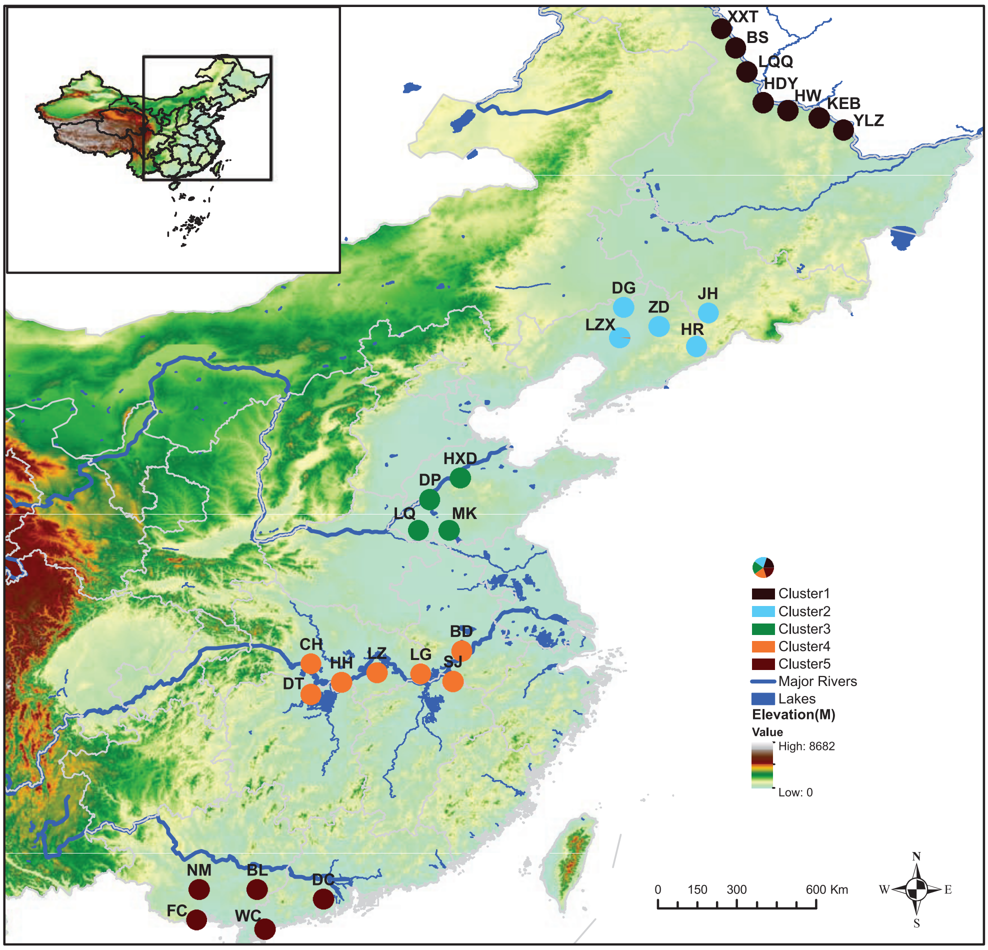


FIGURE 2
Geographical distribution of *Zizania latifolia* samples across the five (I–V) latitudinal regions in China and their respective genetic clusters each denoted by a different color.

was lower compared to within clusters, ranging from 0.0021 to 0.0044 (Supplementary Tables 8, 9).

Environment, geography, and landscape heterogeneity impact on genetic structure based on 23 neutral loci

Based on neutral loci, the IBE model was fully supported with respect to the relative support values of the RCM for the entire study site (Figure 4A). All column values for IBE were positive, while the row values were negative indicating that it explained the genetic structure independent of the alternative hypotheses (IBD and IBR). IBD was the second-best model explaining the genetic structure, with only one negative column value, while IBR was the least supported model. Moreover, the partial Mantel tests between genetic distance and environmental distance controlling for IBR and IBD showed significant positive correlations (Table 5).

It is worth noting that for all loci, neutral, and adaptive loci, IBE was the exclusively supported model. Simple Mantel tests were significant for all models based on the three data sets, but partial Mantel significance differed between the dataset, except for IBE for which all partial Mantels were significant for all loci (Table 5 and Supplementary Tables 10, 11)). The differences between simple and partial Mantels and the estimation of the best model in each of the datasets using RCM indicates its advantage over simple Mantel in landscape genetics.

Based on the neutral loci in each of the clusters, IBE was fully supported in clusters 2, 3, and 5, while in clusters 1 (populations along the Heilongjiang River Basin) and 4 (the Yangtze River Basin), IBD and hydrological connectivity model (IBR_HC) were the fully supported model, respectively. IBR_HC was the second-best model explaining

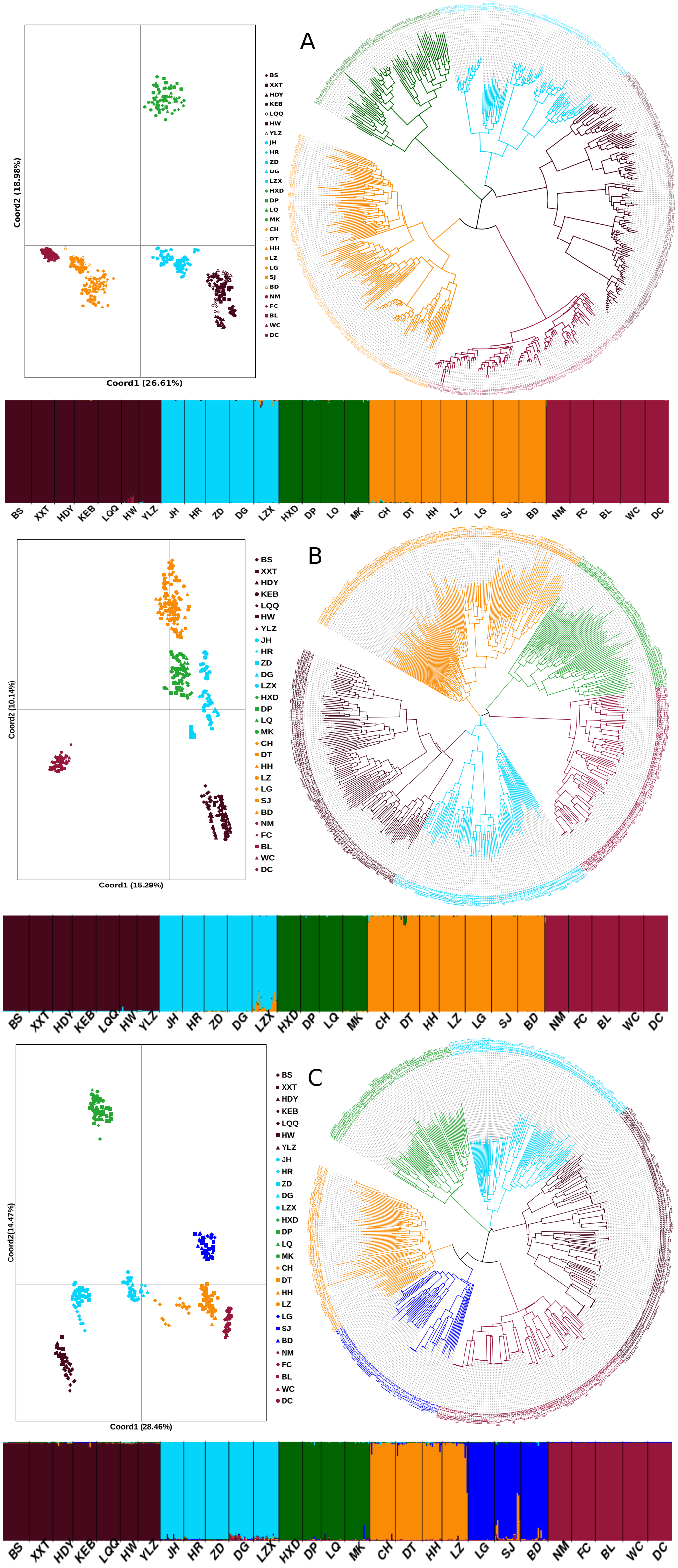


FIGURE 3
Genetic structure of 28 *Zizania latifolia* populations based on STRUCTURE, principal coordinate analysis (PCoA), and neighbor joining tree (NJ). **(A)** based on all loci; **(B)** based on neutral loci; **(C)** based on outlier loci. The colors represent the clusters based on STRUCTURE analysis for the histograms and NJ tree.

TABLE 3 AMOVA results based on 23 neutral loci for the 28 population of *Z. latifolia* across China.

Source of variation	d. f.	Sum of squares	Variance components	Percentage variation	Statistics	*P*
			Among population			
Among populations	27	5,193.553	4.957	65.46		<0.000
Within populations	1,044	2,730.666	2.615	34.54	$\varnothing_{pt} = 0.6546$	<0.000
			Among clusters			
Among groups	4	3,583.867	3.902	47.13		<0.000
Among populations within groups	23	1,609.686	1.761	21.28		
Within populations	1,044	2,730.666	2.615	31.59	$\varnothing_{pt} = 0.6841$	<0.000

TABLE 4 AMOVA results for each of the inferred clusters based on 23 neutral loci.

Source of variation	d. f.	Sum of squares	Variance components	Percentage variation	Statistics	*P*
			Cluster 1			
Among populations	6	565.055	2.572	58.88		<0.000
Within populations	245	440.116	1.796	41.12	$\varnothing_{pt} = 0.5887$	<0.000
			Cluster 2			
Among populations	4	370.708	2.383	52.06		<0.000
Within populations	185	405.997	2.194	47.94	$\varnothing_{pt} = 0.521$	<0.000
			Cluster 3			
Among populations	3	166.675	1.424	27.59		<0.000
Within populations	142	530.949	3.739	72.41	$\varnothing_{pt} = 0.2759$	<0.000
			Cluster 4			
Among populations	6	470.224	1.829	33.00		<0.000
Within populations	279	1036.300	3.714	67.00	$\varnothing_{pt} = 0.330$	<0.000
			Cluster 5			
Among populations	4	37.024	0.192	10.48		<0.000
Within populations	193	317.304	1.644	89.52	$\varnothing_{pt} = 0.1047$	<0.000

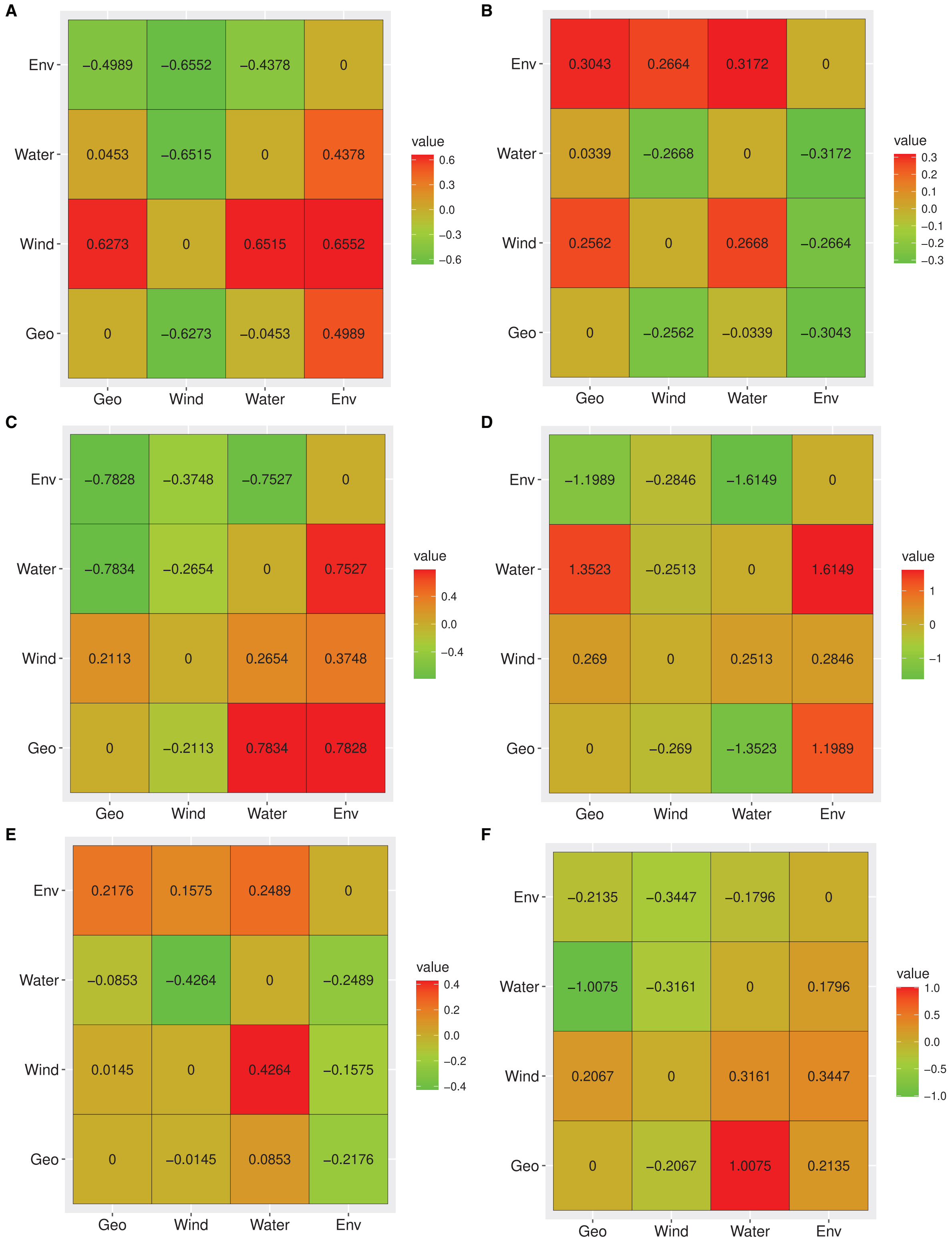
the genetic structure in clusters 1, 2, and 5, while IBD was the second-best supported model in clusters 3 and 4 (Supplementary Tables 12–16).

Environmental influence on genetic diversity based on 23 neutral loci

Generalized linear models showed that 14 of the 21 environmental variables had a significant contribution to the genetic diversity of *Z. latifolia*. Environmental variables, bio_5, bio_10, bio_14, bio_15, and bio_17, were significantly correlated with genetic diversity estimators H_O, H_E, and A (Table 6). Spatially explicit linear mixed models with coordinates as random effect showed that one environmental variable (bio_5) had a significant influence on the genetic diversity estimator H_E. To identify the most important environmental variables for the observed genetic variation, we tested the 14 variables showing a significant contribution to genetic diversity in GLM, for collinearity using VIF analysis (Helsen et al., 2017). Two variables, namely bio_5 (Maximum temperature of the warmest month) and bio_15 (precipitation seasonality), had VIF values below 5 and were therefore considered the best environmental variables explaining the genetic diversity patterns of *Z. latifolia* (Figure 5).

Genetically informed ecological niche modeling

Five environmental variables were left after multi-collinearity analysis (bio3, bio5, bio8, bio15, and bio18) and were used in subsequent analysis. We found significant differences among groups in environmental space using both multivariant and individual-based analyses. The first three principal components accounted for 99.9% of the variation in climate among groups (PC1 = 67.9%, PC2 = 20.1%, and PC3 = 11.9%). Each of the PC axis was highly correlated



A

	Geo	Wind	Water	Env
Env	−0.4989	−0.6552	−0.4378	0
Water	0.0453	−0.6515	0	0.4378
Wind	0.6273	0	0.6515	0.6552
Geo	0	−0.6273	−0.0453	0.4989

B

	Geo	Wind	Water	Env
Env	0.3043	0.2664	0.3172	0
Water	0.0339	−0.2668	0	−0.3172
Wind	0.2562	0	0.2668	−0.2664
Geo	0	−0.2562	−0.0339	−0.3043

C

	Geo	Wind	Water	Env
Env	−0.7828	−0.3748	−0.7527	0
Water	−0.7834	−0.2654	0	0.7527
Wind	0.2113	0	0.2654	0.3748
Geo	0	−0.2113	0.7834	0.7828

D

	Geo	Wind	Water	Env
Env	−1.1989	−0.2846	−1.6149	0
Water	1.3523	−0.2513	0	1.6149
Wind	0.269	0	0.2513	0.2846
Geo	0	−0.269	−1.3523	1.1989

E

	Geo	Wind	Water	Env
Env	0.2176	0.1575	0.2489	0
Water	−0.0853	−0.4264	0	−0.2489
Wind	0.0145	0	0.4264	−0.1575
Geo	0	−0.0145	0.0853	−0.2176

F

	Geo	Wind	Water	Env
Env	−0.2135	−0.3447	−0.1796	0
Water	−1.0075	−0.3161	0	0.1796
Wind	0.2067	0	0.3161	0.3447
Geo	0	−0.2067	1.0075	0.2135

FIGURE 4
Heat map for reciprocal causal model (RCM) values for the entire data set and each of the inferred clusters. **(A)** All population, **(B)** Heilongjiang River Basin, **(C)** Liaohe River Basin, **(D)** Huanghe River Basin, **(E)** Yangtze River Basin, **(F)** Pearl River Basin. Columns indicate test model and rows indicate alternative models. Each value represents the relative support for the test model. The best model has positive column values and negative/zero row values.

TABLE 5 Reciprocal causal modeling, partial, and simple Mantel results for IBD, IBR, and IBE for the 28 populations of *Z. latifolia* and each of the clusters based on 23 neutral loci.

		IBD	IBR_WD	IBR_HC	IBE
		Geo	**Wind**	**Water**	**Env**
I: Reciprocal causal modeling matrix					
IBD	Geo	0	−0.6273	−0.0453	0.4989
IBR_WD	Wind	0.6273	0	0.6515	0.6552
IBR_HC	Water	0.0453	−0.6515	0	0.4378
IBE	Env	−0.4989	−0.6552	−0.4378	0
II: Simple and Partial Mantel correlation matrix					
IBD	Geo	0.6388***	−0.0834	0.0171	0.3167***
IBR_WD	Wind	0.5439***	0.4065***	0.5445***	0.5859***
IBR_HC	Water	0.0624	−0.107	0.6371***	0.3004***
IBE	Env	−0.1822	−0.0693	−0.1374	0.6702***

IBD, Isolation By Distance; IBR, Isolation By Resistance; IBE, Isolation By Environment; Geo, Geographic Distance; Res, Resistance Distance; Env, Environmental Distance.
(I) Reciprocal causal modeling matrix; columns indicate the test model and rows indicate alternative models. Each value represents the relative support of the test model.
(II) Simple and Partial Mantel correlation matrix. Columns indicate test model and rows indicate alternative models. Values are r values for correlations, diagonal values are the simple Mantel test r of a variable.
***P < 0.001.

TABLE 6 Generalized linear model (GML) for the contribution of environmental variables to genetic diversity measures and spatially explicit generalized linear mixed models (spatial GLMMs) for the influence of environmental variables on genetic diversity measures based on 23 neutral SSR loci.

Independent variables	GLM			Spatial GLMM
	Dependent variables			Dependent variables
	H_E	H_O	A	H_E
bio_1	–	0.033	–	–
bio_2	–	–	–	–
bio_4	–	–		–
bio_5	**0.008**	**0.0001**	**0.011**	**0.023**
bio_6	–	0.034	–	–
bio_7		–		–
bio_9	–	–	–	–
bio_10	0.024	0.002	0.027	–
bio_11	–	0.039	–	–
bio_14	0.031	0.008	0.024	–
bio_15	**0.035**	**0.023**	**0.031**	**–**
bio_17	0.031	0.008	0.025	–
bio_18	–	–	–	–
bio_19	0.042	0.012	0.035	–

Values are significant at P ≤ 0.05.
A, Effective Allele Number; H_O, Observed Heterozygosity; H_E, Expected Heterozygosity.

with a single bioclimatic variable (PC1∼bio8, $r = 0.850$; PC2∼bio15, $r = 0.849$; PC3∼bio5, and $r = 0.561$) (Figure 6). Multivariant perMANOVA detected significant niche separation by climate among genetic groups ($p = 0.001$). All genetic groups occupied significantly different environmental niche spaces with respect to each bio-climatic variable as assessed using Turkey's pairwise test (at least $p < 0.01$) and Welch's ANOVA ($p < 0.01$).

Our genetic-informed models (gENMs) showed high discriminatory power. AUC and pROC scores were 0.914–0.987 and 1.879–1.984, respectively. This was an improvement from the species-level model that had AUC and pROC scores of 0.893 and 1.845, respectively (Supplementary Table 17). The percentage contribution of bioclimatic variables to the model building showed high variation across models (Table 7). For instance, bio3 had a 92.3% contribution to the HRB model and 0.1% to the PRB model. Temperature-related variables showed the highest contribution to all models except the YRB, where precipitation seasonality had a contribution of 58.8% (Table 7).

Empirical niche overlap (D) between all model pairs ranged from 0.097 to 0.451. The null hypothesis that any genetic group occupies identical niches was rejected for 5 of the pairwise comparisons, while 5 other comparisons supported niche equivalence (Table 8). However, based on the low observed D values, the gENMs were found to be less similar than expected by chance, supporting the hypothesis of niche divergence among the river basins. The difference in regional habitat available to each population could influence its realized niche. Background similarity test to determine if gENMs were more or less similar than expected showed that D was significantly higher than expected by chance reflecting a degree of niche conservatism (Table 9). This is expected for aquatic plants since they

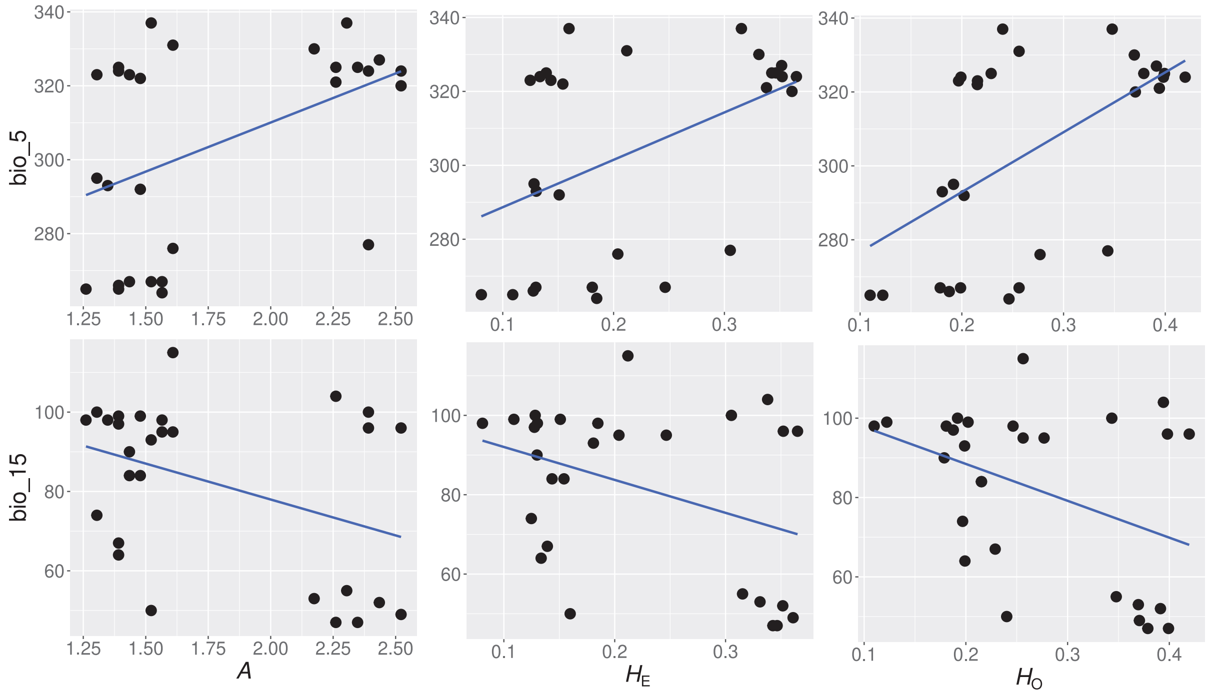


FIGURE 5
Significant relationships between genetic diversity estimators and the two most important environmental variables, based on variance inflation factor (VIF) analysis. *A*, number of alleles per population; H_E, expected heterozygosity; H_O, observed heterozygosity; bio_5; Maximum temperature of the warmest month, bio_15; precipitation seasonality.

persist in limited riparian micro-ecosystems sandwiched among expansive terrestrial ecosystems.

Discussion

Aquatic plants thrive in patchy habitats within the expansive terrestrial ecosystem, and their dispersal mostly depends on water connectivity, wind, and/or birds, generally effective locally. Previous studies have focused on the IBD model to explain the genetic structure, ignoring the influence of other factors, such as climatic, environmental, and landscape heterogeneity. In this study, the evident genetic structure was found for all *Z. latifolia* populations throughout China along the latitudinal gradient. The three models of gene flow (IBE, IBD, and IBR) were tested and showed that IBE was first supported followed by IBD. This indicates that climatic differences, especially bio_5 (maximum temperature of the warmest month) and bio_15 (precipitation seasonality), are more important than the distance between populations. Populations in central China exhibited higher than average genetic diversity followed by populations from the North, and the least diversity was found in Southern populations. This genetic pattern might be impacted by the latitudinal trend.

Comparison of genetic diversity at different latitudes and genetic bottlenecks

Zizania latifolia populations showed relatively low genetic diversity ($H_E = 0.258$). For the 28 *Z. latifolia* populations from the five river basins/regions along the latitudinal gradient, the genetic diversity increased gradually from north to south, culminating in the Yangtze River Basin, and then dropping rapidly to its lowest level in the southernmost populations in the Pearl River Basin. A similar level of genetic diversity was reported in its natural populations across China ($H_E = 0.371$) using limited (three) SSR markers (Xu et al., 2015). Additionally, similar microsatellite diversity of *Z. latifolia* was also found in Northeast China ($H_E = 0.328$; Chen et al., 2017a). In the present study, a higher-than-average genetic diversity was observed in populations along the Huanghe River ($H_E = 0.317$), and along the Yangtze River ($H_E = 0.315$). Similarly, previous studies also reported a relatively high genetic diversity in the Yangtze River Basin (Chen et al., 2012; Zhao et al., 2018, 2019).

Although latitudinal trends in intraspecific genetic diversity have not been fully resolved, the leading hypothesis is that genetic diversity increases along the latitudinal gradient toward the equator, which is explained to be as a result of greater

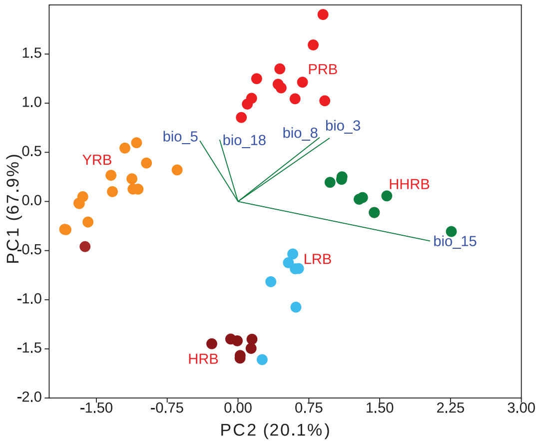


FIGURE 6
Population genetic groups are significantly different in the environmental space. HRB, Heilongjiang River Basin; LRB, Liaohe River Basin; HHRB, Huanghe River Basin; YRB, Yangtze River Basin; PRB, Pearl River Basin.

evolutionary stability in the tropics than in the higher latitudes. Such an intraspecific genetic diversity trend has been generally reported in plants, birds, and vertebrates (reviewed by Hirao et al., 2017). This trend is not completely applicable to the present study. The genetic diversity increased from the Heilongjiang Basin to the Yangtze River Basin, which may mirror the latitudinal trend of genetic diversity. However, the lowest level of genetic variations in the Pearl River Basin broke the trend. Among the five regions studied, the Pearl River Basin has been fully utilized and developed, with little idle land and water area. Furthermore, the residents around rivers in the Pearl River Basin have greatly increased, resulting in more sewage and a poor water environment. Though recent bottlenecks were found in all 28 populations, the harsh habitats in the Pearl River Basin might result in the lowest genetic diversity in this region.

The Bottleneck analysis showed that almost all *Z. latifolia* populations experienced recent demographic bottlenecks, especially under IAM (23 populations) and TPM (17 populations). The microsatellites used in *Z. latifolia* are either dinucleotide perfect repeats or imperfect repeats, both of which tend toward the IAM (Cornuet and Luikart, 1996). Therefore, the IAM might give more accurate estimates. The census population size of species can provide compelling evidence for bottlenecks. Unfortunately, for wild *Z. latifolia*, as a kind of common weed, its census population size is difficult to be obtained. Chinese wetlands, including the current five river basins, have been shrinking seriously in the past few decades (Wang et al., 2011; Huang et al., 2012; Cao et al., 2017; Chen et al., 2017b; Zhang et al., 2019). During this process, we inferred that the habitats of *Z. latifolia* could have been severely disturbed and the population size would be reduced. Besides the general situation, wild *Z. latifolia* populations have continuously suffered from bottlenecks caused by other factors. The factors leading to the bottlenecks were different for populations collected from different habitats. As for the populations from lakes (including all populations from the Yangtze River Basin and DP and LQ from the Huanghe River Basin), they generally grow along the littorals of lakes, and two factors may lead to the bottlenecks: (1) Severe shrinkage of lakeside zones during the dry seasons would result in extensive death of wild *Z. latifolia*; (2) Since people do not need *Z. latifolia* to feed cattle as they did historically, *Z. latifolia* has been removed by machines in some lakes to prevent the process of paludification. As for populations growing in ponds, irrigation ditches, and small rivers, such habitats are usually ephemeral, which would lead to repeated bottlenecks for *Z. latifolia* populations. As we know, *Z. latifolia* cannot withstand drought due to its underdeveloped root systems and recalcitrant seeds (Berjak and Pammenter, 2007).

Historical and contemporary gene flow

In the present study, historical gene flow ($M_H = 0.212$–2.354) was higher compared to contemporary gene flow ($M_C = 0.0021$–0.0044), indicating the effect of wetland fragmentation and subsequent population decline. These findings are supported by observed low genetic diversity, significant bottlenecks, and high population divergence. Unlike the contemporary gene flow, which showed that higher migration was within clusters ($M_C = 0.0112$–0.0247) than among clusters ($M_C = 0.0021$–0.0044), a higher migration rate was found among clusters ($M_H = 0.211$–14.46) than within clusters ($M_H = 0.212$–2.354) for historical gene flow. This could be related to the origin and dispersal history of *Z. latifolia*. Guo and Ge (2005) reported that *Zizania* species originated from the New World and dispersed into North America and then to Asia through the Bering Land Bridge during the Miocene, which is also supported by the timeframe for the diversification of the rice tribe (Wen, 2001; Gutaker et al., 2020). After the introduction of *Z. latifolia* in Northern China, colonization occurred southwards occupying different environmental habitats (Guo and Ge, 2005). During the migration process, the plant could have acquired new mutations, but which were not enough to dilute the shared common ancestry.

IBE, IBD, and IBR

For the first time, IBE and IBR were tested, besides the commonly assessed IBD pattern in the natural populations of *Z. latifolia*. We found that the IBE pattern was exclusively supported by RCM for all populations, and it was also detected

TABLE 7 Percentage contribution of bioclimatic variables across six ecological niche models (ENMs).

Variable	All pops	HRB	LRB	HHRB	YRB	PRB
Isothermality (bio_3)	15.4	92.3	60.9	2.7	16.6	0.1
Max. temperature of the warmest month (bio_5)	13.4	0.4	0	0.2	13.6	0.7
Mean temperature of the wettest quarter (bio_8)	17.3	0.5	0.7	76.4	1	68.8
Precipitation seasonality (bio_15)	1.9	5.5	15.6	19.8	58.8	5.7
Precipitation of the warmest quarter (bio_18)	52	1.3	22.8	0.9	10	24.7

HRB, Heilongjiang River Basin; LRB, Liaohe River Basin; HHRB, Huanghe River Basin; YRB, Yangtze River Basin; PRB, Pearl River Basin.

TABLE 8 Niche identity tests between genetic group pairs.

	HRB	LRB	HHRB	YRB	PRB
HRB		0.342	0.49	**0.156**	**0.255**
LRB	0.256		0.341	**0.097**	**0.166**
HHRB	0.207	0.146		**0.144**	0.341
YRB	0.362	0.313	0.413		0.451
PRB	0.375	0.281	0.112	0.219	

Lower triangle represents 1-tailed t-tests (a = 0.05) while upper triangle represents Niche Overlap (D). The D ranges from 0 (no overlap) to 1 (niche equivalency). Niche divergence was observed in five pairs (bold). Niche equivalency was also observed in five pairs involving HHRB and LRB groups although the D value was substantially low.
HRB, Heilongjiang River Basin; LRB, Liaohe River Basin; HHRB, Huanghe River Basin; YRB, Yangtze River Basin; PRB, Pearl River Basin.

TABLE 9 Background similarity test between genetic clusters with all comparisons being significant at 95 CI level.

	HRB	LRB	HHRB	YRB	PRB
HRB		0.342	0.49	0.156	0.255
LRB	0.021–0.348		0.341	0.097	0.166
HHRB	0.001–0.454	0.032–0.344		0.144	0.341
YRB	0.035–0.132	0.035–0.081	0.068–0.185		0.451
PRB	0.102–0.243	0.053–0.127	0.188–0.347	0.201–0.434	

Lower triangle represents 95% confidence interval while upper triangle represents Niche Overlap (D). The overlap values were within the null distribution, which indicates background similarity showing that the observed niche divergence was not due to difference in habitats available to each group. Focal groups are horizontal while background points are vertical.
HRB, Heilongjiang River Basin; LRB, Liaohe River Basin; HHRB, Huanghe River Basin; YRB, Yangtze River Basin; PRB, Pearl River Basin.

in three regions, including Liaohe River Basin, Huanghe River Basin, and Pearl River Basin. Similarly, previous studies also showed that instead of geographical distance, the environmental variables better explain the genetic patterns of different other plant species [refer to reviews by Shafer and Wolf (2013) and Sexton et al. (2014)]. For example, IBE was reported for the perennial grass species (*Festuca rubra*) of the western Norway alpines and the aquatic species *Ranunculus subrigidus* of the Qinghai-Tibetan plateau (Šurinová et al., 2019; Wu Z. et al., 2019).

The two methods (GLMs and spatial GLMMs) consistently found that the maximum temperature of the warmest month (bio_5) and precipitation seasonality (bio_15) were the best environmental variables responsible for the observed genetic diversity. Environmental association analysis (EAA) based on the outlier loci also showed similar results. Additionally, EAA also detected a significant association between outlier loci and a high number of temperature-related variables, which implied that temperature and precipitation could alter the physiology and phenotypic diversity and subsequently influence plant genetic differentiation and adaptability, which was well-proved by previous studies (Hoffmann and Sgr, 2011; Manel et al., 2012; Wang et al., 2016; Münzbergová et al., 2017). For *Z. latifolia*, the intolerance to drought undoubtedly makes precipitation very important for its growth and population maintenance. Our common garden experiments showed that the individuals exhibited shorter heights and smaller leaves when they were transplanted to a relatively lower latitude region. We also found that with the annual temperature increasing at 1°C, the plant height, leaf length, and width would decrease by 4.65, 2.81, and 0.39 mm, respectively (unpublished data). Furthermore, higher respiration rates were found in the populations from high to low latitude regions compared with those from low to high latitude regions, but all populations showed similar levels of photosynthetic parameters (unpublished data). A previous study suggested that plant respiration often reaches peak at a higher temperature compared to photosynthesis (Atkin and Tjoelker, 2003). Therefore, we inferred that a warmer condition could result in a decrease in biomass accumulation due to higher organic matter degradation. In the present study, the putative roles of the four gene-linked loci (DNA-binding transcription factor activity, protein phosphorylation, RNA splicing, and response to external stimuli) are part of the plant machinery involved not only in normal growth, but also in response to stressors through transcription regulation, RNA modification, and protein metabolism.

Isolation by distance was the second best-supported model for the entire study site. As an anemophilous emergent plant, combined with its edible seeds, *Z. latifolia* can mainly be dispersed by wind, water flow, and waterfowl. These factors work more effectively at close distances, especially in the local area. Furthermore, since the *Z. latifolia* populations in this study were sampled from plains with latitude gradients, geographically distant regions tend to have greater variations due to differences in climate factors. Even though migrating birds can spread seeds over long distances along latitudes, the seeds may unsuccessfully colonize in the new habitat due to unsuitable climatic conditions. In contrast, seeds that spread to nearby regions are more likely to survive due to similar climatic conditions. The contribution of geographical distance to *Z. latifolia* genetic structuring has been reported previously by Zhao et al. (2018, 2019) using a simple Mantel, for which we also found a strong positive correlation ($r = 0.6388$; $P = 0.001$) for the entire data set.

Within regions, IBD was only in the Heilongjiang River Basin where *Z. latifolia* populations grow along the stretch of the Amur River (Fan et al., 2016). When populations reach equilibrium between gene flow and genetic drift, there should be an IBD pattern (Wright, 1943). The IBD pattern in the Heilongjiang River Basin may suggest that the continuous populations along the river in this area are less susceptible to genetic drift.

For aquatic or riparian plants, hydrological connectivity greatly facilitates their dispersal between populations. In the present study, the populations from the Yangtze River Basin were sampled from ponds and small rivers which would connect with the artery of Yangtze in flood seasons. The Yangtze River Basin was a potamo-lacustrine system where lakes (including sampling lakes) were interconnected with the main vein of the Yangtze River by small rivers; thus, hydrological connectivity (IBR_HC) was expected. It is worth noting that of the seven lakes sampled in the Yangtze River Basin, except for Dongting Lake, the water course of the other six lakes was controlled by water gates installed in the 1950s for controlling water levels, generating electricity, and irrigation (Wu Y. et al., 2019). Thus, the hydrological connectivity between lakes depends on the control of lakes by local governments, rather than the connectivity of waterways. Since *Z. latifolia* is a wind-pollinated plant, wind connectivity was also investigated in this study. Previous studies have shown that pollen-mediated gene flow spreads almost exclusively locally, and diminishes logarithmically with increasing distance (Tero et al., 2003). The IBR_WD was not supported in the present study, which proved that the wind pollination of *Z. latifolia* was only effective within a population.

We found that populations within each river basin occupy significantly different environmental spaces, which is expected to facilitate local adaptation. Similar to our results of EAA, where bio_3, bio_5, bio_15, and bio_18 variables were significantly associated with gene-linked loci, these variables proved to be the most influential drivers of niche differentiation. Niche divergence was identified in half of the pairwise comparisons that involved at least each genetic group. Although some comparisons showed evidence of niche equivalency, the niche overlap score was substantially low (0.097–0.451) on a scale of 0 (no overlap) to 1 (niche equivalency). Pairwise background similarity tests showed that gENMs were not significantly different, and thus the observed niche divergence could not be an artifact of difference in habitat availability among the river basins. The IBD model results from geographical isolation, which limits gene flow among populations. Given that the local adaptation could occur in differentiated environment space, the genetic structure pattern could be construed to be shaped by IBD, especially for widespread species. Our gENM results supplement the evidence for IBE being the most important in shaping the genetic structure and distribution of *Z. latifolia*.

In general, the genetic structure of *Z. latifolia* is influenced by the combined effects of the environment, geographical distance, and landscape heterogeneity, with the environment having the highest impact.

Genetic discontinuity and excess heterozygosity

In the present study, the AMOVA based on three data sets consistently showed the strong genetic divergence among *Z. latifolia* populations from different regions/latitude areas, accounting for 45.54–47.13% of the total genetic variations. The results were also supported by the cluster analyses (STRUCTURE, PCoA, and NJ) which showed that the populations from the same regions/latitude areas clustered together. Accordingly, genetic discontinuities were detected between adjacent regions/latitude areas by both *geneland* and BARRIER analysis. The evident genetic differentiation among regions might be explained by a number of factors, such as IBE, IBD, and fragmentation, which were supported by decreased gene flow.

As mentioned above, the IBE and IBD were the two best-supported models for the entire study site. Populations from the same latitude are geographically closer and their climatic conditions are more similar, so populations from the same latitude tend to cluster together with less genetic differentiation.

Habitat fragmentation generally reduces the gene flow among populations by decreasing the habitat size and increasing the isolation between populations, which would eventually increase genetic differentiation. However, Zhao et al. (2013) reported that wild rice *Oryza rufipogon* increased gene flow in fragmented habitats through introgression which is from cultivated populations to wild ones and acts as a bridge to mediate the gene flow among wild populations. In the present study, historical gene flow ($M_H = 0.212–2.354$) was higher than contemporary gene flow ($M_C = 0.0112–0.0247$), which implied that the wetland fragmentation and degeneration were followed by reduced gene flow.

Declining population size and population isolation are expected to result in considerable inbreeding. However, we found negative F_{IS} values for almost all populations. This could be related to the characteristics of *Z. latifolia*, which is a perennial species that reproduces both sexually and clonally. The reproduction system of partial clonality has been shown to influence the heterozygosity rate, with tendencies toward excess, leading to negative F_{IS} values (Stoeckel et al., 2006, 2021). According to Balloux et al. (2003), in clonal/partial clonal species, mutations will accumulate due to irreversible divergence of two alleles of a locus over generations leading to excess heterozygosity and hence the observed negative F_{IS} values for *Z. latifolia* populations.

Implications for conservation and management

Global climate change has brought a tremendous impact on world food production, which highlights the protection and management of the resources of crop wild germplasm increasingly prominent. Knowledge of genetic diversity patterns and local adaptation for the target species is a prerequisite for the effective conservation of its wild germplasm resources.

The wild rice *Z. latifolia* showed a high level of genetic differentiation among the regions along the latitudinal gradient. The genetic discontinuity was attributed to the climate differences (especially temperature and precipitation), the heterogeneous landscape, and the discrete distribution of the natural populations. Given that Chinese wetlands are being increasingly damaged (Liu et al., 2005), both *in-situ* and *ex-situ* conservation should be recommended in the future conservation process. In the process of *ex-situ* conservation, transplanting with a large latitude span is discouraged due to its inadaptability to the local climate. Multiple *ex-situ* conservation sites should be established along the latitude gradient, which can reduce the management cost, and prevent the out-crossing depression caused by the interbreeding of *ex-situ* populations from different latitude regions.

For the same latitude region, obvious genetic differentiation among populations was detected due to wetland fragmentation. Therefore, dredging the watercourses to achieve hydrological connectivity within each wetland is recommended for *in-situ* conservation at the same latitude. This action would increase the gene flow among populations and habitat stability and consequently reduce bottlenecks (Chen et al., 2017a). Additionally, populations with a high level of genetic variation need to be protected as a priority for both *ex-* and *in-situ* conservation, such as populations CH, SJ, and DT in the Yangtze River Basin.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

YC conceived the idea and designed the research project. WL gave suggestions for the design of the study. YC, XF, and WF collected the samples and assembled experiment materials. GW, XF, and MT performed the experiment. YC and GW analyzed the data and wrote the manuscript. All authors contributed to the revision and final editing of the manuscript prior to submission.

Funding

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB31000000), the Talent Program of Wuhan Botanical Garden of the Chinese Academy of Sciences (Y855291), and the CAS-TWAS President's Fellowship for International Doctoral Students.

Acknowledgments

We would like to thank MS. Chen Jian for her valuable comments on the manuscript. This manuscript has been released as a pre-print at https://www.biorxiv.org/content/10.1101/2020.05.29.124685v1 (Wagutu et al., 2020c).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.929944/full#supplementary-material

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frontiers | Frontiers in Environmental Science

TYPE Original Research
PUBLISHED 20 September 2022
DOI 10.3389/fenvs.2022.969980

The combined effects of land use and seasonal environmental factors on stream food web structure

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SPECIALTY SECTION
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Environmental Science

RECEIVED 16 June 2022
ACCEPTED 31 August 2022
PUBLISHED 20 September 2022

CITATION
Wang Y, Li S, Tan X and Zhang Q (2022), The combined effects of land use and seasonal environmental factors on stream food web structure.
Front. Environ. Sci. 10:969980.
doi: 10.3389/fenvs.2022.969980

Land use and seasonal changes in environmental conditions influence biological communities and their trophic interactions in riverine ecosystems. However, how land use and the seasonality of environmental conditions jointly influence the food web structure of riverine ecosystems remains unclear. Here, we conducted a comparative study on basal resources, macroinvertebrates, and fish at woodland and urban sites to explore the combined effects of land use and environmental conditions during spring, autumn, and winter on the food web structure of a subtropical river in China. We used $\delta^{13}C$ and $\delta^{15}N$ to trace consumers' diets and calculate community-level metrics within food webs. At woodland sites, we found that allochthonous sources contributed significantly more to consumers' diets in the high flow season (53%) than in the low flow season (around 30%), but allochthonous sources contributed less than 30% at urban sites. The seasonal flooding facilitated the acquisition of terrestrial sources at woodland sites, while the impervious surface cover at urban sites cut off the influx of terrestrial inputs. The isotopic niche space of basal resources at both woodland and urban sites was significantly higher in the high flow season. However, the isotopic niche space of consumers at urban sites was not significantly different between seasons. Trophic length, niche width, and trophic redundancy showed no seasonal differences at urban sites, but trophic length increased significantly during the high flow season at woodland sites. Our temporal food web study at urban and woodland rivers illustrated the combined effects of land use and seasonal environmental conditions on the food web structure, and highlighted the role of allochthonous carbon in supporting biological communities and the importance of lateral and longitudinal connectivity in river ecosystems.

KEYWORDS

stable isotopes, urbanization, diet tracing, trophic interaction, river wave concept

Introduction

Food web structure within aquatic food webs is a composite of the enormous diversity of species and their trophic interactions (Belgrano et al., 2005), during which energy flow and nutrient cycling occurs. Changes in food web structure can often reflect the alterations in structure and function within natural river ecosystems (Thompson et al., 2012; Fitzgerald et al., 2017; Fugère et al., 2018). Riverine ecosystems have a high level of spatial and temporal heterogeneity with interactive pathways along four dimensions: longitudinal, lateral, vertical, and temporal (Ward, 1989). Thus, riverine ecosystems are subject to numerous environmental alterations (Allan, 2004; Roach and Winemiller, 2015; Brett et al., 2017). A better understanding of how environmental changes affect the energy flows and trophic interactions is essential in aquatic science and has implications for the sustainable management of riverine ecosystems (Wilson et al., 2016; Brett et al., 2017). Stable isotopes have been widely used to estimate community-level metrics, such as isotopic niche, niche width, trophic diversity, and trophic redundancy (Layman et al., 2007; Newsome et al., 2007; Abrantes et al., 2014; Rader et al., 2017). These metrics can provide us with an overview of food web structure properties over a relatively long period (Layman et al., 2012; Jabot et al., 2017), which reflects the response of food web structure when facing environmental changes (Alp and Cucherousset, 2022).

Land uses are major threats to biological communities, as has been observed by their effects on food web structures (Foley Jonathan et al., 2005; Cazzolla Gatti, 2016; Reid et al., 2019). For example, Hette-Tronquart et al. (2018) investigated community-level metrics at sites with contrasting land uses in the Seine River in France and found that urbanization at the local scale affected fish community composition. Price et al. (2019) found that in Zagreb (Croatia), macroinvertebrates in urbanized streams had lower trophic redundancy than counterparts in woodland and agricultural streams. Wang et al. (2021) suggested that food web structure alterations in subtropical streams in the Han River should be attributed to the availability of basal resources across land uses. Among the different land uses, urbanization is the prevalent land use type throughout the world (Paul and Meyer, 2001; Booth et al., 2016). The structure and function of riverine ecosystems can be profoundly influenced by urbanization, even when the urban area is a low percentage of total catchment area. Urbanization effects on riverine ecosystems can be physical (e.g., increased impervious surface cover or decreased shading), chemical (e.g., increased sewage water discharge or other contaminants inputs), and biological (e.g., altered primary production or species diversity) (Paul and Meyer, 2001; Valle Junior et al., 2015). For example, the impervious surface cover can increase water discharge and surface runoff, resulting in increased nutrient inputs and turbidity, thereby degrading water quality in the river channel and decreasing the diversity of aquatic organisms (Paul and Meyer, 2001; Allan, 2004). Additionally, riparian deforestation due to urbanization can reduce the shaded area of the river surface and increase water temperature, which further affects the primary production of river ecosystems, leaf decomposition in the riparian zone, the life history of aquatic consumers (Allan, 2004), and ultimately alters the food web structure within riverine ecosystems (Calizza et al., 2012; Parreira de Castro et al., 2016; El-Sabaawi, 2018; Jackson et al., 2020).

The seasonality in environmental conditions also plays a vital role in influencing the food web structure in riverine ecosystems because there are differences in abiotic (e.g., temperature, precipitation, and light) and biotic (e.g., migration, growth, and reproduction) factors in the different seasons (Jardine et al., 2012a; McMeans et al., 2015; Fernandes and McMeans, 2019; Gibert, 2019). Among those factors, hydrological regimes (e.g., precipitation) can regulate the water discharge among the seasons in river ecosystems (McMeans et al., 2015). The flood pulse concept (FPC) suggests that, in river ecosystems, when the water level increases in the high-flow season, consumers will have more access to terrestrial nutrients in the aquatic and terrestrial transition zone through lateral connectivity (Junk et al., 1989; Jardine et al., 2012a). Thus, the water discharge among the seasons can alter the amount and sources of nutrients available to consumers, and thus change the relative importance of carbon sources that support consumer growth and diversity in rivers (Jardine et al., 2012b; Pease et al., 2020). This change can ultimately alter the food web structure in riverine ecosystems due to the availability of basal resources (Wang et al., 2021). For example, Roach and Winemiller (2015) found that algae contributed more to macroinvertebrates and fish biomass following low-flow periods, and terrestrial plants contributed more following high-flow pulses. Zheng et al. (2018) suggested that allochthonous carbon had an advantage over autochthonous carbon in supporting aquatic food webs in the Irtysh River because allochthonous carbon can subsidize aquatic invertebrates specialized in terrestrial carbon sources and fish consumers during the frozen period.

Recently, the effects of land use on food web structure in rivers have been studied (Hette-Tronquart et al., 2018; Price et al., 2019; Wang et al., 2021). However, there is one critical limitation to these studies in that they failed to recognize the temporal dimension of riverine ecosystems with only a one-time sampling “snapshot” of food web dynamics (McMeans et al., 2015; Hette-Tronquart et al., 2018; Price et al., 2019; Wang et al., 2021). For example, samples were taken only once in July and August in the Orge River (Hette-Tronquart et al., 2018), in early April and May in the Zagreb region of Croatia (Price et al., 2019), and in June in the Han River of China (Wang et al., 2021). Even though several studies have assessed the impact of the seasonality of

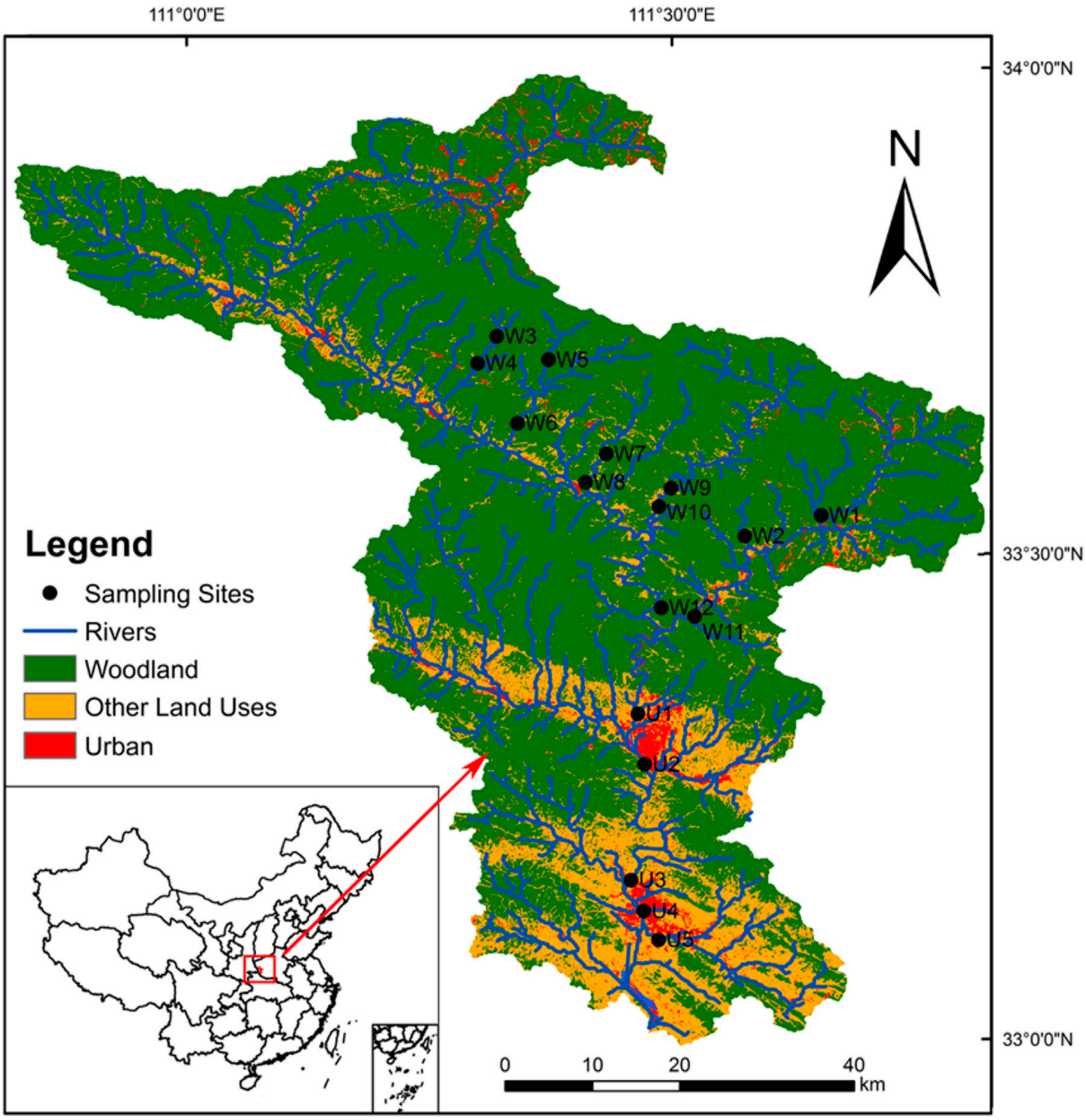

FIGURE 1
Locations of the sampling sites in the Laoguan River.

environmental conditions on the trophic diversity and the relative importance of carbon sources that support fish assemblages in rivers (Jardine et al., 2012b; Pease et al., 2020; Bokhutlo et al., 2021), these studies did not take land use into account. To our knowledge, how land use and seasonality jointly influence the food web structure of riverine ecosystems remains unclear.

Given previous studies on the response of food web structure to either land use or seasonality in river ecosystems, we hypothesized that the impact of urbanization on the food web structure would be exacerbated by the different seasonal hydrological regimes as follows: 1) terrestrial inputs in urbanized rivers would dramatically increase and contribute much more to consumers' diets during the high flow season than aquatic sources; 2) increased terrestrial inputs in urbanized rivers would significantly expand the niche space (or trophic diversity) of basal resources and aquatic consumers; 3) autochthonous sources in urbanized rivers would dominate again when terrestrial inputs decreased during the low flow season. We aimed to shed light on how riverine food webs are structured in spatial and temporal dimensions under changes in environmental conditions.

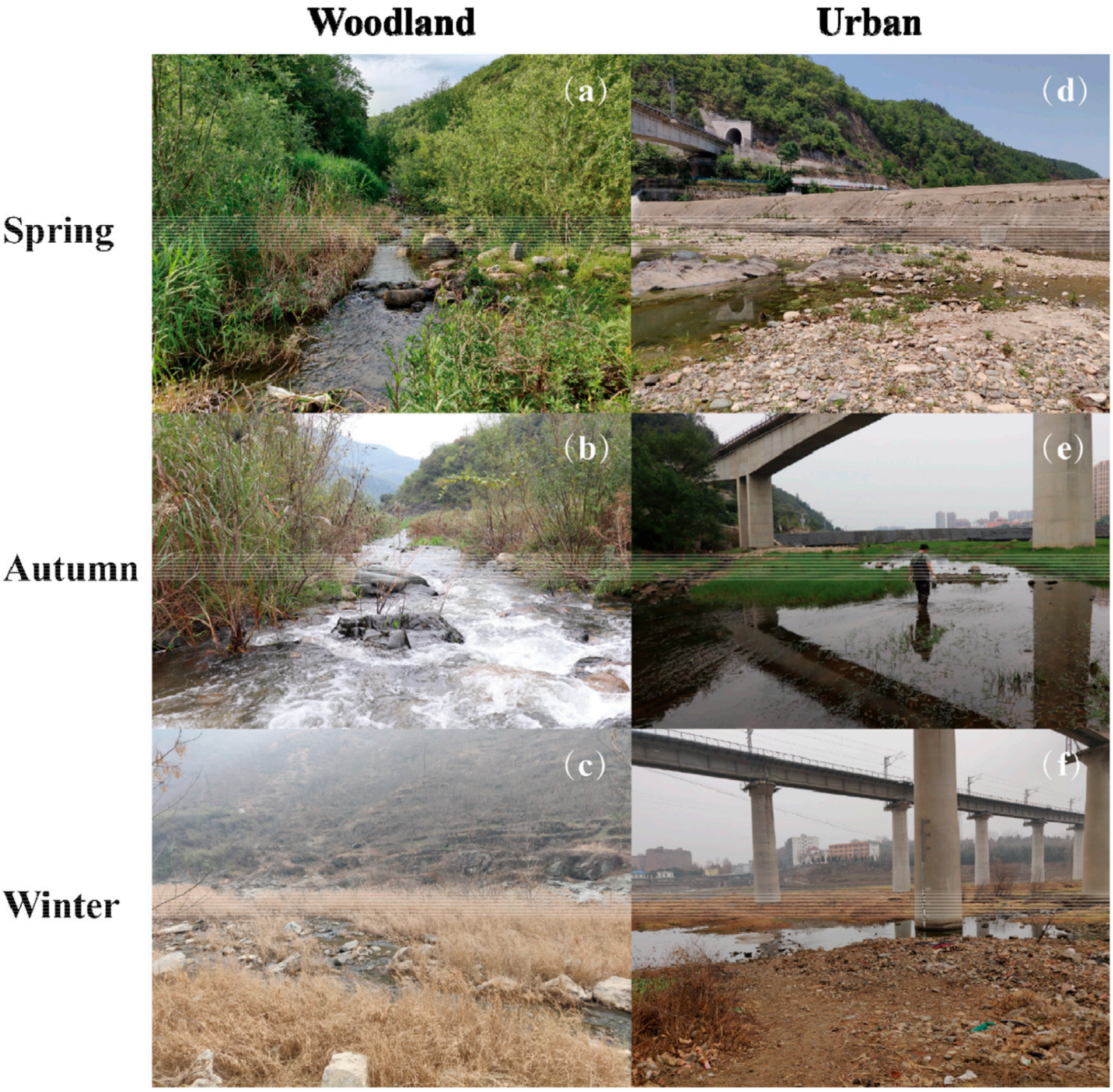

FIGURE 2
Landscapes of a woodland site in **(A)** spring, **(B)** autumn, and **(C)** winter, and an urban site in **(D)** spring, **(E)** autumn, and **(F)** winter of our study area.

Materials and methods

Study area

Laoguan River (E110°17′-111°50′ and N32°55′-34°01′) (Figure 1) is a tributary of the Dan River, which drains into the Yangtze River. The drainage area has a subtropical monsoon climate. The average annual precipitation is approximately 830 mm, and much of the precipitation occurs from July to October (the high flow season) (Li et al., 2017; Yuan et al., 2017). The average annual temperature is approximately 15°C, with a maximum of 31°C in summer and a minimum of −5°C in winter. The river stretch is more than 150 km with an area of around 2,000 km^2. The river channel can be as wide as 30 m with numerous rapids and deep pools. The river network covers a gradient of human disturbances as the river flows from mountainous regions that are less affected by human activities to two counties with nearly 1 million residents (Xixia and Xichuan County).

Land use data

Land use types were derived from Landsat Thematic Mapper imagery with ArcGIS10.0 and ENVI 4.8. Each sample site was defined as the respective land use at the reach scale (which we defined as a 2 km rectangle buffer zone upstream with a 500 m wide strip) when the percentage of a certain land cover exceeded 40% (Wang et al., 2021).

Woodland sites were characterized by dense riparian vegetation and sparse in-stream vegetation in spring (Figure 2A). The riparian vegetation was dominated by either tussock, shrubs, or trees (e.g., *Salix babylonica*). The in-stream vegetation was dominated by submerged plants (e.g., *Myriophyllum verticillatum* or *Potamogeton crispus*). The stream substrate was mainly cobbles and pebbles. There were massive leaf litter inputs during the high flow season (Figure 2B). In winter, the water level and the leaf litter inputs decreased substantially (Figure 2C).

Urban sites were characterized by impervious surface cover (e.g., levees) along the riverbanks where ornamental trees were sparsely planted. There were several railroad and highway bridges crossing the stream. In-stream vegetation was densely distributed with submerged or free free-floating plants (e.g., *Potamogeton* sp. and *Salvinia natans*). The stream substrate was either pebbles or sand and silt.

We chose seventeen sampling sites along the stream with two distinct land use types (urban and woodland) at the reach scale (Figure 2). Those sites were wadable, which allowed for a comprehensive investigation of food web components including basal resources and consumers at higher trophic levels. There were twelve woodland sites and five urban sites with an increment distance of more than 2 km. We conducted three sampling campaigns in early June during baseflow season (spring) in 2019, early October at the end of the high flow season (autumn) in 2020, and January during the low flow season (winter) in 2021.

Physical and chemical parameters

Water temperature (°C), pH, dissolved oxygen concentrations (DO) (mg/L), conductivity (μS/cm), nitrate-N (mg/L), and ammonia-N (mg/L) were measured using YSI 6620 (Yellow Springs, OH, United States) in the field. Water velocity (m/s) was measured using a velocity meter (Global Water, FP201, United States). All the water samples were stored at 4°C before the laboratory analysis. At the laboratory, sub samples were filtered with a GF/F glass microfiber filter (Whatman, 0.7 μm pore size) to measure the total dissolved carbon (TDC, mg/L), total dissolved nitrogen (TDN, mg/L) (Elementar, Vario TOC cube, Germany), and total dissolved phosphorus (TDP, mg/L) (ThermoFisher, X Series 2, United States).

Field sampling and lab processing

Components in the riverine food webs including basal resources (primary producers and other carbon sources) and consumers (macroinvertebrates and fish), were collected at each site. Basal resources differed in amount and types among the seasons and included autochthonous carbon sources, such as filamentous algae (present at several sites), epilithic algae, aquatic macrophytes, fine benthic organic matter (FBOM), and seston, and there were allochthonous carbon sources, including terrestrial insects, terrestrial vegetation, and leaf litter.

Macroinvertebrates were classified into five functional feeding groups (FFGS), which were predators, scrapers, filter-collectors, gatherer-collectors, and shredders (Morse et al., 1994). The sampling method for each food web component was described in detail in the supporting information. A total of 2,353 samples (replicate samples included) at 17 sites in spring, autumn, and winter were collected. For fish, there were 20 species (149 samples) in spring, 28 species (199 samples) in autumn, and 19 species (285 samples) in winter. For macroinvertebrates, there were 70 species (179 samples) in spring, 45 species (356 samples) in autumn, and 39 species (319 samples) in winter (Supplementary Tables S2, S3). For basal resources, there were 231 samples in spring, 398 samples in autumn, and 237 samples in winter.

To identify trophic interactions and infer energy flows, we used carbon and nitrogen stable isotopes to estimate the assimilated fraction of consumers' diets (Fry, 2006; Boecklen et al., 2011; Nielsen et al., 2018). Samples were oven-dried at 60°C for 48 h before being ground into a fine powder before analyzing their isotopic compositions. Carbon and nitrogen stable isotopes were analyzed using the Isotope Ratio Mass Spectrometer (ThermoFisher, Delta V advantage, Germany) and calculated as $[(R_{sample}/R_{standard})-1] \times 1{,}000‰$, where R is the ratio of heavy to light isotope. The isotopic standards are PeeDee belemnite limestone for carbon and atmospheric air for nitrogen (Fry, 2006).

Data analysis

To improve the accuracy of mixing models for tracing consumers' diets, we aggregated basal resources with similar $\delta^{13}C$ isotopic signatures and originality into one group (Nielsen et al., 2018). Basal resources were pooled into three groups: terrestrial sources (leaf litter, terrestrial insects, and vegetation), aquatic macrophytes (submerged and emergent macrophytes), and lower aquatic sources (epilithic algae, filamentous algae, seston, and FBOM). Terrestrial sources were referred to as allochthonous sources while aquatic macrophytes and lower aquatic sources were referred to as autochthonous sources. Macroinvertebrates were pooled into four FFGS, which were predators, collectors (filter-collectors and gatherer-collectors), scrapers, and shredders. According to McCutchan et al. (2003), trophic enrichment factors (TEFs) for the basal resources-macroinvertebrates mixing model were 0.4 ± 0.28‰ ($\delta^{13}C$) and 2.2 ± 0.3‰ ($\delta^{15}N$), and the TEFs for the macroinvertebrates-fish mixing model were 0.6 ± 0.16‰ ($\delta^{13}C$) and 2.4 ± 0.22‰ ($\delta^{15}N$). We applied lipid corrections to consumers in the diet tracing analysis based on the C/N ratio in fish (<4) and macroinvertebrates (>4) measured in the Isotope Ratio Mass Spectrometer (Logan et al., 2008).

Community-level metrics, also known as Layman metrics (Layman et al., 2007), included 1) $\delta^{15}N$ range (NR), which represented the vertical structure within a food web (trophic length) calculated using the distance between two species with the most enriched and depleted $\delta^{15}N$ values; 2) $\delta^{13}C$ range (CR), which represented the niche width at the base of a food web calculated as the distance between two species with the most enriched and most depleted $\delta^{13}C$ values; 3) total area (TA), which represented the convex hull area encompassed by all species in $\delta^{13}C$–$\delta^{15}N$ bi-plot space; 4) mean Euclidean distance of each species to centroid (CD), which represented the average degree of trophic diversity; 5) mean nearest neighbor distance (MNND), which represented the mean Euclidean distances to each species' nearest neighbor in bi-plot space and the overall density of species packing; and 6) standard deviations of mean nearest neighbor distance (SDNND), which represented the evenness of species packing. Those six metrics were calculated at each site.

If more species performed the same trophic function, MNND would be smaller and indicate higher trophic redundancy. If there were more species sharing similar ecological traits, SDNND would be smaller and indicate more evenly distributed trophic niches (Layman et al., 2007). The isotopic variations of the standard ellipse area corrected for the small sample size (SEA_c) of basal resources, macroinvertebrates, and fish were also calculated at each site.

Analysis of variance (ANOVA) was performed to analyze the differences in physical and chemical parameters, isotopic values, and community-level metrics among seasons at a significance level of $p < 0.05$. Relationships among the physical and chemical parameters and community-level metrics were tested using Pearson's correlation with statistical significance at $p < 0.05$. Principal component analysis (PCA) was carried out to explore the key physical and chemical characteristics in relation to land uses and seasons. Statistical analyses were carried out in SPSS 24.0 software and R 3.6.1 (R Core Team, 2019). Diet tracing in consumers was conducted using the simmr package (updated SIAR package) (Parnell, 2019). The community metrics were calculated using the SIBER package which employed Gaussian likelihood and fit the mixing model to the data via Markov chain Monte Carlo (MCMC) (Jackson et al., 2011).

Results

Physical and chemical parameters

At woodland sites, water temperature ($F_{2,32} = 170.433$, $p < 0.01$), water velocity ($F_{2,32} = 3.924$, $p = 0.03$), conductivity ($F_{2,32} = 11.56$, $p < 0.01$), pH ($F_{2,32} = 59.655$, $p < 0.01$), TDC ($F_{2,32} = 9.966$, $p < 0.01$), TDN ($F_{2,32} = 24.515$, $p < 0.01$), TDP ($F_{2,32} = 3.508$, $p < 0.05$), DO ($F_{2,32} = 52.012$, $p < 0.01$), ammonia-N ($F_{2,32} = 45.74$, $p < 0.01$), and nitrate-N ($F_{2,32} = 29.35$, $p < 0.01$) significantly differed among seasons (Table 1).

At urban sites, water temperature ($F_{2,12} = 124.237$, $p < 0.01$), pH ($F_{2,12} = 27.786$, $p < 0.01$), TDN ($F_{2,12} = 5.973$, $p < 0.01$), DO ($F_{2,12} = 12.406$, $p < 0.01$), and ammonia-N ($F_{2,12} = 5.965$, $p < 0.01$), nitrate-N ($F_{2,12} = 10.301$, $p < 0.01$) showed significant differences. There was no significant change in water velocity ($F_{2,12} = 0.001$, $p = 0.999$), conductivity ($F_{2,12} = 0.292$, $p = 0.752$), TDC ($F_{2,12} = 1.253$, $p = 0.32$), or TDP ($F_{2,12} = 2.354$, $p = 0.137$).

Isotopic signatures

At woodland sites, there were significant differences in $\delta^{13}C$ and $\delta^{15}N$ in fish ($F_{2,630} = 41.746$, $p < 0.01$; $F_{2,630} = 103.05$, $p < 0.01$), macroinvertebrates ($F_{2,851} = 67.633$, $p < 0.01$; $F_{2,851} = 18.099$, $p < 0.01$), lower aquatic sources ($F_{2,432} = 4.562$, $p = 0.01$; $F_{2,432} = 129.988$, $p < 0.01$), aquatic macrophytes ($F_{2,137} = 20.818$, $p < 0.01$; $F_{2,137} = 18.05$, $p < 0.01$), and terrestrial sources ($F_{2,288} = 17.303$, $p < 0.01$; $F_{2,288} = 45.184$, $p < 0.01$) (Table 2). Fish and macroinvertebrates had decreasing $\delta^{13}C$ values from spring (-22.63 ± 2.11‰ and −24.02 ± 2.97‰), autumn (-24.29 ± 2.07‰ and −26.10 ± 3.83‰), to winter (−25.36 ± 3.77‰ and −28.28 ± 4.64‰), and higher $\delta^{15}N$ values in autumn (12.43 ± 3.54‰ and 9.00 ± 3.54‰) than spring (9.21 ± 2.67‰ and 6.81 ± 2.77‰) and winter (8.33 ± 3.10‰ and 7.54 ± 5.69‰). $\delta^{13}C$ values for terrestrial sources (−26.66 ± 3.84‰), aquatic macrophytes (−22.33 ± 4.34‰), and lower aquatic sources (−25.22 ± 2.89‰) were highest in spring, and $\delta^{15}N$ values for terrestrial sources (10.85 ± 4.79‰), aquatic macrophytes (11.91 ± 4.06‰), and lower aquatic sources (18.44 ± 8.26‰) were highest in autumn.

At urban sites, $\delta^{13}C$ and $\delta^{15}N$ had significant seasonal differences for fish ($F_{2,199} = 20.296$, $p < 0.01$; $F_{2,199} = 21.699$, $p < 0.01$), macroinvertebrates ($F_{2,189} = 17.913$, $p < 0.01$; $F_{2,189} = 4.464$, $p < 0.05$), aquatic macrophytes ($F_{2,67} = 20.04$, $p < 0.01$; $F_{2,67} = 7.5$, $p < 0.01$), and terrestrial sources ($F_{2,62} = 7.557$, $p < 0.01$; $F_{2,62} = 10.66$, $p < 0.01$). For lower aquatic sources, the $\delta^{15}N$ values differed among the seasons ($F_{2,111} = 57.91$, $p < 0.01$), but $\delta^{13}C$ values were not significantly different ($F_{2,111} = 1.026$, $p = 0.362$) (Table 2). Fish and macroinvertebrates had highest $\delta^{13}C$ values (−23.91 ± 2.37‰ and −23.91 ± 3.72‰, respectively) in spring and highest $\delta^{15}N$ values (14.78 ± 3.42‰ and

TABLE 1 Physical and chemical parameters at woodland and urban sites in spring, autumn, and winter. Temp and Cond are abbreviations for water temperature and conductivity.

Parameters	Urban			Woodland		
	spring	autumn	winter	spring	autumn	winter
Temp (˚C)	24.66 ± 2.67^a	20.2 ± 1.72^b	6.6 ± 0.79^c	19.76 ± 2.88^a	18.63 ± 1.37^b	5.88 ± 1.59^c
Velocity (m/s)	0.32 ± 0.35^a	0.33 ± 0.18^a	0.32 ± 0.22^a	0.18 ± 0.09^b	0.33 ± 0.15^a	0.24 ± 0.11^a
Cond (μS/cm)	460.6 ± 139.61^a	424.54 ± 105.03^a	405.12 ± 100.63^a	312.45 ± 64.53^a	221.93 ± 58.78^b	191.64 ± 63.6^b
pH	8.67 ± 0.39^a	7.6 ± 0.14^b	7.71 ± 0.11^b	8.54 ± 0.29^a	7.76 ± 0.12^b	7.02 ± 0.48^c
TDC (mg/L)	50.56 ± 19.36^a	36.18 ± 12.09^a	41.69 ± 10.46^a	32.80 ± 5.40^a	22.56 ± 8.40^b	20.56 ± 6.74^b
TDN (mg/L)	1.51 ± 0.33^b	9.35 ± 5.66^a	6.28 ± 2.65^a	1.52 ± 0.48^c	6.82 ± 3.00^a	3.80 ± 0.69^b
TDP (mg/L)	0.16 ± 0.07^a	0.16 ± 0.20^a	0.02 ± 0.01^a	0.08 ± 0.05^a	0.03 ± 0.01^b	0.06 ± 0.07^a
DO (mg/L)	6.18 ± 1.82^c	9.66 ± 0.38^a	7.97 ± 0.46^b	5.43 ± 0.68^c	9.56 ± 0.33^a	7.44 ± 1.49^b
Ammonia-N (mg/L)	0.64 ± 0.22^b	0.12 ± 0.02^a	0.41 ± 0.35^b	0.36 ± 0.05^b	0.07 ± 0.02^a	0.67 ± 0.26^b
Nitrate-N (mg/L)	1.18 ± 0.51^a	3.18 ± 1.56^b	0.56 ± 0.21^a	1.08 ± 0.68^b	3.15 ± 1.32^c	0.59 ± 0.14^a

11.72 ± 4.23‰, respectively) in autumn. Terrestrial sources and aquatic macrophytes had the lowest δ^{13}C values (−32.07 ± 7.86‰ and −28.68 ± 2.1‰, respectively) in winter and the lowest δ^{15}N values (8.15 ± 2.46‰ and 9.73 ± 3.48‰, respectively) in spring. Lower aquatic sources had the highest δ^{13}C values (−25.26 ± 3.31‰) in spring and the highest δ^{15}N values (23.11 ± 9.14‰) in autumn.

Relative contributions of autochthonous and allochthonous sources

At woodland sites, the relative contribution of terrestrial sources ($F_{2,31}$ = 3.948, p = 0.03), aquatic macrophytes ($F_{2,31}$ = 4.527, p = 0.019), and lower aquatic sources ($F_{2,31}$ = 16.471, $p <$ 0.01) to macroinvertebrate diets was significantly different among the seasons. In the autumn high flow season, terrestrial sources constituted 53% of the macroinvertebrate diet, which was significantly higher than in spring (36%) and winter (33%). At urban sites, the relative contribution of terrestrial sources ($F_{2,12}$ = 1.942, p = 0.186), aquatic macrophytes ($F_{2,12}$ = 1.146, p = 0.35), and lower aquatic sources ($F_{2,12}$ = 2.646, p = 0.112) to macroinvertebrate diets was not significantly different among the seasons, and the relative contribution of terrestrial sources was around 20% throughout the year. Aquatic macrophytes constituted most of the macroinvertebrate diet in autumn, while lower aquatic sources constituted most of the diet in spring and winter (Figure 3A).

The proportion of predators ($F_{2,31}$ = 0.403, p = 0.672; $F_{2,11}$ = 0.481, p = 0.63), collectors ($F_{2,31}$ = 0.182, p = 0.835; $F_{2,11}$ = 1.418, p = 0.283), scrapers ($F_{2,31}$ = 0.001, p = 0.999; $F_{2,11}$ = 1.969, p = 0.186), and shredders ($F_{2,31}$ = 1.172, p = 0.323; $F_{2,11}$ = 1.424, p = 0.286) in fish diet at woodland and urban sites was not significantly different among seasons. At woodland sites, scrapers (31%) and collectors (~24%) constituted more to the diets of fish than shredders (~15%) throughout the year. At urban sites, scrapers (40%) and collectors (28%) constituted most of the diet in spring compared to predators (24%) and shredders (8%), but scrapers and collectors constituted less (<40%) than predators and shredders in autumn and winter (Figure 3B).

Community-level metrics

At woodland sites, the niche space (SEA_c) of basal resources increased significantly from the spring to the winter and reached the maximum in autumn ($F_{2,29}$ = 66.52, $p <$ 0.01). The niche space (SEA_c) of macroinvertebrates showed a slightly different trend which increased significantly from spring to autumn and winter ($F_{2,29}$ = 10.85, $p <$ 0.01). The niche space (SEA_c) of fish was higher in autumn and winter than in spring ($F_{2,29}$ = 5.84, $p <$ 0.01) (Figure 4). There were no significant differences in niche width (CR) ($F_{2,29}$ = 1.841, p = 0.177), TA ($F_{2,29}$ = 2.584, p = 0.093), CD ($F_{2,29}$ = 1.227, p = 0.308), trophic redundancy (MNND) ($F_{2,29}$ = 2.023, p = 0.151) or SDNND ($F_{2,29}$ = 1.302, p = 0.287), but there was a significant difference in NR among seasons ($F_{2,29}$ = 8.951, $p <$ 0.01). NR was significantly higher in autumn compared to spring and winter (Table 3).

At urban sites, the niche space (SEA_c) of basal resources showed significant differences among the seasons ($F_{2,12}$ = 10.39, $p <$ 0.01) and was higher in autumn and winter than in spring. Nevertheless, SEA_c of macroinvertebrates ($F_{2,12}$ = 1.99, p = 0.18) and fish ($F_{2,12}$ = 0.78, p = 0.48) showed no significant differences among seasons (Figure 4). There were no significant differences in NR ($F_{2,12}$ = 3.475, p = 0.064), CR ($F_{2,12}$ = 3.231, p = 0.075), TA ($F_{2,12}$ = 1.393, p = 0.286), CD ($F_{2,12}$ = 3.679, p = 0.057), MNND

TABLE 2 Mean ± SD of $\delta^{13}C$ and $\delta^{15}N$ isotope ratios of basal resources, macroinvertebrates, and fish at urban and woodland sites in spring, autumn, and winter.

Food web components		Urban			Woodland		
		spring	autumn	winter	spring	autumn	winter
Terrestrial sources	$\delta^{13}C$ (‰)	−24.60 ± 4.83^{a}	−25.55 ± 6.39^{a}	−32.07 ± 7.86^{b}	−26.66 ± 3.84^{a}	−26.48 ± 5.91^{a}	−30.21 ± 5.01^{b}
	$\delta^{15}N$ (‰)	8.15 ± 2.46^{b}	13.92 ± 4.44^{a}	10.28 ± 4.10^{b}	5.56 ± 2.45^{b}	10.85 ± 4.79^{a}	10.72 ± 3.64^{a}
Aquatic macrophytes	$\delta^{13}C$ (‰)	−20.81 ± 4.11^{a}	−25.41 ± 3.81^{b}	−28.68 ± 2.10^{b}	−22.33 ± 4.34^{a}	−25.05 ± 3.4^{b}	−29.28 ± 1.97^{c}
	$\delta^{15}N$ (‰)	9.73 ± 3.48^{b}	13.04 ± 4.13^{a}	13.90 ± 4.61^{a}	8.77 ± 3.09^{b}	11.91 ± 4.06^{a}	13.51 ± 3.98^{a}
Lower aquatic sources	$\delta^{13}C$ (‰)	−25.26 ± 3.31^{a}	−26.47 ± 4.23^{a}	−26.49 ± 3.06^{a}	−25.22 ± 2.89^{a}	−26.94 ± 5.32^{b}	−26.45 ± 2.82^{b}
	$\delta^{15}N$ (‰)	8.04 ± 2.06^{b}	23.11 ± 9.14^{a}	8 ± 5.89^{b}	5.56 ± 2.01^{c}	18.44 ± 8.26^{a}	8.68 ± 5.4^{b}
Macroinvertebrates	$\delta^{13}C$ (‰)	−23.91 ± 3.72^{a}	−27.39 ± 4.83^{b}	−28.69 ± 2.53^{b}	−24.02 ± 2.97^{a}	−26.10 ± 3.83^{b}	−28.28 ± 4.64^{c}
	$\delta^{15}N$ (‰)	9.36 ± 3.00^{b}	11.72 ± 4.23^{a}	12.56 ± 6.42^{a}	6.81 ± 2.77^{b}	9.00 ± 3.54^{a}	7.54 ± 5.69^{b}
Fish	$\delta^{13}C$ (‰)	−23.91 ± 2.37^{a}	−25.56 ± 1.69^{b}	−26.80 ± 3.10^{c}	−22.63 ± 2.11^{a}	−24.29 ± 2.07^{b}	−25.36 ± 3.77^{c}
	$\delta^{15}N$ (‰)	11.08 ± 2.97^{b}	14.78 ± 3.42^{a}	10.72 ± 3.66^{b}	9.21 ± 2.67^{b}	12.43 ± 3.54^{a}	8.33 ± 3.10^{c}

Subscripts a, b, and c denote the significant differences in $\delta^{13}C$ and $\delta^{15}N$ isotopic ratios among different seasons according to ANOVA.

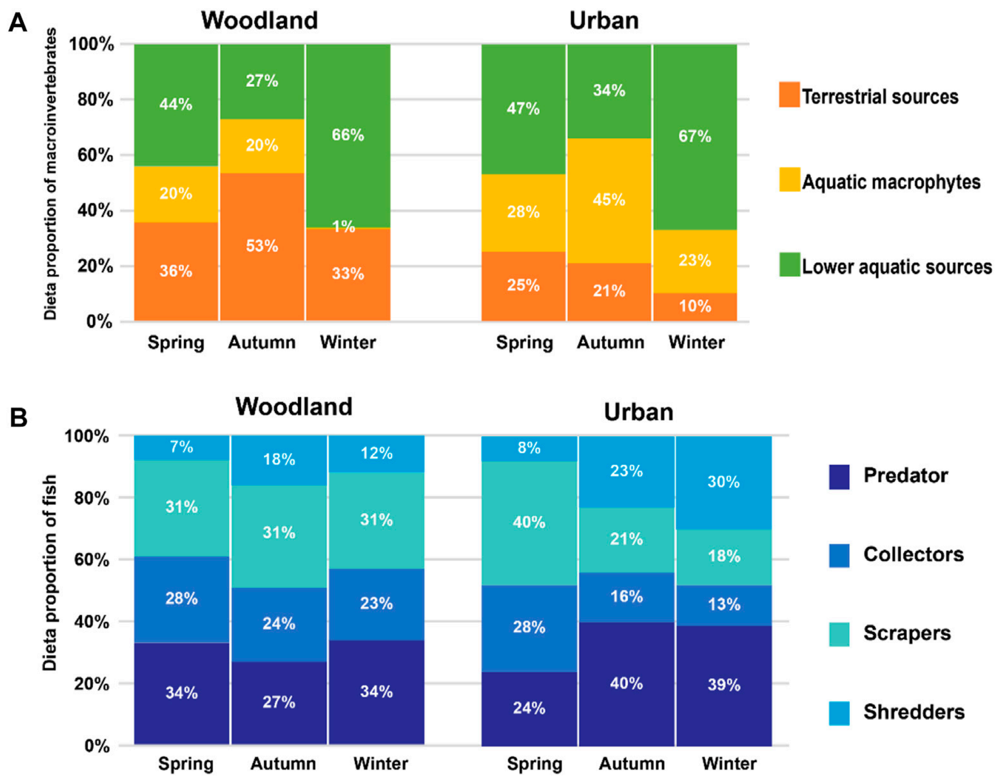

FIGURE 3
The proportions of dietary sources for **(A)** macroinvertebrates and **(B)** fish at woodland and urban sites in spring, autumn, and winter.

($F_{2,12}$ = 3.403, p = 0.067) or SDNND ($F_{2,12}$ = 1.498, p = 0.263) among the seasons (Table 3).

Since there were no significant seasonal differences in the food web metrics at urban sites, we only analyzed the correlations between water quality parameters represented by the principal component 1 (PC1) and food web metrics at woodland sites. PC1 explained 36.6% of the variation in the physical and chemical parameters, and TDC and TDN were the strongest

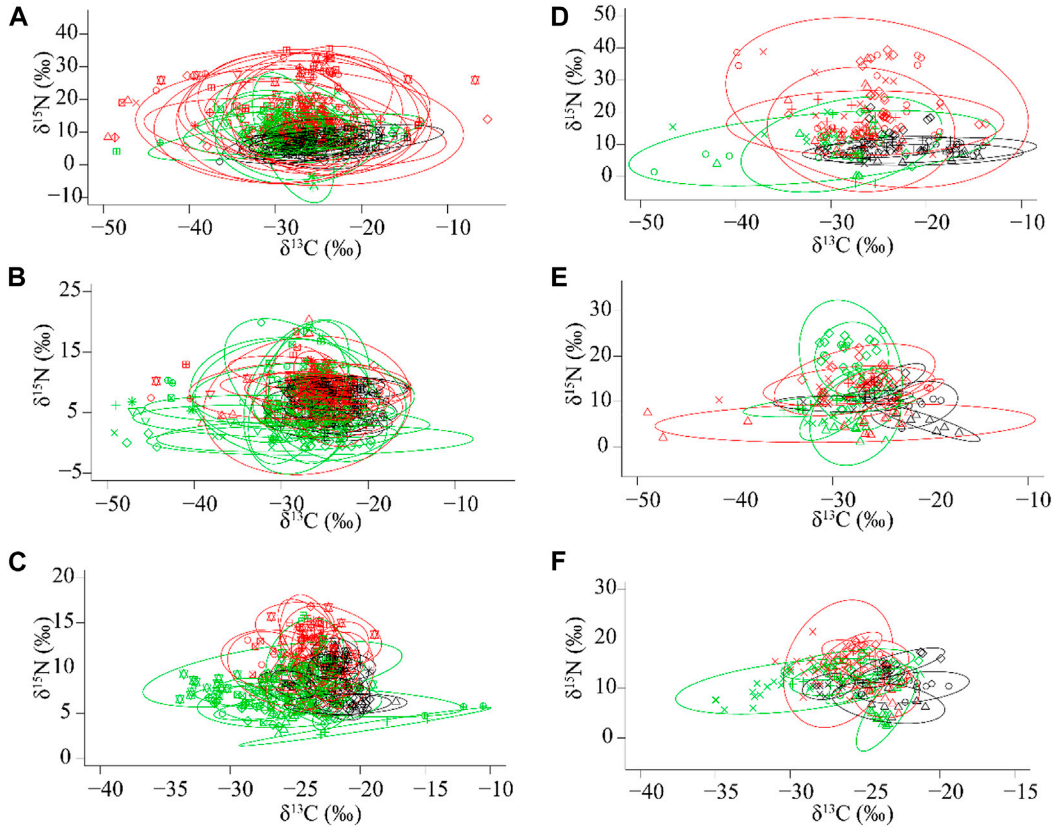

FIGURE 4
The isotopic variances illustrated by standard ellipse area (SEA$_c$, ‰^{2}) for **(A)** basal resources, **(B)** macroinvertebrates, **(C)** fish at woodland sites, and **(D)** basal resources, **(E)** macroinvertebrates, **(F)** fish at urban sites. Black, red, and green ellipses and symbols represented spring, autumn, and winter, respectively.

positive and negative contributors to the PC1 axis, respectively (Figure 5; Supplementary Table S1). PC1 was negatively correlated with the SEA$_c$ of fish, macroinvertebrates, and basal resources (Table 4).

Discussion

Combined effects of land use and seasonality on isotopic signatures

Factors that influence the carbon fixation of primary producers, such as water temperature, eutrophication, pH, or the dissolved CO_2, can be important determinants of their $\delta^{13}C$ values (Finlay, 2001; Chappuis et al., 2017). Lower water temperature in winter could inhibit the respiration of primary producers, which would excrete less ^{12}C (Finlay and Kendall, 2007; Kendall et al., 2008). A decrease in the productivity of primary producers would cause them to sequester less ^{13}C-enriched CO_2 during carbon fixation (Chappuis et al., 2017; Guiry, 2019), and ultimately contribute to the lower $\delta^{13}C$ values in aquatic macrophytes.

In spring, dissolved CO_2 was absent at pH greater than 8, and became available when pH was between 7 and 8 in autumn and winter (Table 1; Chappuis et al., 2017). When there was ample dissolved CO_2 available for photosynthesis, the $\delta^{13}C$ values of aquatic macrophytes decreased when the TDC decreased from spring to autumn and winter (Supplementary Tables S1, S2). In our study area, more than 70% of the runoff was concentrated from July to October (Li et al., 2017; Yuan et al., 2017). Urbanization, together with the increased runoff during the high flow season, could bring more pollutants from the uplands to the river channel, which was reflected in the increased $\delta^{15}N$ values within food webs (Paul and Meyer, 2001; Loomer et al., 2015; Guiry, 2019; Allan et al., 2021). Therefore, in autumn (the high flow season), $\delta^{15}N$ values of lower aquatic sources, including seston and FBOM, increased significantly compared to spring, but then decreased significantly when the runoff was low with fewer nutrients inputs in winter (Table 2). The $\delta^{15}N$ values of aquatic macrophytes increased

TABLE 3 The community-level metrics at woodland and urban sites in spring, autumn, and winter.

Metrics	Woodland			Urban		
	spring	autumn	winter	spring	autumn	winter
NR	2.31 ± 1.09^b	6.09 ± 1.95^a	5.24 ± 2.65^a	2.25 ± 0.74^a	7.25 ± 3.67^a	5.78 ± 3.4^a
CR	5.55 ± 1.67^a	3.76 ± 1.47^a	4.62 ± 2.63^a	1.9 ± 0.92^a	2.54 ± 1.5^a	4.19 ± 1.65^a
TA	3.08 ± 2.12^a	9.35 ± 4.52^a	6.91 ± 8.17^a	0.87 ± 0.65^a	6.94 ± 7.75^a	9.99 ± 11.72^a
CD	2.40 ± 0.56^a	2.96 ± 0.75^a	2.95 ± 1.17^a	1.16 ± 0.23^a	2.97 ± 1.44^a	2.99 ± 1.38^a
MNND	2.93 ± 0.67^a	4.12 ± 1.07^a	3.58 ± 1.7^a	1.58 ± 0.34^a	4.02 ± 1.91^a	4.14 ± 2.08^a
SDNND	1.29 ± 0.97^a	1.04 ± 0.67^a	1.69 ± 0.97^a	0.26 ± 0.06^a	0.78 ± 0.83^a	0.79 ± 0.44^a

Subscripts a and b denote the significant differences in those metrics among the seasons according to ANOVA.

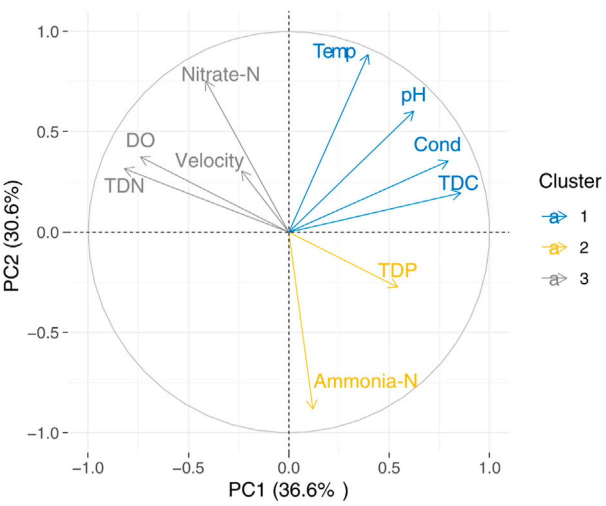

FIGURE 5
Principal component analysis of water quality parameters at woodland sites in spring, autumn, and winter. Temp and Cond are water temperature and conductivity, respectively.

from spring to autumn and winter at both urban and woodland sites, where ^{15}N may accumulate throughout the seasons which could be due to their strategic macrophyte absorption (Chappuis et al., 2017).

Together with residential activities, hydrological regimes among seasons could influence the isotopic compositions of basal resources as well as consumers by altering the water temperature, water discharge, nutrient transport (especially those that are terrestrial-derived), and primary productivity (Bunn et al., 2003; Warry et al., 2016; Guiry, 2019). The differences in consumers' isotopic compositions among seasons were consistent with those of basal resources, which has implications for the bottom-up control of energy flow (Table 2; Shurin et al., 2012). The isotopic compositions of aquatic organisms provide an excellent indicator not only for the presence of human-induced disturbances, but isotopic compositions can also give information about the effects of seasonal environmental factors on the ecology of important species at each trophic level.

Allochthony vs. autochthony in riverine ecosystems

In stream ecology, how aquatic organisms at different trophic levels utilize allochthonous and autochthonous energy has been an important area of research (Paine, 1980; Brett et al., 2017; Twining et al., 2019). Determining the relative importance of autochthonous and allochthonous energy sources can improve our understanding of how energy flows within riverine food webs are affected by anthropogenic activities and/or the seasonality of environmental conditions (Brett et al., 2017). As suggested by the flood pulse concept (FPC), hydrologic alterations in the aquatic and terrestrial transition zone could determine the connectivity between river channels and the floodplains, which was observed to be the main factor in the exchange of water, nutrients, sediments, and biota between aquatic and terrestrial ecosystems (Junk et al., 1989; Bartels et al., 2012).

Allochthonous carbon sources, including terrestrial vegetation, insects, and leaf litter, are the primary carbon sources within riverine food webs (Zeug and Winemiller, 2008; Pingram et al., 2012; Moreira-Turcq et al., 2013; Hayden et al., 2016). As a source of nutrients, their supply plays a key role in affecting aquatic food webs by supporting consumers, such as important fish species (Roach, 2013) or aquatic invertebrates specialized in terrestrial carbon sources (Zheng et al., 2018). Accordingly, we anticipated that in the low flow or baseflow season, autochthonous carbon sources contributed the most to consumers, while allochthonous carbon would contribute the most during the high flow season (Wang et al., 2021). Therefore, it was not surprising that the relative contribution of terrestrial sources to macroinvertebrates at woodland sites was higher than

TABLE 4 Pearson correlation coefficients between water quality parameters represented by the principal component 1 (PC1) and food web metrics at woodland sites.

	PC1	SEA_c of fish	SEA_c of macroinvertebrates	SEA_c of basal resources	NR	CR	TA	CD	MNND	SDNND
PC1	1	−0.37*	−0.53**	−0.63**	0.17	−0.59**	−0.45**	−0.37*	−0.43*	−0.04
SEA_c of fish		1	0.36*	0.40*	−0.27	0.09	−0.02	−0.11	−0.06	−0.15
SEA_c of macroinvertebrates			1	0.08	−0.11	0.18	0.06	0.07	0.07	0.03
SEA_c of basal resources				1	−0.21	0.468**	0.32	0.22	0.32	−0.07
NR					1	−0.14	0.16	0.57**	0.41*	0.43*
CR						1	0.86**	0.72**	0.78**	0.01
TA							1	0.82**	0.92**	−0.03
CD								1	0.92**	0.37*
MNND									1	−0.003
SDNND										1

**and * indicate significance levels of 0.01 and 0.05, respectively.

allochthonous sources in autumn at the end of the high flow season by more than 50%, while terrestrial sources contributed around 30% in spring and winter during baseflow and low flow (Figure 3A).

However, at urban sites, allochthonous carbon contributed much less (around 20%) to macroinvertebrate diets than autochthonous carbon in autumn (Figure 3A). In urbanized rivers, the impervious surface cover, such as dams, weirs, or levees, fragment longitudinal (upstream to downstream) and lateral (river to floodplain) connectivity (Grill et al., 2019; Tickner et al., 2020). The impervious surface cover is the main contributor to the loss of connectivity because they usually alter the water flow which usually travels longitudinally and laterally (Humphries et al., 2014; Jones et al., 2019). Our results could be attributed to the riverbank being concreted at urban sites, which is a common practice globally (Figure 2E; Boggs and Sun, 2011; Valle Junior et al., 2015; Shen et al., 2021). Unlike woodland sites, the river channel was disconnected from the floodplain at urban sites, the terrestrial sources in the high flow season were not fully available to consumers, and thus terrestrial sources contributed much less than autochthonous carbon throughout the year. Our results indicated the combined effects of the impervious surface cover at urban rivers and increased water discharges in high flow season on the relative importance of allochthonous and autochthonous carbon to consumers.

Whether allochthonous carbon is readily consumed depends on the capacity of aquatic consumers to modify their diets and synthesize the new biomass in consumers (Brett et al., 2017). As allochthonous DOC (dissolved organic matter) moved from upstream to downstream, it would be dissected and degraded via the microbial loop and then readily bioavailable to consumers (Brett et al., 2012; Roach, 2013; Baldwin et al., 2016). Those bioavailable carbons that originated from allochthonous carbon could be deposited in the sediment as FBOM or floated in the water column as seston. Technically, allochthonous carbon means sources transported from elsewhere into a particular system (Lau et al., 2009; Brett et al., 2017), but we put seston and FBOM into the category of autochthonous carbon (lower aquatic sources) simply because we sampled them within the river channel, so lower aquatic sources contributed more than 60% to consumers and became the main subsidies at urban sites in the following low flow season (Figure 3A).

When there were fewer terrestrial inputs at urban sites in high flow season, terrestrial nutrients obtained at woodland sites in high flow season can be transported to urban sites downstream via a longitudinal connection (Vannote et al., 1980; Humphries et al., 2014; Ye et al., 2017) and be transformed into aquatic carbon sources at urban sites in the next base flow and low-flow seasons. Therefore, high levels of lateral and longitudinal connectivity along the river networks provided significant opportunities for the exchange and transport of nutrients throughout the high and low flow seasons.

Community-level metrics

How land use and seasonality together influence the food web structure can also be reflected in community-level metrics. Those metrics reflected how the energy flowed to higher trophic levels within the riverine food webs throughout the year. A similar niche width (CR, $\delta^{13}C$ range) within riverine food webs among seasons indicated that multiple basal resources with varying $\delta^{13}C$ values were available to consumers (Wang et al., 2021). The availability of multiple basal resources could also account for a similar trophic diversity (TA and CD) and trophic redundancy (MNND) within riverine food webs among seasons at both woodland and urban sites (Wang et al., 2021).

At woodland sites, the highest niche space (SEA_c, standard ellipse area) of basal resources in autumn proved that the allochthonous carbon was the main subsidy during the high flow season, which could have increased trophic length (NR, $\delta^{15}N$ range) in autumn. When a massive amount of terrestrial carbon was transported into river channels in a short period via increased discharge during the high flow season, the significantly increased niche space (SEA_c) of basal resources during the high flow season could, in turn, lead to the increased niche space (SEA_c) of consumers. When the high flow season ended, much fewer terrestrial nutrient inputs led to decreased niche space (SEA_c) of basal resources in winter, reaching a level close to that of the spring. We confidently suggest that seasonal dynamics of terrestrial subsidies due to the seasonal flooding were the main contributor to the dramatically altered isotopic niche of basal resources. The stable isotopes of consumers often reflect their diets over a long period (Nielsen et al., 2018). The niche space (SEA_c) of macroinvertebrates reached a peak in winter, which indicated their dietary preferences for lower aquatic sources in winter when there was much less primary production and terrestrial inputs. This peak further indicated that lower aquatic sources, especially dissolved organic matter, originated from terrestrial inputs during the high flow season and continued to support the growth of those aquatic insects in winter. Compared to macroinvertebrates, the niche space (SEA_c) of fish were similar in autumn and winter, which indicated that they have a capacity to buffer the disturbances from the massive terrestrial inputs due to their feeding strategies (Price et al., 2019; Wang et al., 2021). The seasonal dynamics of niche space illustrated how the energy flowed in natural riverine ecosystems. Increased water flows during the high flow season resulted in periodic exchanges of nutrients between river channels and floodplains, and ultimately altering the food web structure in riverine ecosystems.

However, it was a different scenario at urban sites. Communities of consumers at urban sites were less affected during the high flow season, as indicated by the niche space (SEA_c) of consumers, which showed no differences among seasons although the niche space (SEA_c) of basal resources was highest in autumn. In the high flow season, when river channels were disconnected from their floodplains at urban sites, the restriction of water flow could not only reduce the terrestrial nutrient inputs (e.g., terrestrial vegetation) but also potential pollution inputs in the riparian zone, which could significantly alter the niche space (SEA_c) of basal resources (as shown at woodland sites), the $\delta^{15}N$ values of consumers (Loomer et al., 2015; Price et al., 2019), and the niche space (SEA_c) of consumers.

Our results suggested that the loss of lateral connectivity could cut off excessive terrestrial inputs at urban sites, and consumers' niche space (SEA_c) was much less affected compared to woodland sites. Also, the combined effects of impervious surface cover in urban areas and altered hydrological regimes due to increased water discharge on the food web structure were represented by those community-level metrics.

It should not be ignored that longitudinal connectivity is essential for the transport of terrestrial carbon sources from upstream woodland sites to downstream urban sites. In our study area, there was one small dam at an urban site (Figure 2D) located in Xixia County, and the dam failed to cut off the water flows longitudinally during the high flow season. Given the role of allochthonous carbon in supporting biological communities in riverine ecosystems, it does not necessarily mean that we should tear down the impervious surface cover in urbanized areas to diminish the impact of urbanization on decreased terrestrial inputs. Alternative measures could be taken in urban areas, such as safeguarding and restoring the longitudinal connectivity, and informing balanced watershed management for the food web structure in urban areas (Auerbach et al., 2014; Reid et al., 2019).

Conclusion

Based on the diet tracing analysis via stable isotopes, a qualitative description of how hydrological regimes among seasons altered the relative contribution of allochthonous and autochthonous carbon to consumers can be applied to natural riverine ecosystems as well as those in urban areas. Niche space (represented by $\delta^{13}C$ and $\delta^{15}N$) occupied by basal resources at both woodland and urban sites was significantly higher in the high flow season. Unlike woodland sites, the impervious surface cover at urban sites disconnects river channels from their floodplains and can cut off the influx of terrestrial carbon sources during the high flow season. This disconnection can cause a lack of seasonal differences in the niche space occupied by aquatic consumers at urban sites.

During the high flow season, increased water discharge can facilitate the acquisition of terrestrial carbon sources for consumers at woodland sites via the lateral connectivity between the river channel and the riparian zone. The terrestrial carbon sources transported downstream can continue to subsidize consumers at downstream urban sites during the low flow season following the high flow season. Our temporal food web study provided a better understanding of the energy transfer and nutrient transport among seasons at urban and woodland rivers and illustrated the combined effects of urbanization and the seasonality in environmental conditions on food web structure in riverine ecosystems. Our findings highlighted the role of allochthonous carbon sources in supporting biological communities and the importance of lateral and longitudinal connectivity in river ecosystems. Such advances in our understanding is critically important for river restoration and management that aims to better protect riverine biodiversity from intensive urbanization along the river network.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

Conceptualization, YW; methodology, YW; software, YW; validation, YW; formal analysis, YW; investigation. YW; resources, YW; data curation, YW; writing—original draft preparation, YW; writing—review and editing, XT, SL, QZ; visualization, YW; supervision, XT, QZ; project administration, XT, QZ; funding acquisition, QZ. All authors have read and agreed to the published version of the manuscript.

Funding

This study was financially supported by the National Natural Science Foundation of China (NSFC grant no. 31720103905, 32030069).

Acknowledgments

We are grateful to Junwei Wei and Ke Zhang from Wuhan Botanical Garden, Chinese Academy of Sciences, Yintao Jia, Kang Chen, Zhengfei Li, Yihao Ge, Zhenyuan Liu, Yang Liu, Ren Zhu, Yihang Jiang, Miss. Yuhan Liu, Miss. Bing Li from the Institute of Hydrobiology, Chinese Academy of Sciences, for their assistance in the field and taxonomy in the lab. We are also grateful to Hongran Li and Miss. Yuxi Tu for their help in GIS plotting. We also thank Russell Doughty for editing this manuscript.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.969980/full#supplementary-material

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Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 23 September 2022
DOI 10.3389/fevo.2022.976448

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to
Conservation and Restoration Ecology,
a section of the journal
Frontiers in Ecology and Evolution

RECEIVED 23 June 2022
ACCEPTED 22 August 2022
PUBLISHED 23 September 2022

CITATION
Zhou Z, Mo L, Li D, Zeng W, Wu H, Wu Z and Huang J (2022) Comparative transcriptomics analyses of chemosensory genes of antenna in male red swamp crayfish *Procambarus clarkii*. *Front. Ecol. Evol.* 10:976448. doi: 10.3389/fevo.2022.976448

Comparative transcriptomics analyses of chemosensory genes of antenna in male red swamp crayfish *Procambarus clarkii*

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The red swamp crayfish, *Procambarus clarkii*, is a globally invasive species and has caused huge damage to aquaculture, biodiversity, and ecology worldwide. Antenna-expressed receptors are important for *P. clarkii* to detect chemosensory cues for mate attraction. In this study, we tested the behavior of male *P. clarkii* to the conditioned water from female *P. clarkii* during the mating and non-mating periods, and performed RNA sequencing to investigate the chemosensory-related genes of the antenna of male *P. clarkii*. The results of the behavioral assay have shown that for the female-conditioned water, male *P. clarkii* within the mating period can be significantly attracted, but not during the non-mating period. This suggested that the expressions of chemosensory-related genes in the antenna of male *P. clarkii* may change significantly with mating seasonal variation. Antenna transcriptomes found that a total of 59,218 unigenes with an average length of 1,056.41 bp, and 4,889 differentially expressed unigenes (DEGs), among which 2,128 were upregulated, while 2,761 were downregulated were obtained. A total of 12 upregulated and nine downregulated DEGs were associated with chemical reception, including four ionotropic receptors (IRs) or ionotropic glutamate receptors (iGluRs), eight G-protein-coupled receptors (GPCRs), five transient receptor potential channels (TRP channels), one sodium–calcium exchanger, one isomerase, and two uncharacterized proteins (chemosensory proteins-like, CSPs). CSPs were preliminarily classified as pheromone receptors in the antenna of male *P. clarkii*. Furthermore, the calcium transduction-related pathways may play an important role in the sex pheromone reception of the male *P. clarkii*'s antenna. The results of quantitative real-time reverse transcriptase PCR (RT-qPCR) showed that the trends of expression of eight selected unigenes were consistent with RNA-Seq results. Our results provide more comprehensive data for chemical communication mechanisms after *P. clarkii* enter the mating period and eventually would develop better control strategies in further.

KEYWORDS

***Procambarus clarkii*, antenna, transcriptome, RNA-Seq, chemosensory, chemical communication**

Introduction

Chemoreception is the dominant sensory modality for most crustaceans due to the complex aquatic environment (Harzsch and Krieger, 2018), which is the base of crustaceans for their daily activities, such as searching for food, reproduction, and communication (Blinova and Cherkashin, 2012). Previous studies suggest that the antenna is one of the predominant chemosensory organs of most crustaceans (Harzsch et al., 2011; Waldrop, 2013; Urbschat and Scholtz, 2019). Crustaceans receive chemical signals through several parallel channels, which can be summarized into two modes of “olfaction” and “distributed chemoreception” (Schmidt and Mellon, 2011). “Olfaction” is mediated by unimodal olfactory sensilla called “aesthetasc,” which are only present on the first antenna of the crustaceans (Harzsch and Krieger, 2018). Aesthetascs are innervated by olfactory sensory neurons (OSNs) (Derby et al., 2016). “Distributed chemoreception” is mediated by various bimodal sensillae in the first antenna (Solari et al., 2017), mouthparts (Garm et al., 2003; Garm et al., 2004), chelipeds, and walking appendages (Altner et al., 1983; Schmidt and Gnatzy, 1984). It is innervated by both chemoreceptor neurons (CRNs) and mechanoreceptor neurons (MRNs). However, there is a gap in the data for chemoreceptors of crustaceans. So far, the predicted chemosensory-related proteins in crustaceans include ionotropic receptors (IRs), ionotropic glutamate receptors (iGluRs), G-protein-coupled receptors (GPCRs), transient receptor potential channel (TRP channel), chemosensory proteins (CSPs), and gustatory receptor (GRs) (Eyun et al., 2017; Kozma et al., 2020a,b). Identification of candidate chemosensory-related genes helps to better understand the molecular basis of chemoreception in pests and eventually develop better control strategies (Wang et al., 2014; Gonzalez et al., 2021).

The red swamp crayfish, *Procambarus clarkii* (Girard, 1852), has its origins in North-eastern Mexico and South America (Shen et al., 2014), and was introduced to China from Japan during the 1930s (Shen et al., 2020). At present, it is an economically important animal (Liu et al., 2021). However, invasive crayfish *P. clarkii* into the natural ecosystems of China has caused huge losses to agriculture, biodiversity, and aquaculture. Moreover, *P. clarkii* is a carrier of the white spot syndrome virus (WSSV) and parasites, whereby it may cause infection and death of other commercial shrimps in case of spread without effective control (Zhu et al., 2009). Therefore, effectively and specifically controlling *P. clarkii* requires more attention. In recent years, control of invasive species using sex pheromone has been adopted for other species of insects (Gherardi et al., 2011; Keller-Costa et al., 2014; Johnson et al., 2015). Previous studies confirm that males can detect sex pheromones through their chemosensory organ uniaxially, and then search for the signal source or perform courtship behaviors (Oyama et al., 2020). In addition, the suitable reproduction temperatures of *P. clarkii* are ranging from 21 to 25°C, and it reduced mating behavior below 15°C (Jin et al., 2019; Dong et al., 2020). The male *P. clarkii* recognizes female mating receptivity by detecting the urinary components of females (Kubec et al., 2019). However, little is known about the chemosensory mechanism of action of these essential chemicals (such as sex pheromone components) in male *P. clarkii*.

Like other crustaceans, the *P. clarkii*’s antenna is a critical chemosensory organ that perceives and locates chemical signals or pheromones released by their mates (Breithaupt, 2011). Although the candidate chemosensory genes of the lateral flagellum of the antennule in *P. clarkii* were reported (Kozma et al., 2020a), the chemosensory genes in the antenna are still unknown. Therefore, to further understand the chemical communication mechanism through chemosensation in male *P. clarkii*, we performed the behavioral assays of male *P. clarkii* on the crude conditioned water from female *P. clarkii* (in the mating period, MP) during the mating (MP) and non-mating period (NMP), and collected the antenna of the male *P. clarkii* in the MP as the experimental group and the NMP as the control group. Subsequently, next-generation sequencing (NGS) and assembly were used to obtain a male antenna transcriptome of *P. clarkii* in MP vs. NMP. Our findings may help better understand the mechanisms of chemosensory responses in *P. clarkii*, and provide a solid foundation for further studies on the relationship between chemosensory-related genes and semiochemicals.

Materials and methods

Animal collection and preparation

Procambarus clarkii (400 males and 20 females) was purchased from Guilin city, Guangxi province, China in

August 2020. The average weight of the mature *P. clarkii* was 16.22 ± 1.82 g. The length of the antenna was longer than 5 cm. A total of 20 *P. clarkii* were cultured in one water tank and fed two times a day on artificial food throughout the experimental period (Li et al., 2012). A total of 20 mature female *P. clarkii* were fasted for 48 h and placed in a water tank with 1 L of ultrapure water (for crude conditioned water collections) for further cultivation for 24 h. The feeding temperature of female *P. clarkii* was between 23 and 25°C. The conditioned water of female *P. clarkii* was centrifuged for 20 min (8000 rpm, 4°C), and removed the insoluble substances to obtain the crude conditioned water (collected in the mating period, for behavior assay). The crude conditioned water samples were stored at −80°C until further behavior assay experiment.

All male *P. clarkii* had no contact with female crayfish or pheromones during the feeding process. We randomly divided male *P. clarkii* into two following groups: 200 individuals were fed until January 2021 (NMP, water temperature <15°C) and another 200 individuals were fed until April 2021 (the initial stage of the MP, water temperatures >22°C).

The behavioral assay of male *Procambarus clarkii* during the non-mating period and mating period

The behavioral assays were performed using the Y-maze (self-made with glass) to determine the sensitivity of male *P. clarkii* to the crude conditioned water (**Figure 1**). There was a drain hole at the start arm of the Y-maze device to keep the water depth at 5 cm. Fifty male *P. clarkii* were tested for behavioral assays in NMP (in January) and MP (in April), respectively. The procedures of behavioral assays were performed as follows: First, 500 ml of ultrapure water was poured into Y-maze and regulated the water flow rate (20 ml/min) of infusion tubes to the same velocity by flow meters. Thereafter, cylindrical sponge blocks with stimulus (1 ml of crude conditioned water, experimental group) and ultrapure water (1 ml, control group) were placed in the end arm, respectively. Finally, the male *P. clarkii* was put into the start arm and observed the arm entry by *P. clarkii*. Each animal was tested for the crude conditioned water only once. At the end of each operation, the Y-maze would be emptied and wiped with alcohol on the surface to prevent leftover interference from the last operation. Each assay lasted 5 min. If no choice was made for more than 5 min, the male *P. clarkii* would be counted as unresponsive individuals, which would be excluded from the analysis. After every five *P. clarkii* were tested, the positions of the experimental group (crude conditioned water) and the control group (ultrapure water) were switched to prevent directional bias. The data were calculated using the Chi-squared test (Barnard et al., 2016) in SPSS v 25.0 at a significant level of $P < 0.05$.

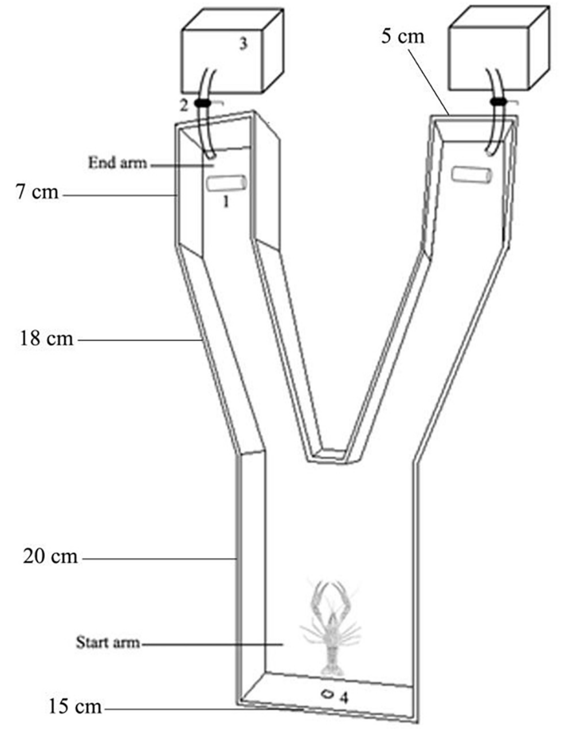

FIGURE 1
The behavioral device: Y-maze. The height of Y-maze is all 5 cm. The Y-maze was symmetrical on both sides. 1: cylindrical sponge block, 2: flow meter, 3: water box, and 4: drain hole.

Total ribonucleic acid extraction and detection

To reveal the difference in male *P. clarkii* responses to female-conditioned water, the antenna transcriptomics of male *P. clarkii* was performed in NMP and MP groups using RNA-Seq technology. When the behavior assay was done, the whole antenna with the hard cuticle of male *P. clarkii* (150 individuals in NMP and MP, respectively) was collected and transferred into sterile 15 ml centrifuge tubes without RNase and DNase (Corning, United States). In order to obtain enough RNA, 50 pairs of male antennae were combined into one sample. NMP and MP groups had three replicate samples, respectively. The samples of NMP and MP groups were frozen in liquid nitrogen and stored at −80°C until the extraction of total RNA. The library construction and sequencing of transcriptomes were performed by Major Technology Co. Ltd. (Shanghai, China). Total RNA extraction was performed using the TRIzol reagent (Invitrogen, Shanghai, China). Both concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Invitrogen, MA, United States). The degradation of extracted RNA was detected on 1% agarose gels and RNA integrity number (RIN) was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies, United States).

Library construction and sequencing

mRNAs were enriched using magnetic beads with Oligo (dT) and randomly fragmented using fragmentation buffer. Under the action of reverse transcriptase, the fragmented mRNAs were used as templates for first-strand cDNA synthesis using random hexamer primers. Subsequently, second-strand cDNA was synthesized using DNA polymerase I and RNase H. We added an end-repair mix (including the end-repair enzyme mix and end-repair buffer) to patch the cohesive ends of the double-strand cDNA, followed by the addition of tail and sequencing adapters. Subsequently, cDNA was amplified by PCR. A total of six cDNA libraries were obtained after purifying the amplification products with AMPure XP beads. Thereafter, QuantiFluor dsDNA System and Quantus^{TM} Fluorometer (Promega, Madison, WI, United States) were used to detect the concentration and inter size of the libraries, respectively. The effective concentration of the libraries was accurately quantified qPCR (quantitative polymerase chain reaction, effective concentration >2 nM) using the Qubit RNA Assay Kit in the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States). Finally, the constructed paired-end libraries were sequenced on the Illumina HiSeqXten/NovaSeq 6000 Sequencing Platform.

de novo assembly and annotation

Using SeqPrep[1] and Sickle,[2] all clean reads from NMP and MP groups were obtained by filtering the sequencing adapters, poly-N, and the low-quality sequences (quality value, QV <20) from raw reads. The assembly of two sets of antenna was combined together and performed by using RNA-Seq *de novo* programs Trinity software (v2.8.5) (Liu et al., 2021). The completeness of the assembly was evaluated using the BUSCO software (v5.3.2). The assembled *de novo* unigenes were obtained, which were the encoding sequences. The redundancy was filtered using the Cd-hit-EST (Foquet et al., 2021). The Transrate (v1.0.3) was used to map reads to contigs and inspect the alignments (Marx et al., 2021).

The unigene sequences were compared using six databases, including the National Center for Biotechnology Information (NCBI) Non-Redundant Protein Sequence Database (Nr),[3] Protein family (Pfam),[4] Gene Ontology (GO),[5] Swiss-Prot,[6] Cluster of Orthologous Groups of proteins (COG),[7] and Kyoto Encyclopedia of Genes and Genomes (KEGG).[8] Functional annotation was processed using the Blast2GO software (Conesa et al., 2005).

Differentially expressed unigene enrichment analysis

The Bowtie software (Langmead et al., 2009) was used to compare clean reads with the unigene library and on combining with RSEM (Li and Dewey, 2011), the expression levels were estimated. The expression of unigenes was calculated using FPKM (fragments per kilobase per million) (Mortazavi et al., 2008) between six libraries. Then, the differentially expressed unigenes (DEGs) were analyzed using the DESeq2 software (v1.24.0) based on the false discovery rate (FDR) correction with Benjamini and Hochberg (BH) method (Varet et al., 2016). The resulting P-values were adjusted using the FDR. The DEGs were filtered using the threshold of FDR <0.05 and $|\log2(\text{fold change})| \geq 1$ (Ma et al., 2020). Subsequently, GO and KEGG pathway enrichment analyses were conducted based on Fisher's precision probability test (BH method, P-adjust <0.05) using the Goatools software (Klopfenstein et al., 2018). All DEGs were mapped to terms in the KEGG and GO databases using the BLAST algorithm with an E-value of $\leq$1E-5, and the significantly ($P < 0.05$) enriched KEGG and GO terms in DEGs were identified compared with the transcriptome background. The data of pathways were analyzed on the online tool of Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/) (Ren et al., 2022).

Real-time quantitative polymerase chain reaction

To verify the results of our sequencing analyses, we selected eight sensory-related DEGs with high expressions in MP or NMP groups (**Supplementary Table 1**) from the male antenna of *P. clarkii* for quantitative real-time reverse transcriptase PCR (RT-qPCR) analysis. The total RNA was reversely transcribed into first-strand cDNA using the PrimeScript^{TM} first-strand cDNA Synthesis Kit (TaKaRa, Shanghai, China), following which the newly synthesized cDNA was used as the template for RT-qPCR. Specific primers were designed using the Primer 5.0 software with a concentration of 10 μmol/L. The primer sequences of eight DEGs used for RT-qPCR are shown in **Supplementary Table 1**. The RT-qPCR was performed on an MA-6000 real-time fluorescence quantitative PCR instrument (Molarray, Jiangsu, China). The RT-qPCR reactions (20 μl) contained 1 μl of cDNA, 0.4 μl of each primer (0.2 μmol/L

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[1] https://github.com/jstjohn/SeqPrep

[2] https://github.com/najoshi/sickle

[3] www.ncbi.nlm.nih.gov/

[4] http://pfam.xfam.org/

[5] http://www.geneontology.org

[6] http://web.expasy.org/docs/swiss-prot_guideline.html

[7] http://www.ncbi.nlm.nih.gov/COG/

[8] http://www.genome.jp/kegg/

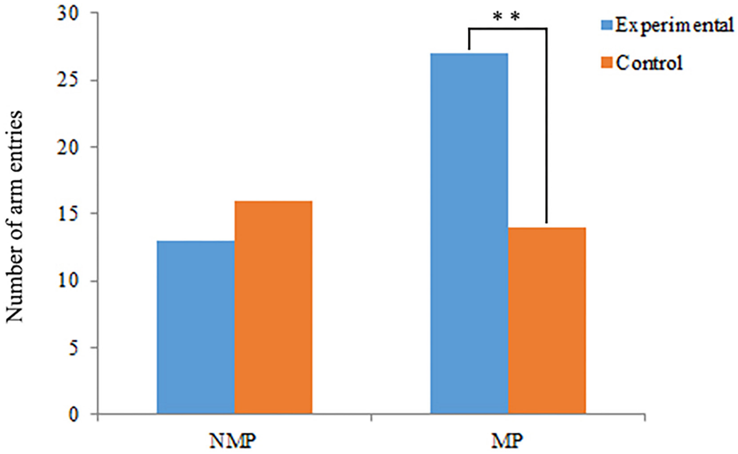

FIGURE 2
Behavioral assays of male *Procambarus clarkii* during the non-mating period (NMP) and mating period (MP). *Represents the significant differences between experimental and control groups ($P < 0.05$, $n = 50$). The numbers of no choice were excluded from the statistical analysis.

in this reaction system), 10 μl of 2 × SYBR real-time PCR premixture (dNTP and buffer), and added the RNase free sterilized ultrapure water. The reaction procedure was as follows: 95°C for 5 min, followed by 40 cycles with 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. To confirm reproducibility, the RT-qPCR reaction for each sample was performed in three technical replicates and three biological replicates based on the MP (>22°C) and NMP (<15°C) groups. The melt curves were used to evaluate the specificity of primers and amplification. All melt curves have a single peak, and PCR efficiency for each primer pair was ranging from 91 to 103%. The level of expression of selected genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and the expression of selected genes was normalized against β-actin expression levels (Yu et al., 2022). Comparative analyses for each target gene among different samples were analyzed using the one-way ANOVA (SPSS, version 25.0).

Results

Behavioral tests of male *Procambarus clarkii* during the non-mating period and mating period

The results of behavioral assays have shown that the conditioned water from female *P. clarkii* can significantly attract the male individuals during the MP ($\chi^2 = 4.12$, $P = 0.04$), but cannot significantly attract the males in NMP ($\chi^2 = 0.31$, $P = 0.58$) (Figure 2 and Supplementary Table 2).

Assembly and splicing

A total of 258.48 million raw reads and 252.98 million clean reads were obtained from six libraries (Supplementary Table 3). The Q20 and Q30 were greater than 97 and 92%, respectively. The GC contents were ranging from 44.38 to 49.05% (Supplementary Table 3). Moreover, all clean reads from NMP and MP groups were combined to *de novo* assemble the unigenes (Table 1 and Supplementary Figure 1). The BUSCO score showed that the transcriptome was with high completeness (single-copy: 91.8%; duplicated: 4.2%). The raw data files have been uploaded to the NCBI sequence read archive (BioProject accession number: PRJNA839237).

Functional annotation and classification of unigenes

To investigate information on gene functions more comprehensively, a total of 57,812 unigenes were annotated using six databases including Nr, Pfam, Swiss-Prot, COG, GO, and KEGG. The results of annotation of the unigenes are as follows: 18,388 (31.81%) unigenes were annotated in Nr; 14,039 (24.28%) in Pfam; 11,986 (20.73%) in Swiss-Prot; 14,479 (25.04%) in COG; 14,129 (24.44%) in GO; and 9,176 (15.87%) in KEGG (Supplementary Figure 3 and Supplementary Table 4). The annotations of unigenes to different databases are shown in

TABLE 1 The length distribution of assembled transcripts and unigenes.

Length (bp)	Transcripts	Unigenes
Total number	78,138	59,218
Max length	34,133	34,133
Min length	201	201
Mean length	1,147.85	1,059.41
N50 length	2,379	2,229
BUSCO score (%)	C: 96 [S: 91.8; D: 4.2]	C: 96 [S: 91.8; D: 4.2]

The “C,” “S,” and “D” in BUSCO score represent the “complete,” “single-copy,” and “duplicate,” respectively.

Supplementary Figure 4. Moreover, a total of 111 unigenes were annotated to chemosensory-related functions, including IRs or iGluRs (43 unigenes annotated), TRP channels (30), CSPs-like (2), GPCRs (17), GR (1), and other olfactory-related proteins (18) (Supplementary Excel Table 1).

Identification and annotation analysis of differentially expressed unigenes

Based on the DESeq2 software, a total of 4,889 DEGs with the FDR <0.05 and $|\log 2$ (fold change) $| \geq 1$ were identified between MP and NMP groups, including 2,128 upregulated and 2,761 downregulated unigenes (Supplementary Figure 4). The fold change (FC) and annotations of all DEGs are shown in Supplementary Excel Table 2. Furthermore, the annotations from the GO analysis of DEGs were classified into 44 GO categories, including BP (18), CC (14), and MF (12). The top three GO terms in CC were cell part (GO:0044464), membrane part (GO:0044425), and protein-containing complex (GO:0032991); in BP were, cellular process (GO:0009987), metabolic process (GO:0008152), and biological regulation (GO:0065007); in MF were, binding (GO:0005488), catalytic activity (GO:0003824), and structural molecule activity (GO:0005198) (Supplementary Figure 5A). Moreover, the KEGG enrichments have shown that the "Ribosome (*P*-value = 5.067E-31, 156 upregulated and 39 downregulated DEGs)" is the most enriched pathway, followed by "Amoebiasis" and "Complement and coagulation cascades" (Supplementary Figure 5B).

Chemosensory-related differentially expressed unigenes and pathway

As shown in Table 2, 21 identified DEGs were related to chemosensory functions. On comparing the MP and NMP groups, nine DEGs were found to be downregulated, while 13 were upregulated, including four IRs or iGluRs, eight G-protein-coupled receptors, five TRP channels, one sodium–calcium exchanger, one isomerase, and two uncharacterized protein (CSP-like). The sequences of these DEGs are shown in Supplementary Excel Table 3. Among these 21 DEGs, the DEG related to sodium–calcium exchanger (solute carrier family, NCX) was involved in the olfactory transduction and calcium signaling pathway (Figure 3).

TABLE 2 Chemosensory or –like regulation-related differentially expressed unigenes (DEGs).

Gene ID	Description	Categories	log2FC	*P*-value
TRINITY_DN21918_c0_g1	Olfactory ionotropic receptor IR93a	IR	−2.306	0.017
TRINITY_DN25415_c0_g2	Ionotropic glutamate receptor subunit	IR	−3.724	4.187E-5
TRINITY_DN20582_c0_g1	Variant ionotropic glutamate receptor	IR	−3.312	3.370E-5
TRINITY_DN21176_c0_g4	Peroxisomal acyl-coenzyme A oxidase 1-like	GPCR	−6.572	0.021
TRINITY_DN521_c1_g2	Metal ion binding*	GPCR	−1.636	2.049E-4
TRINITY_DN1785_c0_g1	Threonine-protein kinase 26-like*	GPCR	−1.247	6.946E-4
TRINITY_DN3158_c0_g2	Crustacean cardioactive peptide receptor, partial	GPCR	−2.250	1.191E-5
TRINITY_DN10908_c0_g3	G-protein coupled receptor GRL101-like	GPCR	−1.762	0.005
TRINITY_DN28245_c0_g1	Transient receptor potential cation channel subfamily A member 1-like isoform X3	TRP channel	−4.519	0.034
TRINITY_DN518_c0_g1	Uncharacterized protein	CSP-like	8.280	6.233E-5
TRINITY_DN7269_c0_g2	Uncharacterized protein*	CSP-like	7.496	1.077E-6
TRINITY_DN16621_c0_g1	Urotensin-2 receptor-like*	GPCR	1.092	0.033
TRINITY_DN22772_c0_g1	G-protein coupled receptor activity	GPCR	1.039	0.010
TRINITY_DN22075_c0_g1	G-protein coupled receptor moody*	GPCR	1.153	0.002
TRINITY_DN14467_c0_g4	Transient receptor potential channel pyrexia OS	TRP channel	3.075	4.122E-4
TRINITY_DN24541_c0_g1	Putative transient receptor potential cation channel subfamily A member 1 isoform X1	TRP channel	1.660	1.279E-4
TRINITY_DN4439_c0_g1	Ankyrin-11-like*	TRP channel	1.605	1.471E-5
TRINITY_DN2621_c1_g1	Transient receptor potential channel pyrexia-like	TRP channel	1.470	3.018E-4
TRINITY_DN5073_c0_g3	Transient receptor potential cation channel pyrexia	TRP channel	2.021	0.01
TRINITY_DN32840_c0_g1	Sodium-calcium exchanger 1*	sodium-calcium exchanger	1.963	2.972E-4
TRINITY_DN4991_c0_g1	Putative E3 ubiquitin-protein ligase TRIM32*	isomerase	2.52	2.847E-6

*Represents the selected DEGs for RT-qPCR. The log2FC < 0 represents the downregulated DEGs, while the log2FC >0 represents the upregulated DEGs.

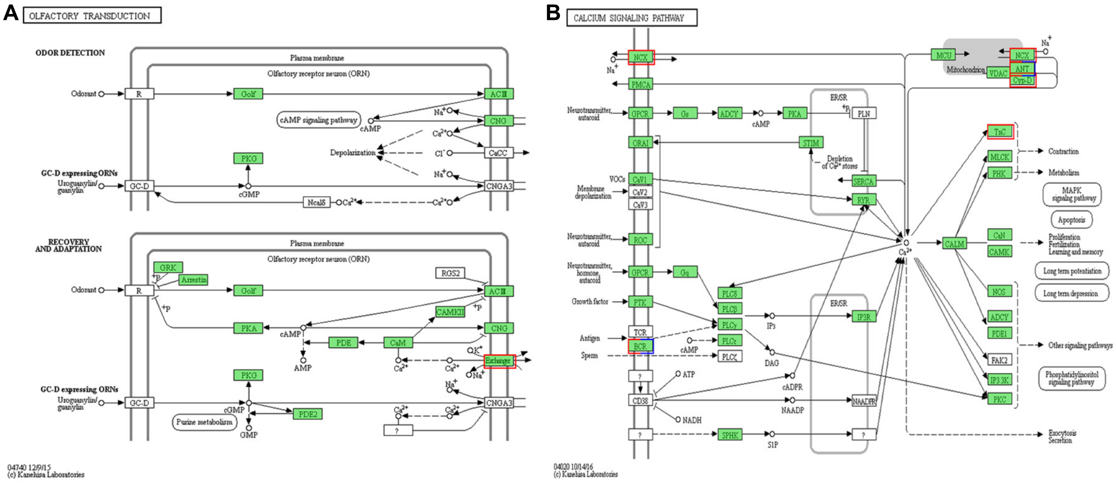

FIGURE 3
The calcium-related differentially expressed unigenes (DEGs) were enriched in **(A)** the olfactory transduction pathway and **(B)** the calcium signaling pathway. The red box represents the upregulated DEGs in mating period (MP) groups. The blue box represents the downregulated DEGs in MP groups, and the box with both red and blue represents that there are both up and downregulated DEGs in MP groups. The green box represents the genes annotated in the pathway without significant difference (Image credits: https://www.kegg.jp/pathway/map=map04740&keyword=olfactory%20transduction and https://www.kegg.jp/pathway/map=map04020&keyword=calcium).

Validation by RT-qPCR analysis

To verify the accuracy of the RNA-Seq data, eight sensory-involved genes were selected from among the DEGs and validated by RT-qPCR analysis. The expression levels of six DEGs were significantly upregulated (GPCR moody, TRIM 32, CSP-like, ankyrin-1, NCX 1, and SSTR 2) in MP groups and two (ATP 1A and STK 26-like) were significantly downregulated in the MP groups. The results of RT-qPCR were consistent with those of RNA-Seq, indicating the reliability of the RNA-Seq data (**Figure 4**, **Supplementary Table 5**, and **Supplementary Excel Table 4**).

Discussion

Numerous behavioral studies indicate that the male *P. clarkii* have "unidirectional receptors" for the sex pheromones released by females during the MP (Monteclaro et al., 2010; Peddio et al., 2019; Oyama et al., 2020). Male individuals can recognize different phases of females by detecting the pheromones and then performing courtship behaviors after checking the sex pheromone levels (Kubec et al., 2019). However, previous research suggests that the frequencies of copulation and oviposition in *P. clarkii* are significantly reduced when the temperature goes below 15°C (Egly et al., 2019). In this study, we found that male *P. clarkii* were attracted to female-conditioned water in MP but not in NMP, which was consistent with previous studies. We speculated that the expression of chemosensory genes in antenna may be altered due to different temperatures and periods. Therefore, we identified the chemosensory genes and pathways in the antenna of *P. clarkii* between MP vs. NMP by RNA-Seq for the first time. However, the information on chemosensory genes was as sparse as expected, presumably since genetic annotation of species closely related to *P. clarkii* is scarce in public genetic databases; in general, the genetic information on chemoreception in crustaceans is lacking. This study is a necessary supplement to the genetic information library for *P. clarkii*.

Procambarus clarkii is one of the main model animals in the study of chemosensory receptors in crustaceans. The previous study had found several types of chemoreceptors in the antennule and legs of *P. clarkii*, including the IRs, iGluRs, TRP channels, and GRs (Kozma et al., 2020a). In our study, we found these four types of chemosensory receptors in the antenna of *P. clarkii* as well, indicating that these chemosensory receptors are not organ-specific, but expressed generally in a variety of sensory organs of *P. clarkii*. Furthermore, we found only one GR in the antenna of *P. clarkii*, which was consistent with the transcriptomic results of the antennule and legs in *P. clarkii* (Kozma et al., 2020a). This suggested that GRs remain low in numbers in *P. clarkii* and other decapod crustaceans. However, compared with the transcriptomic results of antennule and legs in *P. clarkii* (Kozma et al., 2020a), we found that the numbers of TRP channels were more in the antenna than in the two organs (antennule and leg), which we suggested that TRP channels might mediate more chemosensory processes in the antenna of *P. clarkii*. Overall, a lot of research on the chemosensory genes and receptors of crustaceans is still in the recognition stage, and there are still numerous receptor functions that have

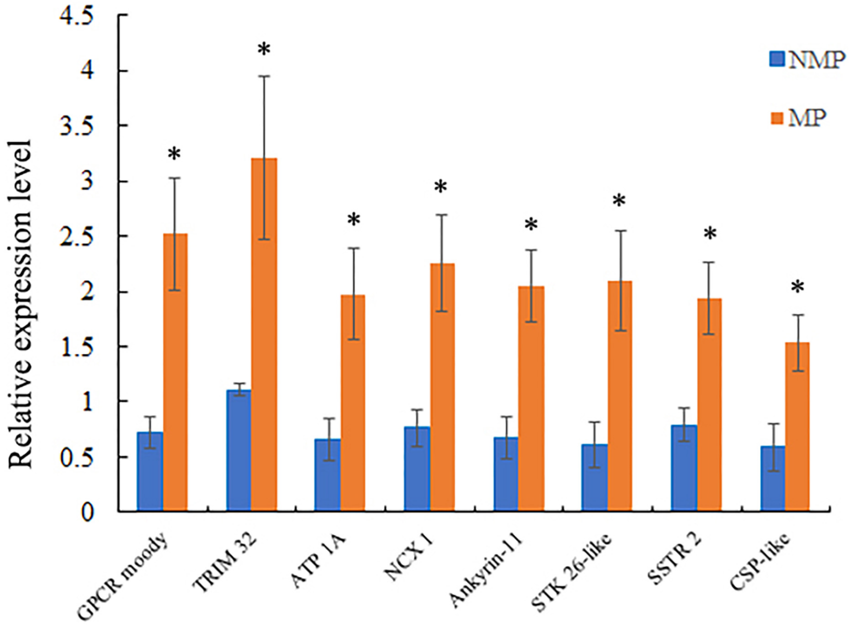

FIGURE 4
Relative levels of selected differentially expressed unigene (DEG) expression by RT-qPCR. Levels were measured using the $2^{-\Delta\Delta CT}$ method; β-actin was the internal normalization control. Data were shown as mean ± SD, $n = 3$; $^*P < 0.05$.

not been studied and reported yet and need more studies to investigate.

Notably, the DEGs related to the TRP channels were typically upregulated. TRP channels are homotetramers or heterotetramers with six transmembrane segments. There are eight subfamilies of TRP channels, including TRPC, TRPA, TRPP, TRPN, TRPV, TRPM, TRPM, and TRPT (Venkatachalam and Montell, 2007; Venkatachalam et al., 2014; Van den Eynde et al., 2021). In this study, six types of TRP channels (TRPA1, Pyrexia TRPA, TRPM2, TRPM3, TRPC4, and TRPV5) were identified. TRP channels participate in many sensory processes, such as vision, olfactory, audition, and temperature sensation, which affect the behaviors of creatures profoundly (Fowler and Montell, 2013). As the climate becomes warmer, more movements are generated by benthos and crayfish (Larson and Magoulick, 2011; Johnson et al., 2014; Wittwer et al., 2018). We speculated that *P. clarkii* could enhance self-abilities for various senses instinctively for environmental adaptation, which include the sex pheromone receptions as well.

Many DEGs have been annotated to uncharacterized proteins in our study, of which two DEGs were annotated to CSPs in other species *via* the Pfam database. The expressions of the two CSPs-like were significantly higher in the MP group than in the NMP group. In addition, the expressions of CSPs were also much higher relative to other unigenes. Previous studies supported that CSPs were sensory-related proteins with pheromone binding function in insects (Bohbot et al., 1998; Jacquin-Joly et al., 2001). Unlike insect-specific proteins (OBPs), CSPs were found in crustaceans such as *Daphnia carinata* and were expressed in ovaries, thoracic limbs, rectum, and second antenna in both sexual and asexual females (Li et al., 2016). This suggested that the CSPs might respond to environmental signals and control the reproductive switch from sexual to asexual reproduction in *D. carinata* (Li et al., 2016). Generally, owing to the lack of OBPs, crustaceans only have 0–2 CSPs (Derby et al., 2016). Our results were consistent. Even though CSPs were broadly reported in insects, crustaceans, and myriapods (Zhou et al., 2006; Chipman et al., 2014; Pelosi et al., 2014), their functions in crustaceans remained unknown. Based on our results, we speculated that CSPs might be one of the dominant signaling receptors of pheromone binding in male *P. clarkii*. More attention to the expressions and changes in CSPs is needed in further studies. Moreover, the functions of lots of DEGs in the antenna of *P. clarkii* have not been annotated in the six databases, and have been classified as uncharacterized proteins, indicating that the information on pheromone-related receptors in *P. clarkii* is very scarce, which needs a lot of research to investigate. We suggested that there are still many candidate sex pheromone receptors in *P. clarkii* that have not been discovered and reported.

The ribosome is the molecular machine for biological protein synthesis (Lv et al., 2017). A previous study has reported that the inactivation of ribosomes can disrupt chemoreception in *Caenorhabditis elegans* (McConnell et al., 2015). Researchers suggested that the ribosomes affect the process of translation, and further affect the biological synthesis of chemosensation

(McConnell et al., 2015). In this study, the ribosome was the most enriched pathway, of which most DEGs related to ribosome were upregulated. In *Apis Mellifera ligustica*, ribosomes were considered to play an important role in olfactory learning behavior, and the learning ability can be significantly reduced after being irritated by insecticide (Hou et al., 2021). Despite the interactive relationship between ribosomes and chemosensory processes in crustaceans remaining unknown, our results can still indicate that biological protein synthesis is more active after the male *P. clarkii* enters the mating period.

Ca^{2+} is the regulator of almost all eukaryotic signaling cascades, and Ca^{2+}-entry is the first step to converting chemical signals into electrical ones (Pyrski et al., 2007). The previous study has shown that the concentration ratio of intracellular Ca^{2+} and cyclic nucleotide levels can mediate the ion channels synergistically (Stengl, 2010). After the sex pheromone binds to the receptor, the latter can activate the type III adenylyl cyclase *via* a GTP-binding protein, resulting in raising the cAMP level (Castillo et al., 2007). High concentrations of cAMP can open the cyclic nucleotide-gated channels, leading to the Na^+ and Ca^{2+} flow into the cell, further leading to depolarization (Firestein and Zufall, 1994). The sodium carrier family (NCX and sodium–calcium exchanger) is one of the membrane transporters, which has been considered the main mechanism for Ca^{2+} efflux in crustaceans (Flik et al., 1994). The NCX has a fundamental role in controlling the intracellular concentrations of Na^+ and Ca^{2+} (Canitano et al., 2002), which is the efficient Ca^{2+} extrusion system in the plasma membrane. In this study, we further found that the DEG related to NCX and sodium–calcium exchanger was significantly upregulated in MP groups and was involved in olfactory transduction and calcium signaling pathways. In the calcium signaling pathway, the upregulation of NCX could further affect the calcium concentrations of mitochondria, which plays a central role in cell signalings (Jacobson and Duchen, 2004). The previous study had shown that the mitochondria acted as the calcium buffer and reacted with the signals *via* upregulating the tricarboxylic acid cycle (Jacobson and Duchen, 2004). The activations of mitochondria might be more active for signal transductions. Therefore, we suggested that the calcium signaling pathway might be involved in sex pheromone signal transductions and the changes in antenna potential to sex pheromone signals were more sensitive when *P. clarkii* entered the mating period.

Conclusion

In this study, we found the sensitivity of male *P. clarkii* to conditioned water was different in the mating and non-mating period, and they were more sensitive in the mating period. Furthermore, a total of 12 upregulated and nine downregulated DEGs were associated with chemosensory-related functions, of which two related to CSPs were remarkably upregulated after *P. clarkii* entered MP. Based on the levels of CSP-related DEG expression, we suggested that CSP might be the key receptor for chemical signals or sex pheromone reception in the antenna of *P. clarkii*. In addition, the calcium-related DEG and signaling pathway might be one of the dominant factors in the differences in chemosensory sensitivity of male *P. clarkii* between the mating and non-mating periods. The results presented herein will be fundamental for future functional studies on chemosensory genes in *P. clarkii*. The findings are expected to clarify the chemical communication mechanisms in *P. clarkii* and provide new targets for invasion management in future.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: National Center for Biotechnology Information (NCBI) BioProject, https://www.ncbi.nlm.nih.gov/bioproject/, PRJNA839237.

Author contributions

ZZ, JH, and ZW designed the study. ZZ, HW, DL, and WZ performed the experiments and data analysis. ZZ drafted the manuscript. ZZ, HW, LM, JH, and ZW discussed the study design and data. JH and ZW revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by grants from the National Natural Science Foundation of China (31960280), Science and Technology Base and Talent Special Project of Guangxi Province (AD20159042), Guangxi Natural Science Foundation of China (No. 2018GXNSFAA294024), Guangxi Universities Young and Middle-aged Teachers Scientific Research Basic Ability Improvement Project (2019KY0055), and Key Research Program of Guangxi Normal University (2017ZD009).

Acknowledgments

We are grateful to the lab members (Tingting Wei, Ronghui Zhao, and Xiufang Nong) for their help in this study.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.976448/full#supplementary-material

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frontiers | Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 26 September 2022
DOI 10.3389/fevo.2022.956555

Spatio-temporal dynamics of fish assemblage in the Datong and Xiaotong rivers, karst tributaries in the upper Yangtze River drainage: Implications for ecological adaptation and conservation of fish in rivers

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to
Conservation and Restoration Ecology,
a section of the journal
Frontiers in Ecology and Evolution

RECEIVED 30 May 2022
ACCEPTED 07 September 2022
PUBLISHED 26 September 2022

CITATION
Xiang P, Wang X, Liu K, Wu B, Liang C and Song Z (2022) Spatio-temporal dynamics of fish assemblage in the Datong and Xiaotong rivers, karst tributaries in the upper Yangtze River drainage: Implications for ecological adaptation and conservation of fish in rivers. *Front. Ecol. Evol.* 10:956555. doi: 10.3389/fevo.2022.956555

The Datong and Xiaotong rivers are karst tributaries in the upper Yangtze River drainage and represent considerable habitat diversity that contributes to high fish diversity and rich fishery resources in the two rivers. During the few decades, fish resources have abruptly declined due to overfishing, water pollution and habitat degradation. In order to further strengthen conservation and restoration of fish resources and provide scientific support for fishery management in these rivers, the temporal and spatial dynamics of fish assemblage in the Datong and Xiaotong rivers were investigated in the present study. A total of 45 species were captured at 30 sample sites during four surveys conducted from March 2016 to January 2017. Species diversity and richness both increased from upstream to downstream in the two rivers. The fish assemblages significantly differed between river reaches, while did not differ in significantly between 4 months. The spatial variations in the abundance of *Rhynchocypris oxycephalus*, *Zacco platypus*, *Triplophysa bleekeri*, *Sinogastromyzon szechuanensis*, *Platysmacheilus nudiventris*, *Sinibrama taeniatus*, *Saurogobio gymnocheilus*, *Pseudorasbora parva*, *Pseudobagrus truncates* and *Opsariichthys bidens* were considered to contribute most to the spatial pattern of fish assemblages. The lack of a temporal pattern of the fish assemblage in the rivers might be attributed to ecological habits of short-distance migration and diversified mesohabitats with riffles and pools for fish spawning, feeding or overwintering within a very short river range. Our results suggest the spatio-temporal dynamics of fish assemblages is highly adapted to the habitat characteristics in the two karst rivers. We recommend that fish conservation of these tributaries be focused on the mesohabitats, particularly the many pools and riffles.

KEYWORDS

fish assemblage, spatial-seasonal dynamics, karst river, upper Yangtze River, habitat with riffles and pools

Introduction

Understanding of spatial and temporal dynamics of fish assemblages is fundamental to interpreting those factors that influence the structuring of fish populations (Silvano et al., 2000). Those variations may occur in fish composition at a scale as small as a microhabitat or as large as a basin and may occur in days or as long as decades (Schlosser, 1982; Adams et al., 2004; Nazeer et al., 2016; Wang et al., 2019). Studying the patterns of such variations has been the major focus of freshwater ecology (Rahel and Hubert, 1991; Covich, 2006). As a high trophic level organism in the aquatic ecosystem, the community structure of fish not only affects ecological functions such as nutrient cycling and food web structure, but also reflects variations in the habitat environment (Kraft et al., 2007; Ives et al., 2019; Fráguas and Pompeu, 2021). Therefore, ascertaining the patterns of the fish assemblages and associated environments is of great significance to the conservation of fish diversity and effective river management (Naiman and Latterell, 2005; Rice, 2005; Erõs et al., 2010).

In terms of spatial distribution, an upstream-downstream gradient in freshwater fish assemblage structure has been extensively studied in freshwater ecosystems (Schlosser, 1982; Poff and Allan, 1995; Liu et al., 2021). Under natural conditions, species distribution within drainages can be explained by the variability in the environment along the flow regime, especially in water temperature or hydro-morphology, creating distinct fish assemblages across the upstream-downstream gradient (Rahel and Hubert, 1991; Ibarra et al., 2005). The difference in fish composition is due to fish diversity constantly changing in response to local habitat diversity. Such upstream-downstream environment-driven changes in species assemblages are consistent with several of the predictions of the River Continuum Concept (Vannote et al., 1980). For example, Wang et al. (2019) found that fish abundance, richness and Shannon-Wiener diversity all increased from upstream to downstream. Liu et al. (2021) found that the Chishui River in China showed natural and continuous changes in geomorphologic structure along its longitudinal gradient, as there are no geographic discontinuities in the mainstream.

In terms of temporal distribution, seasonal changes in fish composition often provide information on the factors regulating immigration and emigration of assemblages (Adams et al., 2004). The seasonal precipitation and runoff in a subtropical monsoon climate region promote the change of seasonal hydrochemistry and directly affect the species richness and distribution (Melcher et al., 2012; He et al., 2017). Fishes will inhabit different habitats of the ecosystem across seasons, depending on their feeding and reproductive strategies as well as on biotic (e.g., avoiding predation) and abiotic influences (suitable environment conditions) (Fernandes, 1997; Rodriguez and Lewis, 1997; Lowe-McConnell, 1999). Moreover, seasonal migrations in potamodromous fish are critical for completing life cycles, particularly in accessing spawning and nursery habitats and refugia (Fraley et al., 2016). River fishes also have evolved several life cycle adaptations to improve their resilience against stochastic disturbances, such as high fecundity, multiple spawning, batch-spawning, a protracted annual spawning season, and long life-time fecundity with multi-cyclic spawning (Wolter et al., 2015). Therefore, they are well adapted to environmental variations driven by hydro-morphological processes in seasons.

In addition to this deterministic effect of the spatial and temporal variability on assemblages, stream fish assemblages are influenced by both natural factors and anthropogenic disturbances (Vannote et al., 1980; Allan, 2004; Kennard et al., 2006). Natural factors include hydro-morphology characteristics, climate, riparian land use and water environmental conditions like salinity, turbidity and temperature that vary in time, largely due to seasonal variations in freshwater input (Cyrus and Blaber, 1987; Barletta-Bergan et al., 2002; Barletta et al., 2003, 2005). Complex factors were identified as anthropogenic disturbances for fish assemblages such as dams, pollution, overfishing and land erosion (Stanford and Ward, 2001). Such stress may cause a decline in fish stocks, habitat destruction, and biodiversity degradation. In addition, biological factors such as predation and competition also directly or indirectly affect the composition of fish (Ostrand and Wilde, 2002).

The Datong River and Xiaotong River originate in Shanxi Province, and flow through the northeast of Sichuan Province, China. The Datong and Xiaotong rivers are tributaries of the upper Qujiang River, a tributary of the Yangtze River. The two rivers are mountain rivers in a karst geological area

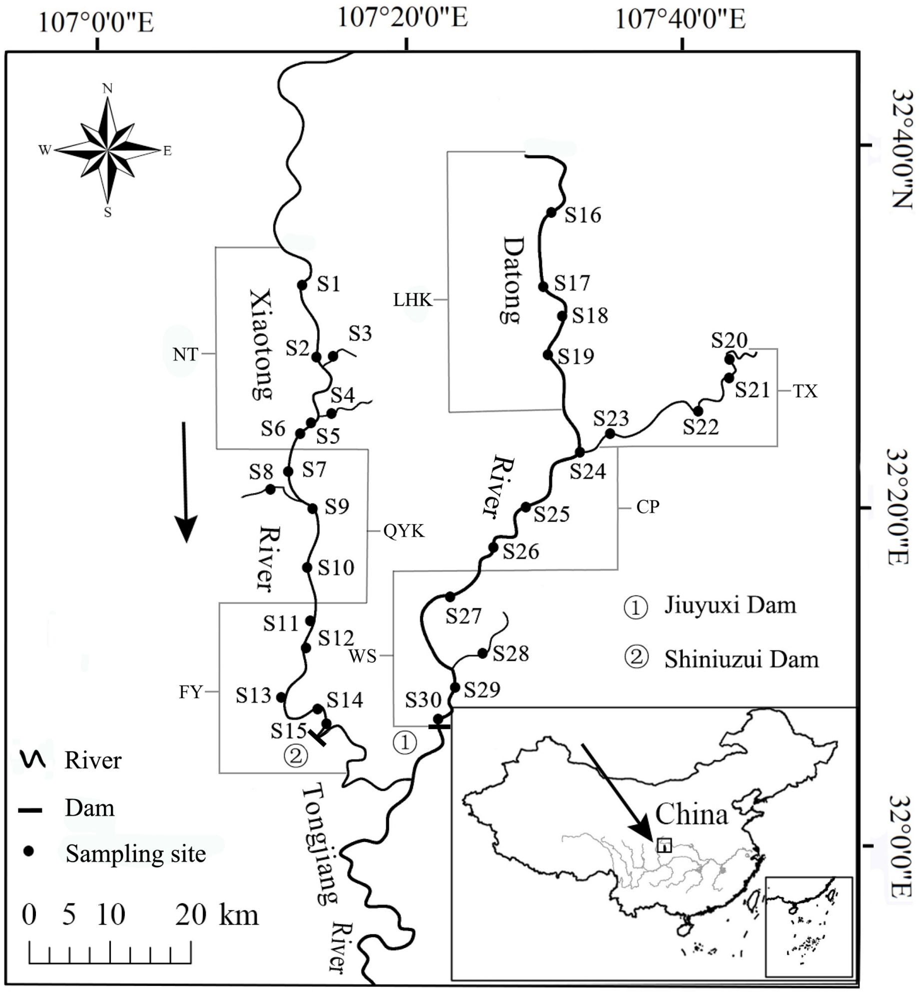

FIGURE 1
Water system map of the Datong and Xiaotong rivers, showing the sampling localities of fish and constructed dams on the rivers. Nuoshui Town reach is represented by NT; Qingyukou reach is represented by QYK; Fuyang reach is represented by FY; Lianghekou reach is represented by LHK; Tiexi reach is represented by TX; Changping reach is represented by CP; Washi reach is represented by WS.

with many rock cavities and swallets, and alternating riffles and pools (Qi, 2018) that provide very important habitats for feeding, breeding and overwintering of aquatic animals. The two rivers' elevations range from 374 to 608 m. Wide and twisting rivers with numerous riffle and pool alternations, huge and small drop-offs, and slightly brisk flow at the riffle and rather calm flow at the pool characterize the area. Mostly sand and gravel make up the river substrate. The region receives a lot of rain each year as a result of the subtropical monsoon climate, and the riversides are covered in lush vegetation. The vegetation in the region consists of subtropical evergreen broadleaf forests, subtropical deciduous broadleaf forests, montane scrub and grasses, which contribute to the protection of soil and water. There are about 57 fish species belonging to four orders, 13 families in the two rivers, according to a recent investigation (Liang et al., 2021). In consideration of the natural aquatic ecological environment and high fish biodiversity, most areas of the Datong and Xiaotong rivers were designated as the "Sichuan Nuoshuihe Precious Aquatic Animal National Nature Reserve" in 2012 (Liang et al., 2021).

However, the ecological environment in the Datong and Xiaotong rivers has been changing due to anthropogenic activities in recent decades, which contributed to the declines of fish resources and diversities in these two tributaries (Liang et al., 2021). Overfishing is one of the major factors attributed to the decline of fish resources in the Datong and Xiaotong rivers before the national nature reserve was established. At the same time, the discharge of urban domestic sewage, coal mining in the headwater area, and the development of tourism had a great impact on water quality. The construction of two dams (Jiuyuxi dam on the lower Datong River and

Shiniuzui dam on the lower Xiaotong River) were also regarded as important anthropogenic factors affecting hydrological regimes. Dams and their associated impoundment posed a significant threat to aquatic biodiversity by altering water flow regimes, fragmenting habitats, modifying channel morphology and riverbed structure (Dudgeon, 2000). In addition, sand excavation in the tributaries has destroyed several important fish habitats, such as spawning and feeding grounds (Liang et al., 2021). Nevertheless, the contemporary and long-term effects of anthropogenic activities on distribution and abundance of native fish in the Datong and Xiaotong rivers remains poorly understood.

We conducted fish resource surveys during 2016∼2017 in the Datong and Xiaotong rivers. The main objectives were: (1) to comprehensively understand the characteristics of the spatial and temporal dynamics of fish assemblage structure in the two karst streams (Xiaotong and Datong rivers), and (2) to provide basic data for conservation and management of fish resources.

Materials and methods

Field sampling

The fish resources in two tributaries, Datong River (DR) and Xiaotong River (XR) of the upper Tongjiang River, a tributary of the Qujiang River, were surveyed in March, May, September, 2016 and January, 2017. A total of 30 sampling sites were set with 15 sites on XR (S1∼S15) and 15 sites on DR (S16∼S30). The sampling sites were classified into seven reaches, which were NT (S1 to S6), QYK (S7 to S10), FY (S11 to S15) in XR and LHK (S16 to S19), TX (S20 to S23), CP (S24 to S26), WS (S27 to S30) in DR (**Figure 1**).

Each sampling was conducted along at least a 500 m length of the river's reach and usually contained all microhabitat types (riffles, pools, torrents and back waters). Fish specimens were caught by a combination of different sampling techniques, due to the heterogeneity of the river habitats. In reaches with shallow but torrential water, specimens were sampled via a battery-powered backpack electro-fisher (16.8 V). In backwater and deep pools, gill nets with a mesh size of 1∼6 mm (20∼50 m long and 1∼2 m high) and fish cages (0.3 m × 0.25 m × 8 m, 4 mm mesh) were used to catch fish. The duration of electrofishing on each sampling site was 40∼60 min. Three gill nets and 3∼6 cages were placed on each survey location. The gill nets and cages were placed at 18:00 in the afternoon, and withdrawn at 6:00 the next morning. The sampling was approved by the Administrative Department of Nature Reserve of Sichuan Province. The collected fish individuals were identified first, then their standard lengths (SL) were measured to the nearest 0.1 mm and body weights (BW) were measured to the nearest 0.1 g immediately after sampling, and finally individuals were released into the water where captured, except for a few specimens that were anesthetized by MS-222 solution and fixed with formaldehyde solution (5∼10%) after death.

Data analysis

We used rank-abundance and occurrence frequency diagrams to exhibit species abundance (Magurran, 2004). We then grouped all the species by their relative abundance (percentage of total fish individuals) and occurrence frequency (percentage of sampling locations in which fish species occurred), then categorized them into dominating species, common species, and rare species. We classified species as dominant, common and rare according to index of relative importance (Liang et al., 2021). Based on their maximum standard body length of less than 100, 100–200, and more than 200 mm, species are categorized as small, medium, and large-sized individuals (Wang et al., 2019). Fish species diversity for each location was calculated using Shannon-Wiener diversity: $H^{'} = -\Sigma P_i ln(P_i)$, $P_i = N_i/N$, where N_i = the total individuals of species i collected in each location; N = the total individuals collected in each location (Magurran, 1988). We used morphological features, living habits and scientific literature to determine main dietary items and then classified the species into different feeding guilds.

One-way repeated measured analysis of variance (ANOVA) was undertaken on data (Shannon-wiener diversity, species richness, evenness, Cypriniformes diversity) to detect differences among the seven reaches (Datong River: LHK, TX, CP, WS; Xiaotong River: NT, QYK, FY) and 4 months (March, May, September, and January). Non-metric multidimensional scaling (NMDS) was performed using abundance data to assess the similarity in patterns between reaches and seasons (Hadley, 2016). Prior to NMDS analysis, the abundance data were log (x+1) transformed and categorized with the Bray-Curtis similarity matrix (Clarke and Warwick, 1994). In order to examine possible seasonal and spatial differences of fish assemblage structure, species abundance was compared between months and reaches using two-way analysis of similarities (two-way ANOSIM). Finally, the similarity percentage procedure (SIMPER) was used to identify fish species contributing most to similarities between reaches within season and between seasons with reaches of the two rivers (Clarke and Warwick, 2001).

The SPSS 23.0 statistics package (SPSS Inc., Chicago, IL, USA) was used to perform one-way repeated ANOVA, and statistical significance was accepted at $P < 0.05$. NMDS and two-way ANOSIM analyses were performed using R software with the package "vegan" version 2.5–6 (Oksanen et al., 2022) and

TABLE 1 Composition of fish species captured from Datong and Xiaotong rivers, with their species code, abundance, relative abundance, occurrence frequency, mean SL, SL range, trophic guild (TG).

Families	Species	Code	Abundance	Relative abundance (%)	Occurrence frequency (%)	Mean SL ± SD (mm)	SL range (mm)	Trophic guild	Datong River	Xiaotong River
Cyprinidae										
	Hemiculterella sauvagei (Warpachowski, 1888)	HSA	37	0.6	13.33	85.6 ± 16.5	56.6–115.3	Omnivore	+	+
	Hemiculter bleekeri (Warpachowski, 1888)	HBL	2	0.03	3.33	122.5 ± 2.1	121.1–124.9	Omnivore	+	
	Rhodeus lighti (Wu, 1931)	RLI	27	0.44	10	47.2 ± 7.5	34.5–67.5	Herbivore	+	
	Hemiculter leucisculus (Basilewsky, 1855)	HLE	44	0.71	3.33	125.5 ± 6.3	110.8–140.1	Omnivore	+	
	Hemibarbus labeo (Pallas, 1776)	HLA	44	0.71	23.33	122.2 ± 46.3	21.5–229.3	Carnivore	+	+
	Squalidus wolterstorffi (Regan, 1908)	SWO	358	5.81	40	63.2 ± 13.0	33.0–158.1	Omnivore	+	+
	Acheilognathus barbatulus (Günther, 1873)	ABA	2	0.03	3.33	78.0 ± 2.8	76.7–80.2	Herbivore	+	
	Abbottina obtusirostris (Wu and Wang, 1931)	AOB	150	2.44	43.33	56.7 ± 11.9	31.3–84.6	Omnivore	+	+
	Rhodeus ocellatus (Kner, 1866)	ROC	1	0.02	3.33	67.1	–	Herbivore		+
	Saurogobio gymnocheilus (Lo, Yao, and Chen, 1998)	SGY	79	1.28	6.67	98.3 ± 11.6	73.7–151.9	Omnivore	+	
	Sarcocheilichthys nigripinnis (Günther, 1873)	SNI	53	0.86	23.33	69.7 ± 7.4	55.3–87.4	Omnivore	+	+
	Carasius auratus (Linnaeus, 1758)	CAU	4	0.06	10	93 ± 51.6	43.3–146.8	Omnivore		+
	Gnathopogon herzensteini (Günther, 1896)	GHE	713	11.57	83.33	65.8 ± 10.9	7.3–105.6	Omnivore	+	+
	Rhynchocypris oxycephalus (Sauvage and Dabry, 1874)	ROX	539	8.75	23.33	57.5 ± 11.4	27.7–95.4	Omnivore	+	
	Acrossocheilus monticola (Günther, 1888)	AMO	309	5.02	70	67.6 ± 19.2	15.7–150.8	Omnivore	+	+
	Zacco platypus (Temminck and Schlegel, 1846)	ZPL	648	10.52	73.33	68 ± 21.2	19.7–163.7	Omnivore	+	+
	Microphysogobio kiatingensis (Wu, 1930)	MKI	304	4.94	53.33	61.3 ± 10.2	22.5–85.4	Omnivore	+	+

(Continued)

TABLE 1 (Continued)

Families	Species	Code	Abundance	Relative abundance (%)	Occurrence frequency (%)	Mean SL ± SD (mm)	SL range (mm)	Trophic guild	Datong River	Xiaotong River
	Cyprinus carpio (Linnaeus, 1758)	CCA	2	0.03	3.33	357 ± 42.4	327–387	Omnivore		+
	Platysmacheilus nudiventris (Luo, Le, and Chen, 1977)	PNU	662	10.75	63.33	54.1 ± 8.9	13.3–106.6	Omnivore	+	+
	Opsariichthys bidens Günther, 1873	OBI	308	5	70	67.8 ± 26.1	15.0–182.2	Carnivore	+	+
	Pseudorasbora parva (Temminck and Schlegel, 1846)	PPA	36	0.58	30	65 ± 12.5	38.5–94.0	Omnivore	+	+
	Saurogobio dabryi (Bleeker, 1871)	SDA	5	0.08	6.67	106 ± 22.0	77.5–126.2	Omnivore		+
	Sinibrama taeniatus (Nichols, 1941)	STA	195	3.17	23.33	86.6 ± 11.7	55.2–110.7	Omnivore	+	+
	Procypris rabaudi (Tchang, 1930)	PRA	1	0.02	3.33	68.7	–	Omnivore		+
	Squalidus argentatus (Sauvage and Dabry de Thiersant, 1874)	SAR	6	0.1	3.33	53.3 ± 10.3	41.1–67.3	Omnivore		+
	Bangana rendahli (Kimura, 1934)	BRE	1	0.02	3.33	176.2	–	Omnivore		+
	Spinibarbus sinensis (Bleeker, 1871)	SSI	2	0.03	6.67	115 ± 108.9	38.9–192.1	Omnivore	+	+
Cobitidae										
	Misgurnus anguillicaudatus (Cantor, 1842)	MAN	23	0.37	13.33	102.7 ± 14.3	64.7–133.3	Omnivore	+	+
	Homatula variegata (Dabry de Thiersant, 1874)	HVA	12	0.19	13.33	82.2 ± 20.6	46.5–113.2	Carnivore	+	+
	Homatula potanini (Günther, 1896)	HPO	214	3.47	50	56.3 ± 12.0	37.3–91.5	Carnivore	+	+
	Triplophysa bleekeri (Sauvage and Dabry de Thiersant, 1874)	TBL	47	0.76	13.33	79.8 ± 13.1	47.4–104.2	Omnivore	+	
	Sinogastromyzon szechuanensis (Fang, 1930)	SSZ	438	7.11	56.67	43.3 ± 14.1	12.3–86.1	Herbivore	+	+

(Continued)

TABLE 1 (Continued)

Families	Species	Code	Abundance	Relative abundance (%)	Occurrence frequency (%)	Mean SL ± SD (mm)	SL range (mm)	Trophic guild	Datong River	Xiaotong River
Bagridae										
	Pseudobagrus emarginatus (Regan, 1913)	PEM	8	0.13	10	75.6 ± 30.1	26.2–102.4	Carnivore	+	+
	Pseudobagrus crassilabris (Günther, 1864)	PCR	213	3.46	30	106.1 ± 22.2	46.2–198.9	Carnivore	+	+
	Hemibagrus macropterus (Bleeker, 1870)	HMA	7	0.11	13.33	130.4 ± 52.1	52.6–195.7	Carnivore	+	+
	Tachysurus fulvidraco (Richardson, 1846)	TFU	4	0.06	3.33	127.3 ± 2.6	125.1–131.2	Carnivore		+
	Pseudobagrus vachellii (Richardson, 1846)	PVA	7	0.11	6.67	103.6 ± 10.6	95.4–122.5	Carnivore	+	+
	Pseudobagrus pratti (Günther, 1892)	PPR	3	0.05	3.33	133.7 ± 43	95.3–180.9	Carnivore		+
	Pseudobagrus truncatus (Regan, 1913)	PTR	347	5.63	66.67	81.8 ± 30.4	19.5–197.4	Carnivore	+	+
Siluridae										
	Silurus meridionalis (Chen, 1977)	SME	2	0.03	3.33	347.5 ± 3.5	345.1–350.5	Carnivore		+
	Silurus asotus (Linnaeus, 1758)	SAS	7	0.11	6.67	281.4 ± 72.1	198.2–419.4	Carnivore		+
Sisoridae										
	Glyptothorax fokiensis (Rendahl, 1925)	GFO	142	2.31	60	62.9 ± 13.8	30.2–104.1	Carnivore	+	+
Amblycipitidae										
	Liobagrus marginatoides (Wu, 1930)	LMA	38	0.62	20	71.9 ± 14.0	40.8–101.2	Carnivore		+
Gobiidae										
	Rhinogobius giurinus (Rutter, 1897)	RGI	115	1.87	23.33	45 ± 11	26.3–84.4	Carnivore	+	+
	Siniperca knerii (Garman, 1912)	SKN	1	0.02	3.33	246.2	–	Carnivore		+

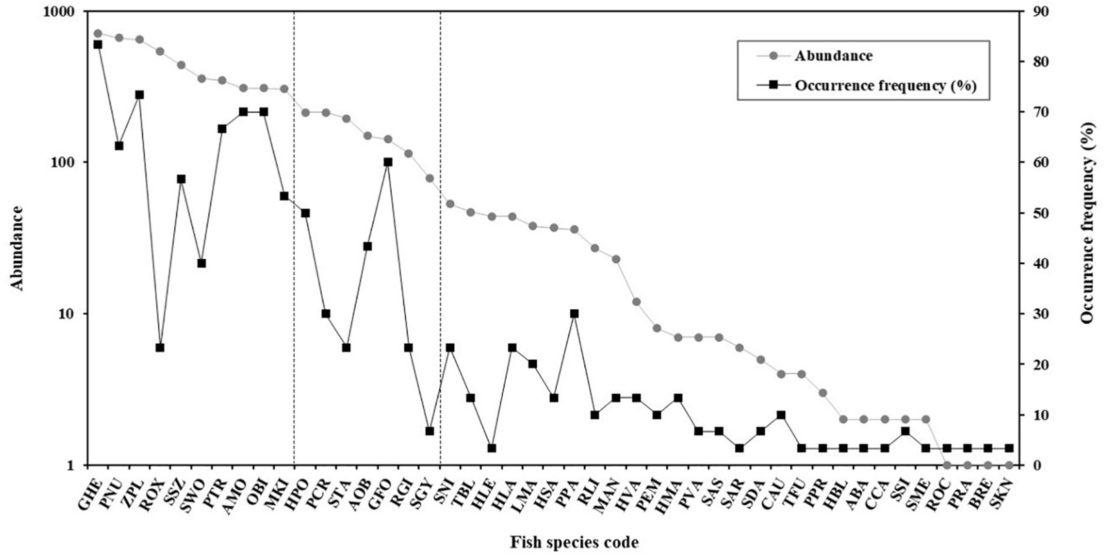

FIGURE 2

Rank-abundance (total number of individuals captured) and occurrence frequency (percentage of sampling locations in which the species occurred) of the 45 species captured from the Datong and Xiaotong rivers. Fish species codes are shown in Table 1.

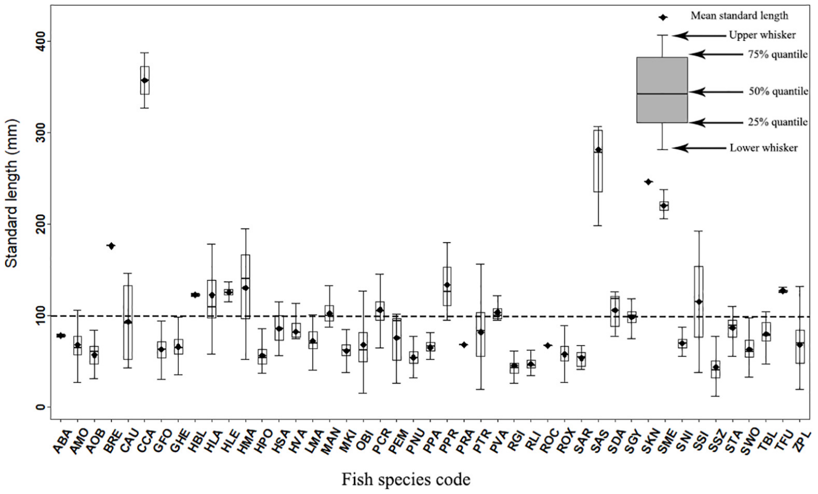

FIGURE 3

Box-Whisker plots of standard length for 45 species captured from the Datong and Xiaotong rivers.

"ggplot2" version 3.2.1 (Wickham et al., 2016). SIMPER analysis was conducted in PEIMER version 5 (Clarke and Warwick, 2001).

Results

Fish composition

A total of 6,160 individuals belonging to 45 fish species in 35 genera, seven families, and three orders were collected from the Datong and Xiaotong rivers. Cypriniformes and Siluriformes were the most representative orders, with 32 and 11 species, respectively, and together represented more than 95% of the species. Cyprinidae was the dominant family, comprising 22 genera and 27 species, followed by Bagridae (7 species), Cobitidae (5 species), Siluridae (2 species), Serranidae (2 species), Sisoridae (1 species), and Amblycipitidae (1 species). Altogether 32 species were captured in the Datong River, and 38 species in the Xiaotong River, respectively (Table 1).

A species rank-abundance and occurrence diagram indicated that the 45 species were classified into three

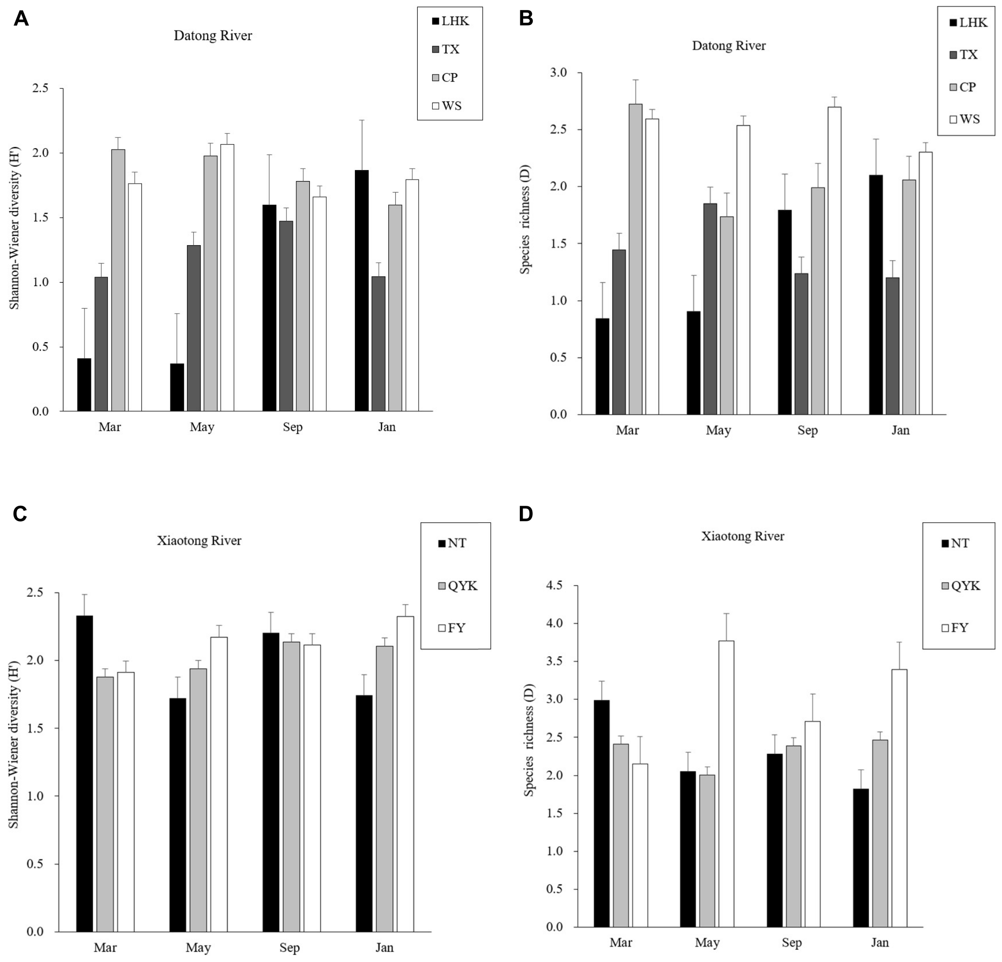

FIGURE 4

Mean value and standard deviation (SD) of Shannon-Winener diversity (H'), species richness (D) at seven reaches (NT, QYK, FY, LHK, TX, CP, WS) in 4 months. **(A)** Shannon-Wiener diversity (H') of Datong river, **(B)** species richness (D) of Datong river, **(C)** Shannon-Wiener diversity (H') of Xiaotong river, **(D)** species richness (D) of Xiaotong river.

groups, according to their relative abundance and occurrence frequency (Figure 2 and Table 1). Dominant species included *G. herzensteini*, *P. nudiventris*, *Z. platypus*, *R. oxycephalus*, *S. szechuanensis*, *Squalidus wolterstorffi*, *P. truncatus*, *Acrossocheilus monticola*, *O. bidens*, *Microphysogobio kiatingensis* and accounted for 75% of the total individuals captured (Figure 2 and Table 1). These dominant species were characterized by high relative abundance (from 4.94 to 11.57% of the total individuals captured) and high occurrence frequency (from 23.33 to 83.33% of the 30 sites). The dominant species *R. oxycephalus* was characterized by the highest relative abundance (8.75% of the total individuals captured) and lowest occurrence frequency (only 23.33% of the 30 sampling locations). The common species were *Homatula potanini*, *Pseudobagrus crassilabris*, *S. taeniatus*, *Abbottina obtusirostris*, *Glyptothorax fokiensis* and *Rhinogobius giurinus*, exhibiting moderate relative abundance (between 1.87 and 3.47%) and moderate occurrence frequency (from 23.33 to 60%). These common species accounted for 16.54% of the total individuals captured. The remaining 29 species were considered as rare species with a relative abundance below 1.28% and an occurrence frequency less than 23.33%, which contributed only 8.20% of the total individuals captured.

Of the 45 species, 39 species were classified as small-sized species because their first maturity ages were less than 2 years (Ye et al., 2006) and maximum standard lengths were small (Table 1). Only six large and medium-sized species (maturity over two years) were recorded and they were *Procypris rabaudi*, *Silurus meridionalis*, *Spinibarbus sinensis*, *Siniperca knerii*, *Bangana rendahli* and *Cyprinus carpio*. The standard lengths of the 45 fish species ranged from 12 to 419 mm (Table 1). Twenty-nine species (64.44% of the total fish species) were below 100 mm (mean SL), 12 species (26.67%) between 100 and 200 mm, and the other four

TABLE 2 One-way repeated measured ANOVA of effects of seven reaches (NT, QYK, FY, LHK, TX, CP, WS) in 4 months (March, May, September, January) on species diversity, richness, evenness and Cypriniformes diversity.

	F	*P*-value
Species diversity		
Between-subjects (range)	9.834	**<0.001**
Within-subjects		
Month	0.752	0.527
Month × range	2.208	**0.018**
Species richness		
Between-subjects (range)	4.032	**0.015**
Within-subjects		
Month	1.568	0.211
Month × range	1.832	0.054
Evenness		
Between-subjects (range)	0.297	0.928
Within-subjects		
Month	3.263	**0.031**
Month × range	2.737	**0.004**
Cypriniformes diversity		
Between-subjects (range)	8.743	**<0.001**
Within-subjects		
Month	0.594	0.622
Month × range	3.042	**0.04**

P-values less than 0.05 are bolded.

species (8.89%) had mean SL above 200 mm (Table 1 and Figure 3).

The 45 fish species were classified into three trophic guilds: omnivore, herbivore, and carnivore (Table 1). The trophic guild with the highest proportion in the total catch was omnivore (53.33% of the total fish species; e.g., *G. herzensteini*, *P. nudiventris*, *R. oxycephalus*), followed by carnivores (37.78%), herbivores (8.89%) and detritivores (6.67%).

Spatial variations

Overall, both the Datong River and Xiaotong River had high species diversity and richness (Figure 4). Spatially, in the Datong River, species diversity was lower both in the upstream (LHK) and tributary (TX) than in the midstream (CP) and downstream (WS). In the Xiaotong River, there were slight differences in diversity between the upstream (NT), midstream (QYK) and downstream (FY), but they also showed a trend of increasing gradually from upstream to downstream. Species richness was the lowest in the upstream (LHK) of the Datong River, followed by the midstream (CP) and tributaries (TX), and the highest in the downstream (WS). Similar to the Datong River, species richness gradually increased from upstream to downstream in the Xiaotong River.

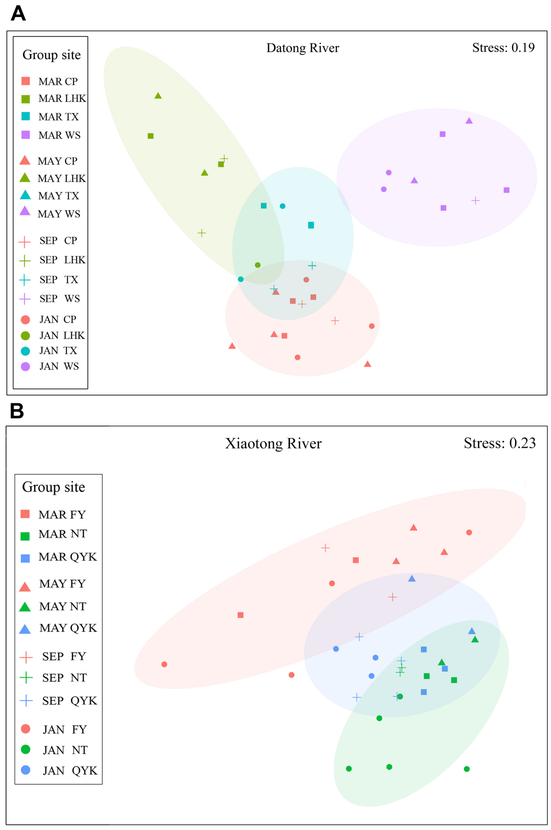

FIGURE 5
Non-metric multi-dimensional scaling (NMDS) by reaches (Datong River: LHK, TX, CP, WS; Xiaotong River: NT, QYK, FY) and months (March, May, September, January) for the abundance data of the Datong **(A)** and Xiaotong **(B)** rivers. Each symbol represents a month (dot, January; square, March; triangle, May; cross, September). Each color represents a range (orange, CP and FY; green, LHK and NT; blue, TX and QYK; purple, WS).

One-way repeated-measured ANOVAs revealed that Shannon-Wiener diversity, species richness and Cypriniformes diversity significantly differed between the seven reaches ($P < 0.05$), but species evenness was not significantly different between reaches ($P > 0.05$; Table 2).

NMDS revealed clearly spatial groups of the sampling sites in the Datong and Xiaotong rivers. The sites in the downstream of the Datong River (WS) were clustered on the right, the ones in the upstream (LHK) were clustered to the left, while those in the midstream (CP) and the tributary (TX) were clustered at the bottom and middle, respectively (Figure 5). The sites in the upstream (NT), midstream (QYK) and downstream (FY) of the Xiaotong River were clustered at the top, middle and bottom, respectively (Figure 5).

Similar to the NMDS results, two-way ANOSIM results indicated that there were significant spatial differences in fish

TABLE 3 Two-way ANOSIM results of month and reach in Datong and Xiaotong rivers.

River	Factor	R	p	Permutation N
Datong River	Month	0.17	0.0875	9,999
	Reach	0.78	**0.0001**	
Xiaotong River	Month	0.15	0.0826	
	Reach	0.39	**0.001**	

P-values less than 0.05 are bolded.

community structure between the four reaches in the Datong River (Global $R = 0.78$, $p = 0.0001$) and between the three reaches in the Xiaotong River, although there was a clear spatial overlap in community structure (Global $R = 0.39$, $p = 0.001$; Table 3).

Analyses of similarity percentage (SIMPER) indicated that *R. oxycephalus* was a typical species of the upstream (LHK) and tributary (TX) reaches, contributing, respectively, to 29.76 and 12.48% of the similarity of the upstream (LHK) and tributary (TX) in the Datong River (Table 4). *T. bleekeri* was plentiful in the upstream (LHK) and contributed 11.91% to the similarity of the upstream (LHK). Moreover, *Z. platypus* was widely distributed in the tributary (TX) and midstream (CP) and contributed to 14.09 and 7.02% of the similarity. *S. szechuanensis* and *P. nudiventris* were widely distributed in the midstream (CP) and contributed to 10.69 and 7.19% of the similarity. *S. taeniatus*, *S. gymnocheilus* and *P. parva* were mainly distributed in the downstream (WS), contributing, respectively, to 15.08, 13.34, and 11.41% of the similarity of the downstream (WS).

In the Xiaotong River, *G. herzensteini* was the most common species, being widely distributed throughout all the river reaches. However, *P. truncatus* was more abundant in the midstream (QYK) and downstream (FY), contributing, respectively, to 8.01 and 6.27%. *O. bidens* and *P. nudiventris* were more plentiful in the upstream (NT) of the Xiaotong River, contributing to 18.73 and 7.61% of the similarity of the upstream (NT).

Temporal variations

Generally, the species diversity and richness of the Datong River and Xiaotong River did not change significantly between seasons (Figure 4). Seasonally, species diversity and richness did not differ significantly between the 4 months in the midstream (CP), tributary (TX) and downstream (WS) of the Datong River, whereas they were lower in March and May than in September and January in the upstream (LHK). In the Xiaotong River, the species diversity in the midstream (QYK) and downstream (FY) also did not vary significantly across the 4 months. The upstream (NT) had higher diversity in March and September, and lower diversity in May and January. The variation of richness with season was not obvious in the upstream (NT) and midstream (QYK) of the Xiaotong River, whereas the downstream (FY) reach had lower richness in March and higher richness in May, September and January.

One-way repeated-measured ANOVAs showed that there were no significant differences in Shannon-Wiener diversity, species richness and Cypriniforms diversity between the 4 months ($P > 0.05$), while species evenness significantly differed between months ($P < 0.05$; Table 2). The interactions of reach × month were also significant in fish diversities except species richness ($P < 0.05$; Table 2). Whereas, there was no clear clustering of sampling sites according to the different months in NMDS (Figure 5). Two-way ANOSIM results also indicated that there were no seasonal variations of the fish assemblages in each reach and the community structure overlapped significantly in the Datong River (Global $R = 0.17$, $p = 0.0875$) and Xiaotong River (Global $R = 0.15$, $p = 0.0826$; Table 3).

SIMPER analysis revealed that *R. oxycephalus* dominated the upstream (LHK) across the 4 months in the Datong River, and *T. bleekeri* was common in May, September and January (excluding March; Table 4). In the tributary (TX), *Z. platypus* and *R. oxycephalus* were abundant across the 4 months, *P. potanini* was abundant in March and May. *S. szechuanensis* dominated the midstream (CP), *P. nudiventris* was abundant in March, May and September (excluding January) and *Z. platypus* was common in May, September, January (excluding March). In the downstream (WS), *S. taeniatus* was abundant across the 4 months, *S. gymnocheilus* was abundant in March, September and January (excluding May) and *P. parva* was common in March, May and January (excluding September).

In the Xiaotong River, *G. herzensteini* dominated the upstream (NT), midstream (QYK) and downstream (FY) across the four seasons. *O. bidens* was plentiful in May, September and January (excluding March) and *P. nudiventris* was common only in March and September in the upstream (NT). In the midstream (QYK), *P. truncatus* was abundant in March, September and January (excluding May). *P. truncatus* was common in March, May and September (excluding January).

Discussion

Size and trophic guild changes due to the anthropogenic disturbances

The frequency distribution of species length showed that the Datong and Xiaotong rivers were dominated by small-sized individuals, which accounted for 99.81% of the total individuals and 91.11% of the total species. Instead, the large and medium-sized fishes such as *S. asotus*, *S. meridionalis*, *C. carpio* and *S. knerii* had a narrow geographic distribution and low quantities. It is likely to have been caused by frequent fishing that targeted the more commercially valuable large and medium-sized species. Some studies have also shown that smaller fish

TABLE 4 Results of SIMPER analysis identifying the contribution of fish species to differences among the seven reaches (LHK, TX, CP, WS, NT, QYK, FY) in four months (March, May, Sep, Jan) in Datong and Xiaotong rivers.

River	Reaches	Four seasons Av. Sim	Av. Abu.	March Av. Sim.	Av. Abu.	May Av. Sim.	Av. Abu.	September Av. Sim.	Av. Abu.	January Av. Sim.	Av. Abu.
Datong River	**Upstream (LHK)**	48.09%		38.81%		70.15%		31.54%		24.31%	
	Rhynchocypris oxycephalus	29.76%	45.38	38.81%	53.00	60.72%	101.00	20.47%	20.50	14.2%	7.00
	Triplophysa bleekeri	11.91%	6.25			9.42%	1.50	11.07%	18.00	10.11%	4.00
	Tributary (TX)	55.96%		88.25%		54.46%		50.52%		47.04%	
	Zacco platypus	14.09%	34.38	10.43%	6.00	9.74%	49.00	7.74%	11.50	28.02%	47.05
	Rhynchocypris oxycephalus	12.48%	11.75	11.14%	8.00	13.56%	5.67	17.51%	19.00	7.36%	17.00
	Paracobitis potanini	6.23%	5.00	10.43%	8.50	12.89%	5.33				
	Midstream (CP)	48.12%		22.89%		48.54%		68.32%		45.02%	
	Sinogastromyzon szechuanensis	10.69%	16.08	7.46%	1.33	11.01%	17.67	17.3%	51.33	20.97%	4.33
	Platysmacheilus nudiventris	7.19%	14.00	3.82%	4.33	4.35%	7.33	18.14%	42.00		
	Zacco platypus	7.02%	10.75			11.81%	17.67	7.89%	12.67	10.67%	7.85
	Downstream (WS)	52.3%		52.8%		39.83%		66.57%		67.46%	
	Sinibrama taeniatus	15.08%	21.57	12.36%	41.50	16.91%	28.50	13.72%	4.50	8.53%	2.50
	Saurogobio gymnocheilus	13.34%	16.29	17.7%	18.50			20.59%	9.00	33.33%	23.00
	Pseudorasbora parva	11.41%	1.86	7.58%	2.50	6.02%	1.50			8.53%	2.00
Xiaotong River	**Upstream (NT)**	48.94%		38.77%		71.6%		63.89%		56.55%	
	Opsariichthys bidens	18.73%	20.50			20.73%	23.00	10.02%	16.00	38.06%	24.25
	Gnathopogon herzensteini	16.47%	22.00	21.92%	21.00	18.44%	16.00	13.89%	51.00	14.72%	11.00
	Platysmacheilus nudiventris	7.61%	17.40	16.85%	14.00			17.66%	59.00		
	Midstream (QYK)	40.48%		64.94%		32.61%		56.89%		37.74%	
	Gnathopogon herzensteini	16.85%	22.64	21.40%	5.00	26.6%	22.33	18.53%	39.00	7.11%	7.00
	Pseudobagrus truncatus	8.01%	7.82	16.26%	4.67			5.9%	5.50	21.84%	7.00
	Downstream (FY)	25.43%		49.72%		28.78%		53.15%		33.46%	
	Pseudobagrus truncatus	6.27%	11.11	12.27%	16.33	13.32%	12.50	13.68%	6.50		
	Gnathopogon herzensteini	4.97%	13.67	17.83%	25.00			18.31%	19.00		

dominating the catch are often the signs of overfishing (Beverton and Holt, 1993; Marcos and Christopher, 2004).

According to the River Continuum Concept, the upstream fish assemblage was expected to be dominated by herbivore, insectivore and frugivore fish since food sources are mainly from riparian vegetation and its associated fauna (Vannote et al., 1980; Araújo et al., 2009; Wang et al., 2019). In comparison, fishes from downstream were predicted to be dominated by carnivorous, omnivorous and detritivorous species. In the Datong and Xiaotong rivers, most of the fish were omnivorous and carnivorous species, which accounted for 91.11% of the total species. However, the resources of herbivorous species such as *Onychostoma simum* and *Sinilabeo redahli* have declined. In addition, the large- or medium-sized carnivorous species such as *Silurus asotus, S. meridionalis and S. knerii,* have declined but the small-sized carnivorous species such as *P. crassilabris*, *Pseudobagrus truncatus* and *Liobagrus marginatoides* still have some resources. One reason for this may stem from anthropogenic disturbances such as overfishing.

Spatial variations in fish assemblages along the upstream to downstream

We found significant longitudinal variation in fish assemblages in the Datong and Xiaotong rivers. Overall, our findings match general patterns for fish communities in riverine systems (Welcomme, 1985; Cowx and Welcomme, 1998; Vila-Gispert et al., 2002), where species are replaced gradually along the longitudinal gradient rather than abruptly. One-way repeated-measured ANOVA and related diversity indices in the Datong and Xiaotong rivers indicated that species richness, diversity and abundance gradually increased from upstream to downstream. Longitudinal position has been extensively studied as a major predictor of fish assemblage structure in riverine ecosystems. In accordance with a previous study in an undisturbed river ecosystem, aquatic animals tend to vary along an upstream to downstream gradient that implies the current velocity, deepness and the substrate control the spatial distribution of fish assemblages (Vila-Gispert et al., 2002). In our study, the variation of altitude, water depth, current velocity and substrate heterogeneity may be responsible for generating longitudinal variation in fish community structure in the Xiaotong and Datong rivers.

The two rivers are karst rivers which have karst landform characteristics. Although those two karst rivers were separated by the dams, they had diverse habitats such as riffle and pool mesohabitat and therefore the impact of Jiuyuxi and Shiniuzui dams was limited for the fish community structure. In addition, the similar results were found in some other karstic landsform rivers (White et al., 2009; Pedro et al., 2018). Even in karst landscapes, where stream continuity is naturally interrupted by obstacles such as subterranean stretches, the river continuum is still maintained (Vannote et al., 1980; White et al., 2009; Pedro et al., 2018). This suggested that karst geomorphic rivers may be somewhat resilient to disturbance (Kollaus et al., 2015). Nevertheless, despite fish community dynamic systems being resilient, karst river systems are not immune to anthropogenic disturbances such as overfishing (White et al., 2009). Therefore, in future studies, we need to be more focused on the influence of anthropogenic disturbances on the longitudinal structure of fish community in karst rivers.

No temporal variations in fish assemblages attribute to diverse mesohabitat

We found there was no significant seasonal variation in fish assemblages in the Datong and Xiaotong rivers. Species richness and Shannon-Wiener diversity of fish also did not change over the four seasons. Fluvial systems are dynamic and fish species diversity and assemblage are known to seasonally change in temperate rivers (Deudero et al., 1999; Carpentier et al., 2004) and tropical rivers (Dansoko et al., 1976; Laë, 1992; Garcia et al., 2003; Rueda and Defeo, 2003) in association with the flood periodicity and the availability of breeding habitat and food items (Winemiller and Jepsen, 1998; Junk and Wantzen, 2006). In the Datong and Xiaotong riverine system, a large number of pool and riffle mesohabitats alternated, that facilitated fish short-distance reproduction, feeding and overwintering migration, which accounted for the lack of temporal variance. The channel morphologies were predominantly categorized into cascade, step-pool, plane-bed, pool-riffle, and dune-ripple by Montgomery and Buffington's (1997). The Datong and Xiaotong rivers are located in the karst landscape area of southwest China, and they not only have 128 karst caves and numerous underground rivers (Qi, 2018), but also have a variety of channel morphologies such as riffle and pool. Although we found no long-distance migratory fish in the Datong and Xiaotong rivers, most species had short-distance migration habits such as spawning, feeding and overwintering. Fishes migrate relatively short distances because adequate habitats exist in close proximity (Makrakis et al., 2012). The benefits would be that fish may not need to migrate long distances to spawning, feeding or overwintering grounds. We speculated that it may be a consequence of the diverse habitats caused by large distribution of pool-riffle mesohabitats, resulting in no significance of seasonal variation in fish community.

Similar findings have been found in freshwater rivers in China, such as the Zhougong River (Wang et al., 2019). The findings indicated that no seasonal change in fish assemblage were partially attributed to the physicochemical changes caused by cascade hydroelectric dams (e.g., channel blocking, hydrological alterations), pollution (e.g., phosphorus) and it is also likely that overfishing has exacerbated impacts (Kadye and

Marshall, 2010; Wang et al., 2019). While Araújo et al. (2009) found seasonal differences in fish community structure in a large tropical river with no habitat diversity in southeastern Brazil. Therefore, when evaluating the seasonal variation of river, it is important to consider habitats, such as pool and riffle mesohabitats, as fish assemblages may be influenced by habitat diversity (Gorman and Karr, 1978; Schlosser, 1982; Martin-Smith, 1998; Goldstein and Meador, 2004; Erõs and Grossman, 2005; Erõs et al., 2010; Troia and Gido, 2015; Wolff and Hahn, 2017).

Conservation implications

Our findings indicated that large and medium-sized individuals, carnivorous and herbivorous species in the Datong and Xiaotong rivers had decreased due to overfishing. Due to water pollution, overfishing and dam construction, the Yangtze River faces a depletion of fishery resources (Liu et al., 2019). However, fishing has been prohibited in the Yangtze River basin since 2020, which includes the Datong and Xiaotong rivers, and fish population stocks have gradually recovered. We consequently suggest the government conduct an efficient, long-term evaluation of the river fishing ban's effectiveness. In several countries, over-exploitation of fish caught for bait has been controlled through three types of action: (1) bans on fishing, (2) limits on the number of fish captured, (3) regulation of the type of equipment used (Pipitone et al., 2000; Bartholomew and Bohnsack, 2005; Cooke et al., 2012). The ban on the number of individuals caught is effective for population recovery in several areas ensuring the sustainability of fishing (Wynberg and Branch, 1991; Anderson et al., 2011). Nevertheless, population restoration was quite slow, thus we suggest that releasing mature and large-sized native fishes in the Datong and Xiaotong rivers would accelerate wild population recovery.

The spatial results indicated that fish communities had a continuous variation from upstream to downstream in the two rivers and are easily disrupted by anthropogenic perturbations. Therefore, protecting single sections of the two rivers is insufficient for conserving the entire rivers' fish assemblage, and the managers should protect the whole river and maintain connectivity. Moreover, it is important in fish conservation to identify areas of high fish biodiversity and select nature reserves to mitigate the loss of fish biodiversity (Fu et al., 2003). Fortunately, most of the Datong and Xiaotong rivers have been incorporated into the "Sichuan Nuoshuihe Precious Aquatic Animal National Nature Reserve" in 2012 by the government (Liang et al., 2021). The Chishui River in China is a free-flowing river which varies gradually in geomorphologic structure along its longitudinal gradient, meanwhile, it is located in "National Nature Reserve for the Rare and Endemic Fishes in Upper Reaches of the Yangtze River" (Wu et al., 2011; Liu et al., 2021). As a result, the fish resource in the Chishui River has now been restored to a certain extent (Liu et al., 2021). Therefore, we consider that designating the Datong and Xiaotong rivers as a nature reserve currently is effective for protecting the longitudinal structure of the fish community.

In terms of temporal dynamics in fish community structure of the two rivers, the following measures should be considered to reduce adverse impacts. (1) Protect the riffle and pool Mesohabitat diversity and reduce the damage to fish diversity caused by human disturbance. (2) Loss of connectivity between riffles and pools may lead to native fish extinctions, maintenance of migration routes of fish is essential for conservation of river fish in karst rivers (Chakona et al., 2022). Thus, it is necessary to take measures such as banning water resources exploitation when flows are below a certain level to keep connectivity between different habitats. (3) Sand excavation activities, particularly in those areas that provide for fish spawning, feeding or overwintering in the Datong and Xiaotong rivers, should be prohibited by the government. Currently, although these mesohabitats are relatively well-protected, there are also potential threats to fish mesohabitat from sand excavation. Moreover, a finding in an African karst river with seasonal variation in fish communities showed that damming and impoundment have negative impacts on fish and improvements in the design of dams to allow fish migration, regulation of the controlled use of the river water would help for fish diversity and abundance (Bem et al., 2021). Therefore, we also suggest that the water-related projects be limited, and the short and long-term biological impacts of dams be monitored to avoid the temporal structure of fish communities being disrupted by human factors.

Conclusion

In summary, we found that the Datong and Xiaotong rivers were dominated by small-sized individuals and few large-sized fish. Additionally, the population of herbivorous and large or medium-sized carnivorous species has declined. The results showed that fish assemblages in two rivers varied significantly along the longitudinal gradient, while there were no significant differences between the four seasons. The spatial differences and no seasonal change in fish assemblage were partially attributed to the diverse habitats of riffles and pools within a relatively short reach of the karst streams and the ecological habit of most fish species with short-distance migration for spawning, feeding or overwintering. Understanding the spatial and temporal dynamics of fish assemblages and what anthropogenic disturbances have contributed to these changes is of great significance to formulate efficient management policy.

Nevertheless, the findings indicated that fish assemblages have been impacted by overfishing. Therefore, we suggest that the managers should take measures such as conducting long-term investigation, releasing mature and large-sized native fishes to restore populations and protecting the riffle and pool mesohabitats and channel connectivity.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the animal study because All individuals were released into the water where captured, except for a few specimens that were anesthetized by MS-222 solution and fixed with formaldehyde solution (5～10%) after death.

Author contributions

PX: investigation, writing, analysis, software, and revision. XW: investigation and writing advice. KL and BW: investigation. CL: writing advice. ZS: revision and writing advice. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by scientific research project of the Bureau of Aquatic Products of Sichuan Province.

Acknowledgments

We thank Qinchao Wen, Yi Lei, Shu Li, and Maohua Li for their assistance in the field sampling and Megan Price for her help in revising the manuscript.

Conflict of interest

BW was employed by Chengdu Jena Environmental Protection Technology Company.

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

Publisher's note

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frontiers | Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 11 October 2022
DOI 10.3389/fevo.2022.990835

Environmental factors indirectly reduce phytoplankton community stability *via* functional diversity

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SPECIALTY SECTION
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

RECEIVED 10 July 2022
ACCEPTED 20 September 2022
PUBLISHED 11 October 2022

CITATION
Zhang X, Tan L, Cai Q and Ye L (2022) Environmental factors indirectly reduce phytoplankton community stability *via* functional diversity. *Front. Ecol. Evol.* 10:990835. doi: 10.3389/fevo.2022.990835

The biodiversity-stability relationship is a fundamental subject of ecological research. Considerable evidence demonstrates that biodiversity can either increase or decrease stability. Most relevant research mainly focuses on grassland and forest ecosystems. The biodiversity-stability relationship in aquatic ecosystems and the underlying mechanisms remain poorly understood. To fill the gap, we conducted a year-long study on the phytoplankton of reservoir ecosystems in the Xiangxi Bay of Three Gorges Reservoir (TGR) to test the following hypotheses: (H1) phytoplankton species richness and functional diversity directly reduce phytoplankton community stability in reservoir ecosystems; (H2) nutrient enrichment and water temperature increasing directly reduce phytoplankton community stability; and (H3) nutrients and water temperature indirectly reduce phytoplankton community stability *via* biodiversity. The structural equation model (SEM) found that functional diversity (community-weighted means of traits and functional divergence) had significant negative correlations with phytoplankton community stability ($p < 0.05$), while the species diversity had no significant correlation with phytoplankton community stability ($p > 0.05$). This finding partially supported the hypothesis H1, which suggested that the functional diversity had a closer tie with stability than the species diversity. SEM did not find any direct effect of environmental factors on phytoplankton community stability, which rejected our hypothesis H2. Instead, SEM found that water temperature and phosphate decreased phytoplankton community stability by increasing the first principal component of the community-weighted means of traits (CWM_PC1), which supported hypothesis H3. Further analysis showed that the increased water temperature and phosphate concentration can promote "r-strategists" species (larger CWM_PC1), which are less resistant to environmental disturbances, therefore decreasing the phytoplankton community stability. Our study highlights the importance of functional diversity in maintaining the relationship between biodiversity and stability in the phytoplankton community, which may provide a mechanistic understanding of the biodiversity-stability relationships in aquatic ecosystems.

KEYWORDS

biodiversity, community-weighted means of traits, functional diversity, ecosystem function, community stability, Three Gorges Reservoir

Introduction

The relationship between biodiversity and ecosystem functioning is a central topic in ecological research (Ptacnik et al., 2008; Filstrup et al., 2014; Ye et al., 2019). A particularly pressing scientific challenge in the context of global change is to understand and predict the impact of biodiversity loss to ecosystem functions, because biodiversity is being lost at an unprecedented rate (Diaz et al., 2007; de Mazancourt et al., 2013; Leps et al., 2018). As one of the fundamental ecosystem functions, ecosystem stability is defined as the ability of a community to restore its original structure and functional state after disturbance (Loreau and de Mazancourt, 2013). The ecosystems that have higher stability are generally supposed to have a high ability to buffer the effects of environmental disturbances (de Bello et al., 2021). Therefore, understanding the drivers that promote ecosystem stability is essential to maintain biodiversity and ecosystem stability, and mitigate the adverse impacts of global change on ecosystems and biodiversity (de Bello et al., 2021; Van Meerbeek et al., 2021).

The relationship between biodiversity and stability is one of the long-term research interests for ecologists (MacArthur, 1955; McCann, 2000; de Bello et al., 2021). Both theoretical and empirical studies have shown that species diversity can either increase or decrease stability, depending on different intrinsic mechanisms (McCann, 2000; Ives and Carpenter, 2007). For example, Craven et al. (2018) reported that grassland community stability increases with grassland diversity increasing due to compensatory dynamics across species. Yet, for the ecosystems in which compensatory dynamics is not the mechanism for diversity dependence, a negative biodiversity-community stability relationship was also observed (Pfisterer and Schmid, 2002). So far, most knowledge on the biodiversity-stability relationship comes from grassland ecosystems and forest ecosystems (Craven et al., 2018; Bongers et al., 2021; Schnabel et al., 2021). Diffusion barriers in aquatic ecosystems are usually weak and energy and material flow within and between habitats are relatively rapid (Giller et al., 2004). Thus, it is usually considered that the dispersal rates of plankton in aquatic ecosystems are faster than other species in terrestrial ecosystems (Ptacnik et al., 2010), implying that aquatic organisms have a higher community turnover rate and may have different biodiversity-stability relationships comparing to terrestrial ecosystems. Exploring the biodiversity-stability relationships in aquatic ecosystems can help us deeper understand the mechanism of community maintenance in aquatic ecosystems, which is crucial for the future development of conservation strategies for aquatic ecosystems (Baert et al., 2016; Danet et al., 2021; Xu et al., 2021).

Beyond taxonomic diversity, there is a growing interest in investigating the relationship between traits-based functional diversity and ecosystem functioning (Ye et al., 2013, 2019; Gross et al., 2017; Bongers et al., 2021). Functional diversity is based on the various traits of species and their adaptations and responses to environmental change. Compared to taxonomic diversity, trait-based functional diversity can better reflect species resource acquisition and niche complementarity (Mason et al., 2005; Poos et al., 2009). Recently, ecologists have begun to focus on the effects of functional diversity and traits on stability (Mouillot et al., 2013; Oliver et al., 2015; de Bello et al., 2021). Theoretically, the dominant species effect, compensatory dynamics, and insurance effect are three of the major mechanisms explaining biodiversity-stability relationships (de Bello et al., 2021). Specifically, the dominant species effect is generally used to explain the relationships between stability and functional diversity; while, the theories of compensatory dynamics and insurance effect are applicable for explaining the relationships between stability and taxonomic and functional diversity (de Bello et al., 2021). The theory of the dominant species effect is derived from the mass-ratio theory (Grime, 1998) and holds that the resistance and recovery of community biomass after disturbance is related to the traits and functional roles of dominant species in the community, rather than to species diversity itself. The compensatory dynamics theories hypothesized that community species have countervailing responses to environmental change (Gonzalez and Loreau, 2009). While the theory of insurance effect emphasizes that the species with the same of similar effects on ecosystem functions have functional redundancy to maintain the community stability (Yachi and Loreau, 1999). Therefore, an interesting question is whether taxonomic or functional diversity is more closely related to the stability in aquatic ecosystems and the mechanism behind it.

Recently, several studies have shown the significance of functional trait-based life history strategies in understanding the functional diversity-stability relationships (Craven et al., 2018; Li et al., 2021; Schnabel et al., 2021). It is generally accepted that "K-strategists" species with larger individuals and more conservative resource acquisition show greater resistance (ability to withstand perturbation) but less resilience (recovery from perturbation) to a disturbance (MacGillivray et al., 1995; Karlowsky et al., 2018). On the contrary, "r-strategists" species with smaller size and rapid resource uptake can recover rapidly after a disturbance and show greater resilience. However, "r-strategists" species are less resistant in the face of environmental disturbances (Pianka, 1970). The "r-K strategists" continuum based on phytoplankton traits in freshwater ecosystems was proposed by Wentzky et al. (2020), who argued that a trait-based rather than taxonomic approach to classifying phytoplankton into "r-K strategists" species is more helpful in the understanding of how phytoplankton communities respond to changes in environmental conditions. Specifically, the "r-strategists" species were characterized by smaller cell size, stronger motility and clustering tendency, ability to take mixed nutrients, silicon-free access capability, and the "K-strategists" species were characterized by larger cell size, weaker motility and clustering tendency, lack of ability to take mixed nutrients, ability to obtain silicon. Previous studies on the phytoplankton "r-K strategists" continuum generally focus on how phytoplankton community structures in response to environmental factors (Wentzky et al., 2020). In this study, we try to investigate the mechanisms underlying the relationship between phytoplankton functional diversity and community stability from the conception of the phytoplankton "r-K strategists" continuum.

Phytoplankton is the basis of food webs in aquatic ecosystems (Litchman and Klausmeier, 2008), accounting for about 50% of total global primary production (Field et al., 1998). The phytoplankton diversity and dynamics are closely related to the biogeochemical cycling processes of elements (Falkowski et al., 2004). Therefore, studying the phytoplankton diversity-community stability relationship is essential for the development of conservation strategies in aquatic ecosystems. Most current research on phytoplankton diversity-community stability relationships is based on the compensatory and insurance effect (Ptacnik et al., 2008; Tian et al., 2017a,b). For example, Tian et al., 2017a,b reported that phytoplankton taxonomic diversity increases phytoplankton community stability due to compensatory effects. On the contrary, the dominant species effect in maintaining the phytoplankton diversity-stability relationship was seldom reported yet. Actually, in some dynamic ecosystems (e.g., reservoir ecosystems), the small-size phytoplankton likely dominate the community (Wang et al., 2011b; Pęczuła et al., 2014). In this case, the dominant species effect may be the main mechanism explaining the relationship of phytoplankton diversity-stability. Therefore, following the theory of the dominant species effect, we hypothesized that phytoplankton diversity directly reduces phytoplankton community stability in reservoir ecosystems because of the dominance of small-size phytoplankton.

In contrast, the impact of changes in environmental factors on biodiversity-stability relationships has received extensive attention from scholars (De Laender et al., 2016; Craven et al., 2018; Li et al., 2021; Schnabel et al., 2021). Phytoplankton community stability is not only affected by diversity but also directly affected by the environment (Hillebrand et al., 2018; Polazzo and Rico, 2021). Research showed that warming will increase the proportion of small-size species in aquatic ecosystems (Daufresne et al., 2009). In the light of this research, we hypothesized that water temperature increase will result in the dominance of small-size phytoplankton species ("r-strategists" species), therefore reducing the phytoplankton community stability. Meanwhile, nutrient enrichment has been recognized as the major factor causing cyanobacterial blooms (Lurling et al., 2018), which was generally constituted by small body-size cyanobacteria (e.g., *Microcystis* sp.) (Filstrup et al., 2014). Therefore, we also hypothesized that nutrient enrichment would reduce the phytoplankton community stability because of the increment of the proportion of small body-size cyanobacteria.

We conducted a year-long study on the phytoplankton of natural aquatic ecosystems in the Xiangxi Bay of Three Gorges Reservoir (TGR). Using the weekly dynamic time series of phytoplankton communities and environmental variables, the causal network between environmental variables, biodiversity, and community stability was reconstructed using piecewise structural equation modeling, which examines the relationship between environmental variables, biodiversity, and community stability. Specifically, we tested the following hypotheses:

> H1: Phytoplankton species richness and functional diversity directly reduce phytoplankton community stability in reservoir ecosystems.

> H2: Nutrient enrichment and water temperature increase directly reduce phytoplankton community stability.

> H3: Nutrients and water temperature indirectly reduce phytoplankton community stability *via* biodiversity.

Materials and methods

Study area

The field sampling site was set up in the middle region of Xiangxi Bay of the TGR (**Figure 1**). The TGR is located in Chongqing and Hubei Province in the upper reaches of the

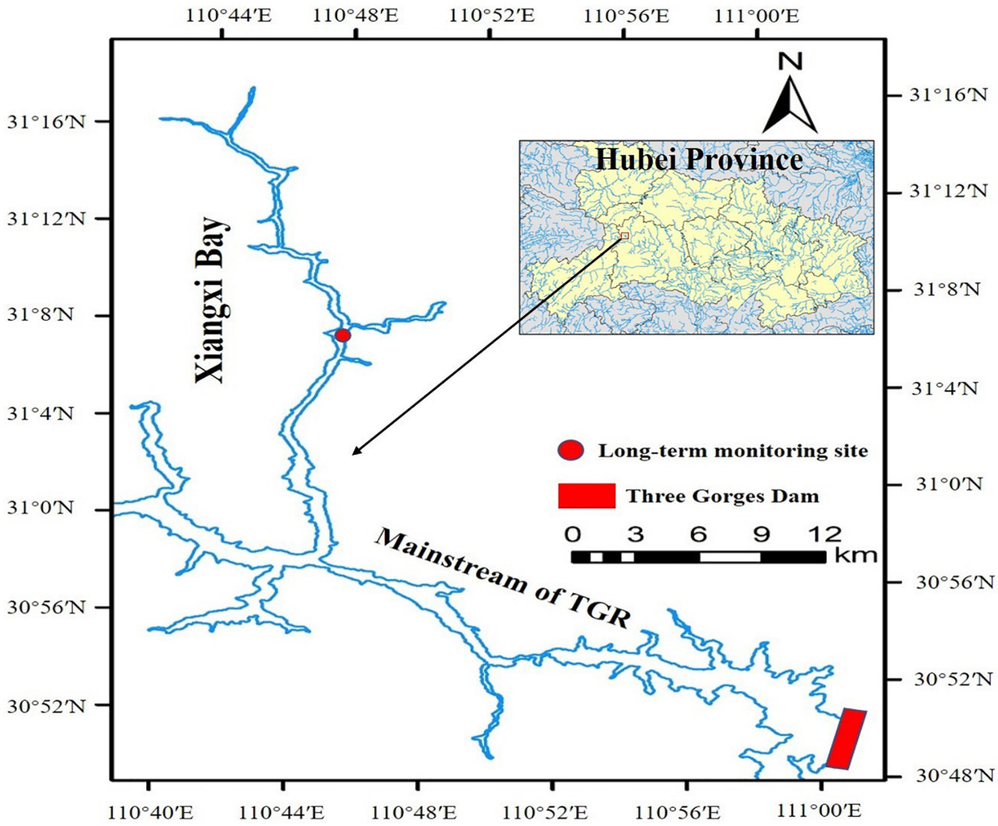

FIGURE 1
Geographical location of sampling site in the Xiangxi Bay of Three Gorges Reservoir (TGR).

Yangtze River, with a total water surface area of 1,080 square kilometers and a total storage capacity of 39.3 billion m^3 (Ye et al., 2022). The TGR is the most giant strategic reservoir of freshwater resources in China and has huge benefits for water resource allocation (Ye et al., 2016). Xiangxi Bay is the largest tributary of the TGR in Hubei Province. After the TGR was impounded, the downstream section of the river for dozens of kilometers became a lake-like slow-flowing water body, called Xiangxi Bay (Ye et al., 2007). We choose Xiangxi Bay as the study site because the previous study showed that small-size species dominate the phytoplankton community (Wang et al., 2011b), which provides an excellent natural study site to test our research hypotheses. The local climate is subtropical monsoon, characterized by warm winters and hot summers, cold early spring and autumn, cloudy and breezy, high humidity, heavy rain, and little frost (Wang et al., 2011a).

Data

The phytoplankton and related environmental data used in this study were obtained from the Xiangxi Bay Ecosystem Station of TGR. Specifically, weekly data from 29 February 2008 to 3 April 2009, for the site in the middle of Xiangxi Bay (**Figure 1**) was selected to test our research hypotheses. During the study period, the weather in the area was typical, with no unusual occurrences of hotter or wetter weather than the annual average (Wang et al., 2011a). The details for the field sampling, phytoplankton, and environmental factors analyses can be found in Ye et al. (2007) and Wang et al. (2011b). In simple terms, the water temperature (WT) was measured *in situ* by a multi-parameter water quality sonde (YSI 6600, YSI Inc., Yellow Springs, OH, United States). The water samples were collected at the surface layer (0.5 m depth) for water quality analyses and phytoplankton identification and counting. The water quality variables, including dissolved inorganic nitrogen (DIN, the sum of the ammonia nitrogen, nitrite nitrogen, and nitrate nitrogen), phosphate (PO_4-P), dissolved silicate (DSi), were analyzed by the segmented flow analyzer (Skalar SAN++, Netherlands) in the lab. Phytoplankton was counted in the Fuchs–Rosenthal chamber by a microscope (Olympus CX21, Japan) at 10 × 40 magnification. The phytoplankton species identification was referred to by John et al. (2002) and Hu and Wei (2006). The summary statistics of the above environmental variables including nutrients and water temperature could be found in (**Supplementary Table 1**) in the supporting information.

Phytoplankton functional and taxonomic diversity

The diversity indices include, species richness (SR), community-weighted means (CWM) of traits (Lavorel et al., 2008), and functional divergence (FD_{iv}) (Villeger et al., 2008) were used to investigate the biodiversity-stability relationship. CWM represents the effect of dominant species in a community on ecosystem stability (de Bello et al., 2021). FD_{iv} represents the degree of ecological niche complementarity among community species (Lavorel et al., 2008). The CWM and FD_{iv} are selected to investigate whether the dominant species effect or the insurance effect is the mechanism determining the relationship between phytoplankton diversity and stability in our study.

Following Weithoff (2003) and Ye et al. (2019), the functional diversity indices were calculated based on five functional traits, including (1) motility, (2) cell size, (3) silica use, (4) mixotrophy, and (5) chains or colonies. Specifically, Motility is classified as 0, no motility; 0.5, motility regulated by air bubbles in the body; and 1, flagellated and able to move actively in water. The cell size takes values ranging from 0.1 to 1. The last three functional traits are binary variables (0, 1), with 1 for having the ability and 0 for not having the ability (Ye et al., 2019).

To reduce the dimension of community-level traits, we performed principal component analysis (PCA) on the community-weighted means eigen values CWM of these five traits. The first principal component explained 73.41% of the total variation of the community-weighted means eigen values of the five traits (CWM_PC1) (Craven et al., 2018). The Bi-plot of principal component analysis (**Supplementary Figure 1**) and its loadings of community weight mean of five functional traits (**Supplementary Table 2**) suggest an "r-K strategists" continuum in life history. Specifically, a larger value of CWM_PC1 corresponds to "r-strategists" species, characterized by smaller cell size, stronger motility and clustering tendency, ability to take mixed nutrients, and silicon-free access capability. Whereas a smaller value of CWM_PC1 corresponds to "K-strategists" species, characterized by larger cell size, weaker motility and clustering tendency, lack of ability to take mixed nutrients, and ability to obtain silicon (Wentzky et al., 2020). PCA was performed with the "PCA" function in the "FactoMineR" package (Lê et al., 2008). Calculation of functional diversity using the "FD" package (Laliberté and Legendre, 2010; Laliberté et al., 2014).

Community stability

The temporal stability of phytoplankton is calculated as the Bray–Curtis similarity (BC) between phytoplankton community samples sampled in two adjacent weeks (Ptacnik et al., 2008; Filstrup et al., 2014; Tian et al., 2017b). The values of BC were calculated by Equation 1. Specifically, in Equation 1, W_{i1} and W_{i2} are the biomass of phytoplankton species i in the two samples; n is the total number of phytoplankton species present in the two sampling samples. The numerator of the above equation represents the summed variation of biomass; the denominator represents the total biomass. Exploring the changes in the numerator and denominator can provide further insight into the mechanisms of community stability changes (Tian et al., 2017b). The BC value is ranged from 0 to 1, and a higher BC value represents higher community stability (Filstrup et al., 2014).

Statistical analysis

To test our hypotheses, we used piecewise structural equation models (SEMs) to analyze the direct and indirect causal relationships between community stability and environmental and biotic variables. SEM is widely used in the field of ecology to investigate direct or indirect relationships between study variables (Schnabel et al., 2021; Li et al., 2022). Following Craven et al. (2018) and Schnabel et al. (2021), we fitted the piecewise SEM using the linear regression models. Based on prior knowledge (Ptacnik et al., 2008; Xu et al., 2021), we hypothesized that biodiversity diversity directly improved community stability and its components (i.e., total biomass and summed variation of biomass) and that these indicators are regulated by environmental variables. In other words, environmental variables can affect the stability of the phytoplankton community, either directly or indirectly, by influencing biodiversity. We built our SEMs using the "piecewiseSEM" package (Lefcheck, 2016).

The relative importance of the two components of stability (i.e., summed variation of biomass and total biomass) in explaining community stability was investigated using linear regression and variance partitioning analysis (VPA). VPA was performed with 999 permutation tests to obtain significance. VPA used the "varpart" function in the "vegan" package (Oksanen et al., 2019). All data analysis was performed on R 4.1.2 (R Core Team, 2021). In all analyses, environmental factors, biodiversity (except CWM_PC1), community stability and phytoplankton biomass were log2 transformed to meet normality assumptions (Craven et al., 2018; Schnabel et al., 2019).

Results

Phytoplankton community stability

The weekly dynamics of phytoplankton community stability calculated by the Bray–Curtis similarity were presented in **Figure 2A**. It can be seen from the figure that though

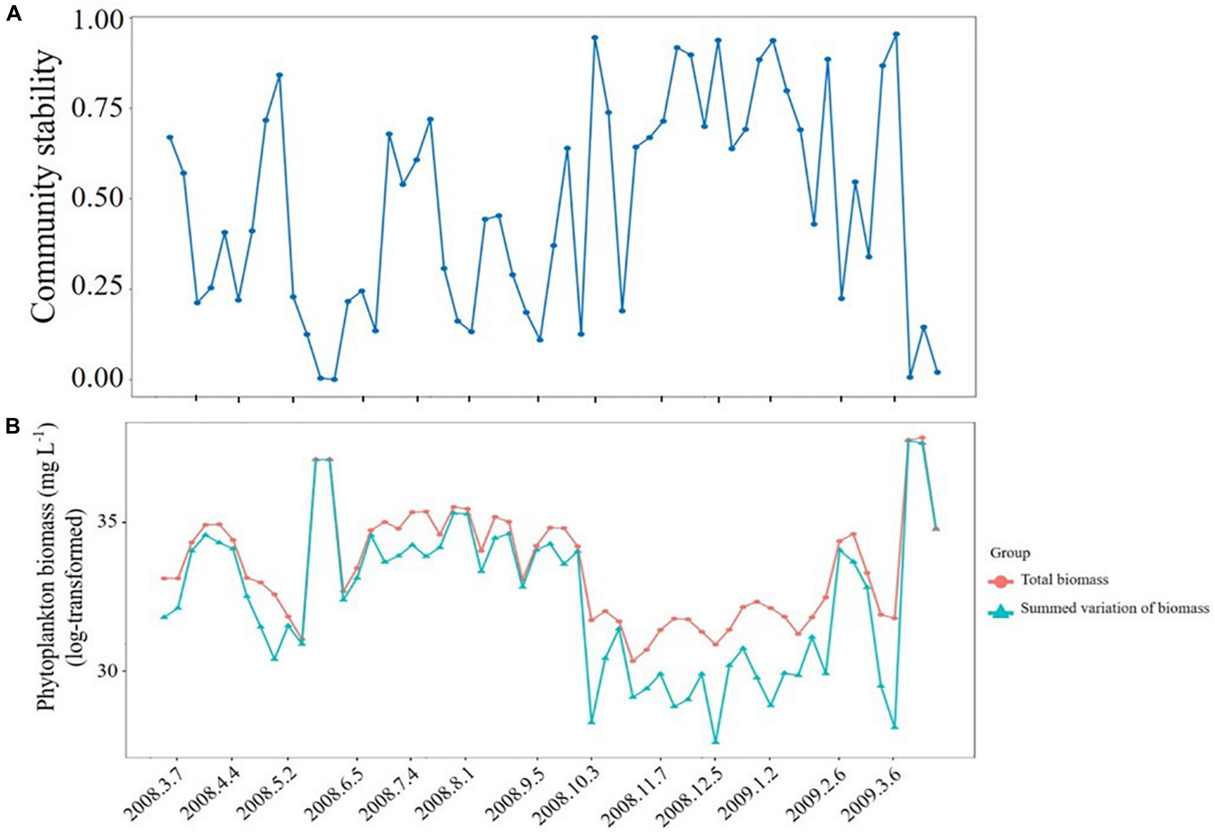

FIGURE 2
Weekly dynamics of phytoplankton community stability (Bray–Curtis similarity) **(A)** and the components of Bray–Curtis similarity shown in panels **(A,B)** in the Xiangxi Bay of Three Gorges Reservoir (TGR).

there was substantial month-to-month variation, community stability was generally lower from March 2008 to October 2008, compared to November 2008 to January 2009. And the community was most stable on 6 March 2009, and the community stability decreased from 6 March 2009, onward. This suggests that the phytoplankton community is more stable from late autumn to early winter compared to spring and summer, but longer-term testing would be needed to confirm this. Components of community stability whose weekly dynamics of summed variation of biomass trended in the opposite direction to that of community stability (Figure 2B). That is, both summed variation of biomass and total biomass were significantly and negatively correlated with phytoplankton community stability (Figures 3A,B), suggesting there was a correlation between high community stability and lower total phytoplankton biomass variation from the points of 3 October 2008, 5 December 2008, and 6 March 2009. Summed variation of biomass and total biomass explained 51 and 23% of the phytoplankton community stability, respectively, and together they explained 14% of the variation in community stability (Figure 3C).

The drivers of the phytoplankton community stability

Our structural equation modeling provides insight into the relationship between biodiversity, environmental factors, and phytoplankton community stability, which provides strong evidence that biodiversity is a key mediator regulating the environmental factor-phytoplankton community stability relationship, and that biodiversity is driven by environmental factors. Overall, our model achieved an acceptable fitness (Fisher's $C = 3.661$, and p-value $= 0.454$) (Figure 4A). All variables together explained 33% of the variation in community stability ($R^2 = 0.33$). The pathway with the greatest impact on community stability was (standardized path coefficient of direct effect $= -0.50$) CWM_PC1, which directly reduced community stability, and FD_{iv} had a similarly strong negative effect on community stability (standardized path coefficient of direct effect $= -0.37$). Direct pathways of SR effects on community stability were not significant. Furthermore, we found that PO_4-P and WT affected stability through indirect pathways, i.e., WT and PO_4-P enhanced CWM_PC1 to reduce

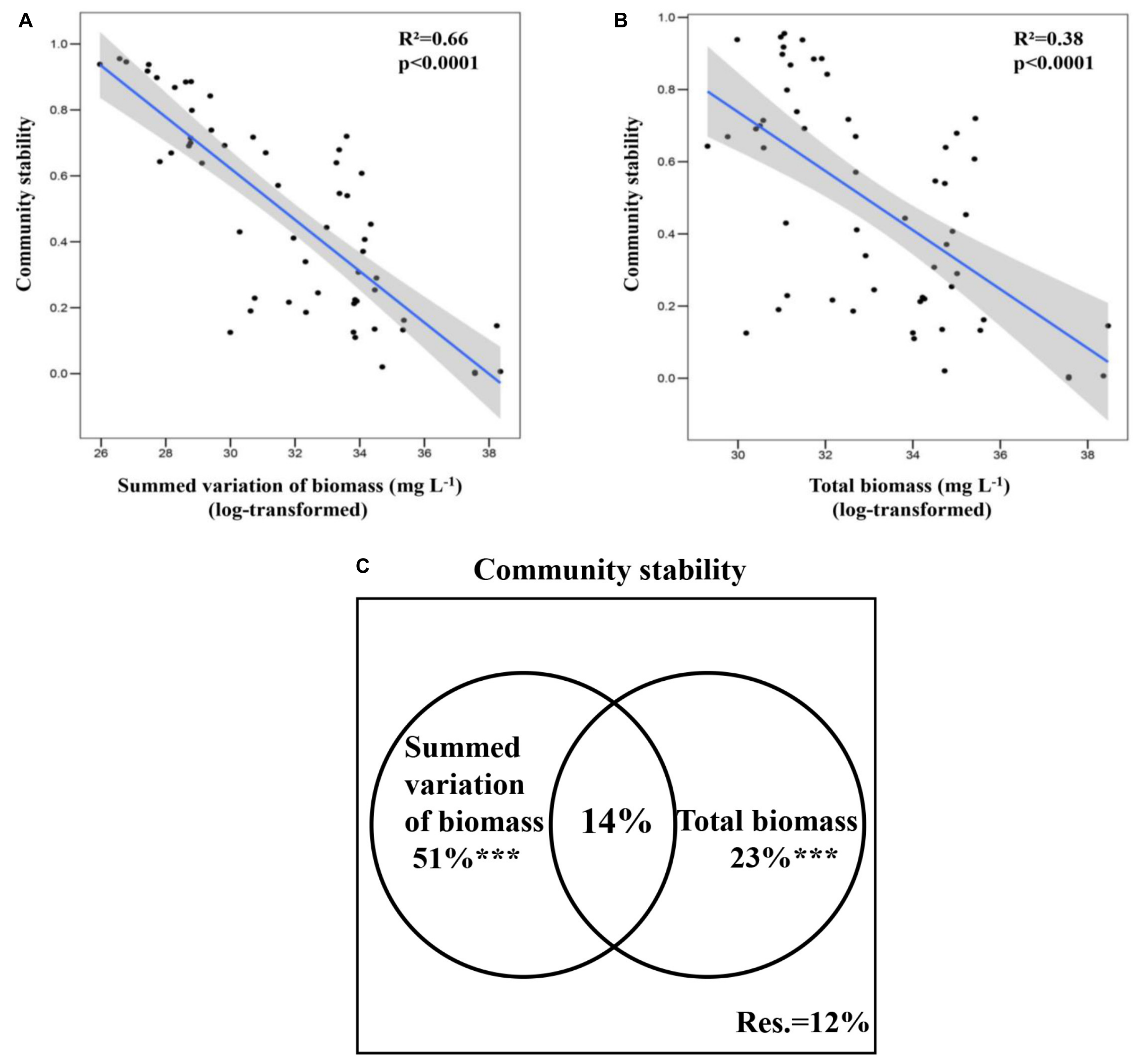

FIGURE 3
Linear relationships between phytoplankton community stability (Bray–Curtis similarity) and summed variation of biomass **(A)** and total biomass **(B)**. **(C)** Shows the variance partitioning result for phytoplankton community stability. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

community stability. The direct pathways of PO_4-P, WT, DIN, and DSi on community stability were not significant (Figure 4A).

Further, we investigated how environmental factors and diversity affect the community stability for a deeper understanding of the mechanisms behind the biodiversity-stability relationship (Figure 4B). All variables together explained 40% of the total biomass ($R^2 = 0.40$) and 44% of the summed variation of biomass ($R^2 = 0.44$). We can see that all the paths that affect the total biomass are not significant (Figure 4B), and the path that has the greatest impact on the summed variation of biomass (standardized path coefficient of direct effect = 0.48) is that CWM_PC1 directly increases the summed variation of biomass, which indicates that CWM_PC1 decreases community stability by increasing summed variation of biomass. Similar to CWM_PC1, species richness also improved the summed variation of biomass (standardized path coefficient of direct effect = 0.47). Environmental variables affect the summed variation of biomass through direct or indirect pathways, WT increases the summed variation of biomass by promoting CWM_PC1 and species richness, thereby reducing the community stability. PO_4-P positively acting on CWM_PC1 increased the summed variation of biomass, while negatively acting on SR decreased the summed variation of biomass. DIN can reduce the

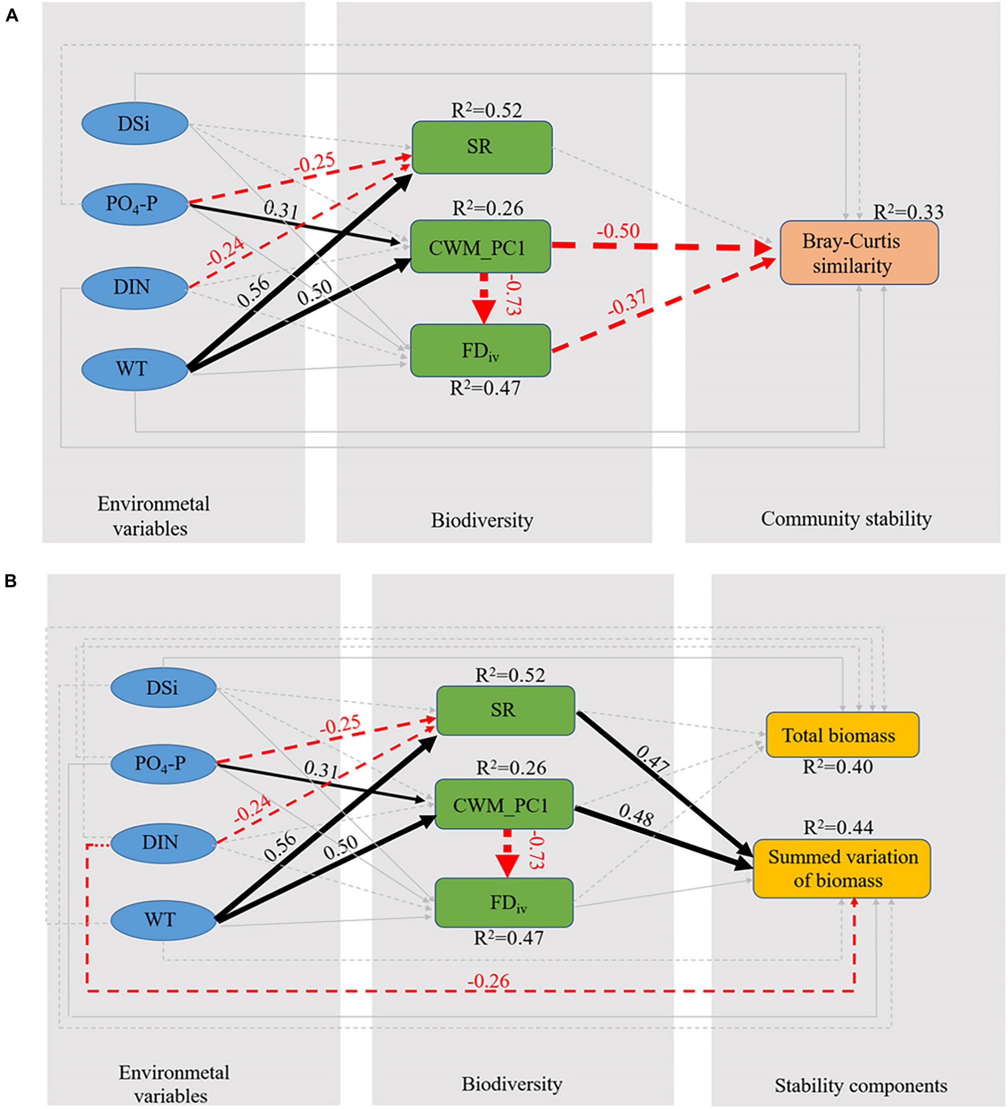

FIGURE 4
Piecewise structural equation model (SEM) depicts the relationships between the environment variables, biodiversity and community stability **(A)**. Overall, the model fits the data well: Fisher's C = 3.661, AIC = 59.661, and p-value = 0.454. Piecewise SEM depicts the relationships between the environment variables, biodiversity and community stability components **(B)**. The model fit the data well: Fisher's C = 3.661, AIC = 79.661, and p-value = 0.454. The solid and dashed arrows represent positive and negative relationships, respectively, and the light-gray arrows represent non-significant relationships ($p > 0.05$). Dissolved silicate (DSi), phosphate (PO$_4$-P), dissolved inorganic nitrogen (DIN), water temperature (WT), phytoplankton species richness (SR), the first principal component of the community-weighted means of traits (CWM_PC1), functional divergence (FD$_{iv}$), Bray–Curtis similarity between phytoplankton community samples sampled in two adjacent weeks (Bray–Curtis similarity), the total biomass of phytoplankton samples from two adjacent weeks (Total biomass), summed variation of biomass of phytoplankton samples from two adjacent weeks (Summed variation of biomass).

summed variation of biomass directly or by reducing SR (Figure 4B).

Discussion

Relationship between biodiversity and phytoplankton community stability

We found that phytoplankton community stability had no significant correlation with the species richness but had a significant negative correlation with CWM_PC1 (Figure 4A). This finding revealed that CWM_PC1 is the most important biotic factor driving phytoplankton community stability (Figure 4A) and supported our hypothesis that functional diversity directly reduced phytoplankton community stability in reservoir ecosystems. In our case, larger values of CWM_PC1 indicated that the phytoplankton community was more unstable because the community was dominated by "r-strategists" species (characterized by smaller cell size, stronger motility and clustering tendency, ability to take mixed nutrients, and silicon-free access capability). Conversely, smaller values of CWM_PC1 indicated that the phytoplankton community dominated by "K-strategists" species (characterized by larger cell size, weaker motility and clustering tendency, lack of ability to take mixed nutrients, and ability to obtain silicon), which can maintain a more stable community. We also found that CWM_PC1 reduced community stability by increasing summed variation of biomass (**Figure 4B**). When communities were dominated by "r-strategists" species, they were less resistant to environmental stresses and thus showed greater variation in community biomass, which reduced community stability (Donohue et al., 2013).

Our study is consistent with a recent study conducted in grassland ecosystems (Craven et al., 2018). Craven et al. (2018) found that communities dominated by "slow" species (low specific leaf area, low leaf N and P, and high leaf dry matter content) are more stable than those dominated by "fast" species (high specific leaf area, high leaf N and P, and low leaf dry matter content) because "slow" species can enhance ecosystem stability (the ratio of the mean and standard deviation of biomass) by increasing mean above-ground biomass production. When considering different disturbances, communities species dominated by "r-strategists" species can maintain community stability when a system is subjected to persistent environmental fluctuations (environmental stochasticity) due to stochastic factors in the biotic or abiotic environment due to the ability to recover quickly, while communities dominated by "K-strategists" species may maintain community stability in the face of pulse perturbation (Donohue et al., 2016; Kefi et al., 2019). The "r-strategists" species should be more resilient, while the "K-strategists" species should be more resistant (Kefi et al., 2019). Previous studies showed that water level fluctuations will affect the dynamic of phytoplankton in the TGR (Wang et al., 2011a,b; Ye et al., 2022). Therefore, how water level fluctuations influence the phytoplankton diversity-stability relationships is an interesting research question and should be explored in future studies.

Structural equation model found a significant negative relationship between FD_{iv} and community stability (**Figure 4A**). This is contrary to some previous studies on the phytoplankton diversity-stability relationships (Ptacnik et al., 2008; Tian et al., 2016, 2017b). However, the negative correlation between phytoplankton biodiversity (evenness) and phytoplankton community stability was supported by Filstrup et al. (2014). Filstrup et al. (2014) studied the lakes are eutrophic and the phytoplankton communities were dominated by a few dominant species of the cyanobacteria, especially the *Microcystis* sp., limiting the growth and reproduction of other phytoplankton species. The reduction in phytoplankton community turnover in cyanobacteria-dominated ecosystems indicates an "inhibition model" of succession (Connell and Slatyer, 1977), similar to the formation of closed canopy shrubs in terrestrial ecosystems, and inhibits the growth of trees. *Microcystis* blooms can also reduce weekly community turnover by competitively excluding or inhibiting other taxa through the efficient use of TP supply (Reynolds, 2006) or the chemosensory effects of secondary metabolites (Pflugmacher, 2002). Previous studies showed that Xiangxi Bay is facing a serious eutrophication problem because of the pollutants in its watershed and the associated phytoplankton bloom in the bay (Wang et al., 2011b; Ye et al., 2014, 2022). Specifically, in our study, cyanobacterial biomass accounted for 51% of the total phytoplankton community biomass, and the cyanobacterial was dominated by *Microcystis* sp. and *Anabaena* sp., which is consistent with the study reported by Filstrup et al. (2014), suggesting the biodiversity-stability relationship was also determined by the dominant species effect.

We note that CWM_PC1 has a significant negative effect on FD_{iv} (standardized path coefficient of direct effect = −0.73) (**Figure 4A**). FD_{iv} describes the formation of species clusters in the character space, where a higher degree of divergence indicates that species groups are distributed at the edges of the character space (Mason et al., 2005; Villeger et al., 2008). For example, divergence is lower when the most abundant species have functional traits close to the center of the functional trait range; conversely, divergence is higher when the most abundant species have extreme functional trait values. In multivariate environments, functional divergence is related to the distribution of abundance within the functional trait space occupied by the species (Villeger et al., 2008). CWM is defined as the average of the values present in the community, weighted by the relative abundance of taxa with each value (Lavorel et al., 2008), Lavorel et al. (2008) argue that both CWM and FD_{iv} measures require characterization of taxa and information on their relative abundance, and thus both CWM and FD_{iv} depend on the characterization values among the more abundant species within the community distribution of the two indices, and there is some similarity in the properties of these two indices. We also found that an increase in the CWM_PC1 index caused a decrease in the FD_{iv} index, i.e., the dominance of "r-strategists" species led to a decrease in FD_{iv}, and since FD_{iv} has a negative effect on stability, CWM_PC1 indirectly maintained phytoplankton community stability by decreasing FD_{iv}.

Our study has not found a significant relationship between species richness and phytoplankton community stability (**Figure 4A**). Functional diversity reflects the differentiation of functional traits of community species, and trait-based functional diversity better explains the variation of phytoplankton community stability through ecological niche differentiation (Mouillot et al., 2013; de Bello et al., 2021). The functional diversity index was suggested to be superior to species diversity in explaining the stability of the phytoplankton community.

Environmental variables indirectly reduce phytoplankton community stability *via* functional diversity

Our SEM found that water temperature and phosphate indirectly reduced phytoplankton community stability *via* functional diversity, which supports hypothesis H3. The direct effects of the four environmental factors on phytoplankton community stability were not significant. It is widely considered that warming will affect the size structure of organisms in ecosystems (Daufresne et al., 2009) and thereafter changes ecosystem function directly or indirectly (Burgmer and Hillebrand, 2011; Yvon-Durocher et al., 2015). Our study shows that WT increases summed variation of biomass by increasing CWM_PC1 values (**Figures 4A,B**) (i.e., increasing temperature promotes the growth of "r-strategists" species and suppresses the growth of "K-strategists" species), thus reducing community stability. At the same time, our study is consistent with previous findings that elevated temperatures affect biodiversity, which disrupts ecosystems and increases the volatility of phytoplankton community biomass (Beninca et al., 2011; Bestion et al., 2021). Wentzky et al. (2020) found that spring phytoplankton communities with lower temperatures were dominated by "r-strategists" species, and summer phytoplankton communities with higher temperatures were dominated by "K-strategists" species. Our results differ from the study of Wentzky et al. (2020), and further comparisons show that the lakes studied by Wentzky et al. (2020) differed not only in temperature but also in nutrients between spring and summer, i.e., higher nutrient content in spring and lower nutrient content in summer lakes. Differences in nutrient content between seasons are also factors to be considered in causing a species shift in the "r-K strategists."

PO_4-P was positively correlated with CWM_PC1, indicating that PO_4-P promoted the growth of "r-strategists" species and increased summed variation of biomass, thus reducing community stability (**Figures 4A,B**). According to previous studies in Xiangxi Bay, phosphorus was found to be the main limiting nutrient for algal growth and blooms in this bay (Huang et al., 2020). According to the Redfield ratio (Redfield, 1958), the stoichiometric ratio of 16N: 1P (in atoms) is a widely used criterion in determining nutrient limitation in aquatic ecosystems. According to our study, we found that the N/P ratio was greater than 16 in 42 of 57 samples, indicating that the phytoplankton in Xiangxi Bay may be limited by phosphorus resources. Nutrient inputs are considered to be important drivers of species turnover (Jochimsen et al., 2013) and diversity (Lehtinen et al., 2017), suggesting that diversity may be a necessary mediator of nutrient effects on stability. The study by Jochimsen et al. (2013) also reported that community changes were closely related to changes in phosphorus, i.e., as nutrients increased, increases in some taxa were offset by decreases in others, thus altering the resilience of phytoplankton community biomass. Therefore, the increase in effective phosphorus concentration in Xiangxi Bay may increase the risk of autumn blooms, suggesting that management strategies need to pay special attention to phosphorus loading and control.

Conclusion

In this study, we investigated how environmental factors, species and functional diversity affect phytoplankton community stability. We found that the functional diversity indices, community-weighted means of traits and functional divergence, had significant negative correlations with phytoplankton community stability ($p < 0.05$), while the species richness had no significant relationship with the phytoplankton community stability ($p > 0.05$). This indicated that functional diversity can better explain phytoplankton diversity and community stability than species richness. Meanwhile, we found that the environmental factors have no direct impact on stability, but indirectly reduce phytoplankton community stability through changing functional diversity. Specifically, water temperature and phosphate are the most important environmental factors indirectly affecting phytoplankton community stability. We found that increasing water temperature and PO_4-P will promote the first principal component of the community-weighted means of traits (CWM_PC1), and thereafter decrease phytoplankton community stability. Further analysis found that the increased water temperature and PO_4-P concentration promoted the growth of "r-strategists" species and inhibited the growth of "K-strategists" species, thereby reducing phytoplankton community stability. Our study suggests that global warming and eutrophication will bring significant negative effects on phytoplankton community stability and highlights the important role of functional diversity and traits in maintaining the stability of aquatic ecosystems.

Equations

$$\mathrm{BC} = 1 - \frac{\sum_{i=1}^{n} |W_{i1} - W_{i2}|}{\sum_{\mathrm{i=1}}^{\mathrm{n}} (W_{i1} + W_{i2})} \tag{1}$$

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author contributions

XZ was responsible for the analyses and interpretation of the data and wrote the original draft. QC was responsible for the conception. LT provided the methodology and conducted the sample analysis. LY was responsible for the administration and supervision of the project and writing—review and editing and contributed to the conception. All authors read and approved the final manuscript.

Funding

This study was supported by the National Key R&D Program of China (2018YFD0900902) and the National Natural Science Foundation of China (31670534 and 31300392).

Acknowledgments

This manuscript was inspired by discussions with ecologists in the field of ecology, we are grateful to Zhouyuan Li (Beijing Forestry University) and Wang Tian (North China Electric Power University) for providing manuscript ideas and theoretical support. We also thank two reviewers for their valuable comments and suggestion in improving this manuscript.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.990835/full#supplementary-material

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frontiers | Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 10 January 2023
DOI 10.3389/fevo.2022.1062612

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to
Conservation and Restoration Ecology,
a section of the journal
Frontiers in Ecology and Evolution

RECEIVED 06 October 2022
ACCEPTED 12 December 2022
PUBLISHED 10 January 2023

CITATION
Feng K, Yuan J, Zhang Y, Qian J, Liu J, Li Z, Lek S and Wang Q (2023) Application of artificial spawning substrates to support lacustrine fish recruitment and fisheries enhancement in a Chinese lake. *Front. Ecol. Evol.* 10:1062612. doi: 10.3389/fevo.2022.1062612

Application of artificial spawning substrates to support lacustrine fish recruitment and fisheries enhancement in a Chinese lake

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Lake littoral zones with abundant submerged vegetation are considered to harbor diverse biotic communities and additionally serve as spawning grounds for important fishery resources. In recent decades, due to various human activities submerged vegetation in lakes have declined dramatically resulting in the function loss of spawning grounds. In order to enhance the recruitment of lacustrine fish (mainly *Carassius auratus* and *Cyprinus carpio*) and the proliferation of fishery resources, we explored a method of constructing artificial spawning ground based on artificial spawning substrate in lake. The results of present study indicate that the artificial substrates with complex and dense three-dimensional structure which have enough space to enable material exchange and oxygen circulation could be efficient to enhance fish recruitment. The water temperature of spawning peaks of artificial substrates were within the range of 20~25°C, and the water temperature provides a basis for the deployment period of artificial spawning grounds. The spawning substrate in the upper layer of water column was more attractive for fish to spawn. The above results provide guidance for the deployment of artificial spawning grounds from both temporal and spatial scales. Observations on the behavior of aggregations found that spawning activities mainly occurred at night and in the early morning. The abundance of larger individuals (>20cm), as potential spawning groups, increased during this period. The above findings are informative to the construction and management of artificial spawning ground in lakes.

KEYWORDS

artificial spawning ground, Liangzi Lake, spawning behavior, phytophilic spawners, fishery resources, habitat restoration

1. Introduction

Submerged vegetation in lake littoral zones is often considered to provide important structure habitat for many invertebrate and fish species (Pawlikowski and Kornijów, 2019; Bolduc et al., 2020). It has been extensively verified that the decline of submerged vegetation is an important cause of loss of aquatic biodiversity and degradation of ecosystem function in lakes (von Nordheim et al., 2018; Pawlikowski and Kornijów, 2019). The decline in structural complexity of submerged vegetation may directly compromise the nursery functions of many invertebrate and fish species, including spawning, foraging, and overwintering (Heck et al., 2003; Knaepkens et al., 2004; Churchill et al., 2016). One of the most important ecological functions of aquatic vegetation is to provide spawning substrates for a wide range of phytophilic spawners (Heck et al., 2003; Miano et al., 2019). Although the function of supporting spawning is common knowledge in aquatic ecology, this important ecosystem service has been rarely studied in details and receives little attention in current lake ecosystem management programs (Marsh et al., 2022). However, fish dependent on submerged vegetation for spawning are important component of the food web and important fishery resources, such as cyprinids in lakes (such as common carp, *Cyprinus carpio*; and crucian carp, *Carassius auratus*). The fecundity of phytophilic cyprinids in lakes is thought to be closely dependent on the availability of submerged vegetation (Liu and He, 1992).

However, lakes have suffered a significant loss of submerged vegetation in the past decades due to human activities such as reclamation of paddy fields and aquaculture ponds, anthropogenic regulation of water levels, over-stocking of herbivorous fish, urbanization, and eutrophication (Du et al., 2011; Wang et al., 2022). These human activities have led to an increase in water turbidity and resulting sharp shrinkage in the distribution of submerged vegetation and shift in species composition from diverse species to homogeneous and/or seasonal species such as *Potamogeton crispus* (Liu and Wang, 2010; Zhang et al., 2018). In this regard, the impairment of natural spawning ground for phytophilic spawners in lakes is strongly associated with the decline of submerged vegetation in the littoral zones (Kanstinger et al., 2018). However, restoration of submerged vegetation is a lengthy and expensive process and is often not feasible in hypereutrophic lakes (Zeng et al., 2017; Slagle and Allen, 2018). In recent years, restoration of degraded fish spawning habitats has become a priority practice for fisheries development and biodiversity conservation (Palm et al., 2007; Manny et al., 2014; Riebe et al., 2014). Specifically, artificial spawning substrates such as artificial macrophytes, shrubs and nets and so on have been used as medias to assist fish spawning and improve spawning habitat quality to increase spawning and restore fish populations (Gillet and Dubois, 1995; Nash et al., 1999; Lehtonen et al., 2006; Čech et al., 2012; Crane and Farrell, 2013; Malinovskyi et al., 2018).

Nowadays, the effectiveness of artificial spawning substrates in enhancing fish recruitment and the construction and management of artificial spawning grounds are increasingly being studied. Among them, the selection of artificial spawning substrate materials and structures is the most widely reported (Gillet and Dubois, 1995; Nash et al., 1999; Lehtonen et al., 2006; Čech et al., 2012; Crane and Farrell, 2013; Malinovskyi et al., 2018; von Nordheim et al., 2018). Complex and rigid substrates are the better choice for constructing artificial spawning grounds (Gillet and Dubois, 1995; Nash et al., 1999). Artificial materials are superior to natural materials in providing a healthy incubation environment for eggs, especially the provision of adequate oxygen (Čech et al., 2012; Malinovskyi et al., 2018; von Nordheim et al., 2018). Environmental factors such as water temperature, depth, waves, flow velocity, and sunshine duration can all affect the efficiency of artificial spawning substrates (Gillet and Dubois, 1995; Nash et al., 1999; Čech et al., 2012). Therefore, it is very important to determine the timing and location of artificial spawning grounds. Proper placement and regular maintenance can maximize the effectiveness of artificial spawning substrates (Nash et al., 1999). Artificial spawning substrate is an ideal material for studying fish reproduction behavior, and imaging sonar is considered to be the most effective, safe and environment-friendly observation method for fish reproduction behavior (Pavlov et al., 2009; Langkau et al., 2016; Smith et al., 2021). The reproductive behavior of fish is a guideline for developing strategies for the protection and management of artificial spawning grounds. However, very little knowledge exists about the effects of artificial spawning substrates on lacustrine phytophilic spawners.

This study aimed to (1) according to the effects of spawning substrates of different structures and materials on egg adhesion and egg hatching, explore effective materials for constructing artificial spawning grounds; (2) according to the influence of water depth and water temperature on the effect of artificial spawning substrates, explore the effective deployment period and water column position of artificial spawning grounds; (3) according to the diurnal rhythm patterns and spawning population composition of fish in artificial spawning grounds, explore the effective management and protection strategies on spawning population of artificial spawning grounds. The results of this field study can provide insights in managing these phytophilic spawning populations and can guide habitat restoration or conservation efforts in lakes.

2. Materials and methods

2.1. Study area

The study was carried out in Liangs Lake (Figure 1, 114.34 ~ 114.85°E; 30.04 ~ 30.43°N) which is a typical shallow lake with a surface area of 271 km^2 in the Yangtze River basin. It is the also largest impounded lake in Hubei Province and extensively covered with macrophytes (such as *Potamogeton maackianus, Vallisneria natans*) until the extreme flood in the Yangtze River basin in 2010 (Wang et al., 2019).

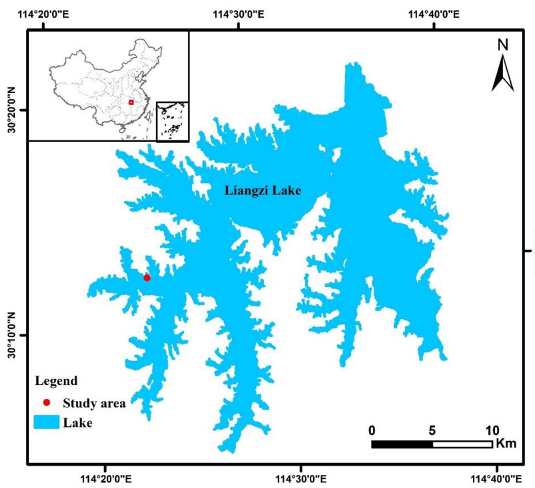

FIGURE 1
Map of Liangzi Lake and study site.

2.2. Artificial substrates

Three types of artificial substrates with different morphologic complexity and material were used in current study (Figure 2A; Figure A1 in Appendix). These artificial substrates were palm sheet (PAS; length: 450 mm, width: 300 mm), polyethylene filament (PEF; diameter: 0.3 mm, length: 350 mm) and polyethylene net (PEN; mesh size: 2.5 mm, length: 300 mm, width was 75 mm), respectively. PAS is a kind of natural material and has the highest complexity of three materials. The structure of PEF in water is more complicated than that of PEN, although they are all man-made materials. Each type of artificial substrate was used to make three-layers of circular spawning nests (diameter: 300 mm; height: 1500 mm; vertical interval between each layer: 500 mm). Each layer of circular spawning nest was consisted of four bundles of the same substrates fixed at four corners on a steel ring. One bundle of PAS has 3–4 pieces, PEF has about 1,000 filaments, PEN has 6–8 pieces. Each unit of three-layers of circular spawning nests was installed with a colored float and steel weight. The vertical height between the spawning nests from top to bottom and water surface were 200 mm, 700 mm, 1,200 mm, respectively (Figure 2A).

The artificial spawning grounds were deployed in the littoral zone with an average water depth of 2.5 m in Liangzi Lake on March 21, 2018. For each type of artificial spawning substrate, there are 90 units of artificial spawning nets which were arranged in 5 lines at 10 m intervals. In total, 18 units of the same artificial spawning substrates were connected at 2 m intervals between each unit using 20 mm diameter nylon rope following the water depth contour in Liangzi lake. Moreover, there was an interval of 20 m between adjacent types of artificial spawning grounds (Figure 2B). It should be noted that, in practical applications, the artificial spawning substrate must exist in the form of artificial spawning grounds, that is, it will not exist as a separate unit. Therefore, this study chose to build three artificial spawning grounds in similar locations with relatively homogeneous water environment. The difference between these three is only the difference of the spawning substrate material used. And there is a certain interval between the spawning grounds of different materials. In this way, the difference of artificial spawning substrates of different materials in practical application (used in combination to construct spawning grounds rather than isolated) can be studied. Therefore, this study did not adopt the method of random arrangement of artificial spawning matrix units of different materials.

2.3. Sampling strategy

The *in-situ* monitoring experiment of spawning activities was conducted daily between March 21 and June 7, 2018. According to records, the breeding of common carp and crucian carp in the Liangzi lake starts in April and basically ends in June. The main spawning period of the studied species was therefore basically included in the study period. Although the spawning time of a few of their individuals may be delayed until the end of June, but the high temperature will make the number of spawning eggs very small (Department of Ichthyology, Institute of Hydrobiology, CAS, 1976). Viable and dead eggs on the three types of spawning substrates were counted daily, which could be determined by the natural transparency of originally vital eggs and cloudiness of dead eggs (white color) (von Nordheim et al., 2018). Egg retention was randomly inspected daily on one unit of spawning nests along the five lines, hence there were five replicates for each type of artificial spawning ground. And the exact same relative position was sampled in 1 day in the spawning grounds composed of three materials, so as to ensure the comparability of the data of artificial spawning substrates made of different materials. Three layers of circular spawning nests from top to bottom were counted, respectively. The specific method of egg counting is to take a fixed number of pieces or filaments spawning substrates out of the water surface and count the attachment of eggs on the spot. To verify field data and reduce counting errors, digital images of sampled substrates on every surface were taken, and the viable and dead eggs were counted with an imaging software Image J (version 1.38x, Wayne Rasband, National Institute of Health, 2008). The sampled surface area of each material was calculated.

A certain number of viable eggs were sampled together with spawning substrates and used for hatching experiment to estimate hatchability when the spawning peak occurred in the field. Incubation experiments are carried out at the station near the lake and use lake water for incubation to ensure the environment is close to the lake environment. Subsequently, the hatched fry was reared for 1 week to calculate survival rate. When the fry has grown to a size that can identify the species, they are sampled and identified under a microscope based on morphology. According to

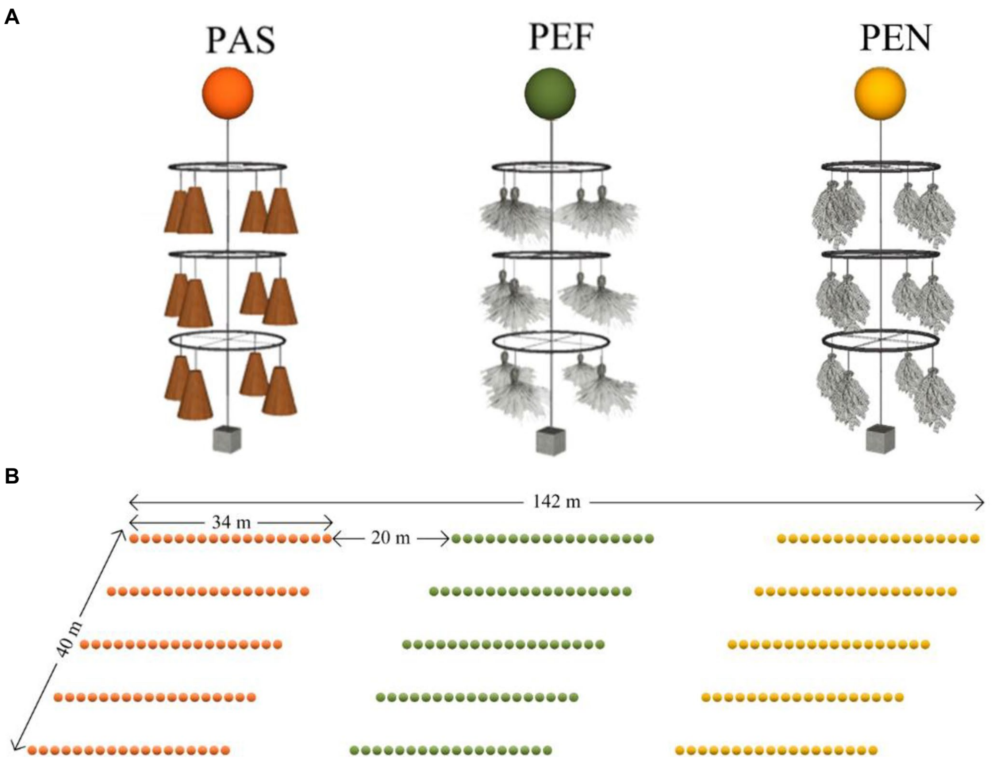

FIGURE 2
The structure of artificial spawning substrates **(A)** and arrangement of artificial spawning grounds **(B)**. PAS, palm sheet; PEF, polyethylene filament; PEN, polyethylene net.

microscopic identification, the fry hatched from eggs attached to the artificial substrates were dominated by crucian carp (*Carassius auratus*) and common carp (*Cyprinus carpio*).

2.4. Spawning monitoring

Dual-frequency identification sonar (DIDSON, ARIS Explorer 3,000, Sound Metrics) was used to observe the spawning behavior, monitor the spawning peak and measure the body length of spawning groups on the artificial spawning grounds. It is worth mentioning that this method cannot identify species. DIDSON operates at two frequencies, providing two modes. Identification mode, which provides high resolution (operating frequency is 3.0 MHz) and low range (nominal effective range is 5 m). Detection mode, which provides lower resolution (operating frequency is 1.8 MHz) and high range (nominal effective range is 15 m). In this study, we used the detection mode to broaden the observational range in the artificial spawning ground. The beam array was 128 beams at the low-frequency setting, with beamwidth of 0.3°H by 14°V. This array of beams is assembled into a sector along the narrow dimension of the beams to constitute a 30° × 15° field of view. The downrange resolution is 0.003 m. The max frame rate is 4–15 frames/s. The probe was about 20 cm below the surface and the field of vision was horizontal. In this way, the probe was at the same water depth as the upper rings. Through early surveys, the PAS was the favored artificial spawning substrates. Therefore, the observation site of DIDSON was arranged in the PAS spawning ground. The probe was fixed on a boat and the observation distance was set to 0.7 ~ 11.5 m. The direction of the probe was almost coincided with the first row of the PAS spawning ground. As the result, there were 5 rings of artificial spawning substrates in the monitor vision of DIDSON. The spawning peak and body length of spawning groups were monitored by DIDSON during April 8 to April 12, 2018. From the perspective of the whole study period, the spawning monitoring period was in the third spawning peak (Figure 3). Although the spawning intensity of fish at each spawning peak was different, according to the distributison of fish spawning periods in Liangzi Lake, common carp and crucian carp were still the main spawning species during this monitoring period (Department of Ichthyology, Institute of Hydrobiology, CAS, 1976). Therefore, the fish behavior and spawning assemblage composition observed during the monitoring period are representative of the study.

2.5. Water temperature monitoring

To study the relationship between fish spawning behavior and water temperature, water temperature of artificial spawning

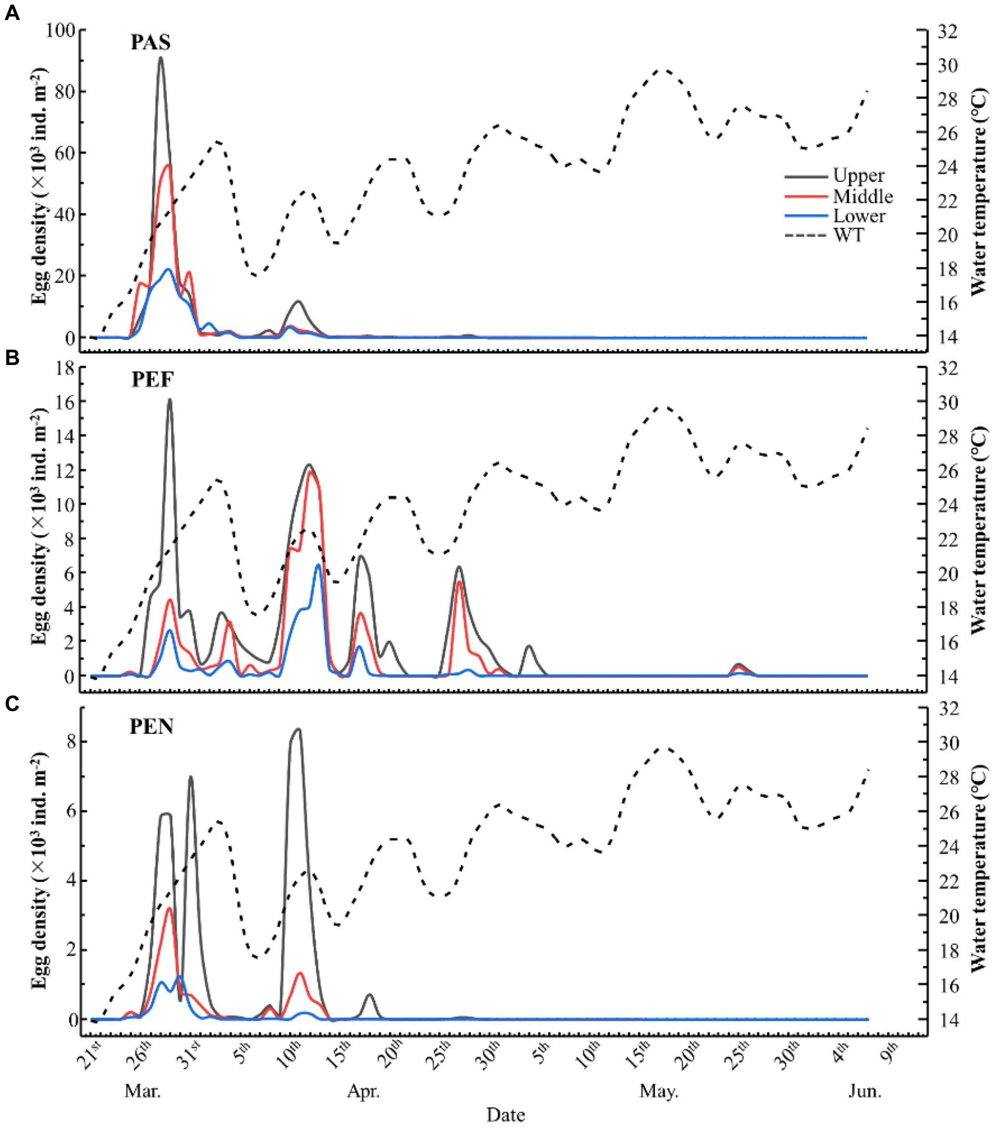

FIGURE 3
The relationship between daily water temperature and egg density among substrate groups and water layers. PAS, palm sheet; PEF, polyethylene filament; PEN, polyethylene net.

ground was recorded every 2 h under 0.5 m from the water surface using water temperature recorders (HOBO, Onset Computer Corporation) during study period.

2.6. Data analyses

2.6.1. Egg deposition and mortality

Egg deposition was determined by counting the eggs attached to the artificial substrate and measuring the surface are of artificial substrate. Each artificial spawning substrate had five replicates in each water layer (upper, middle, and lower). Egg density on artificial spawning substrate was calculated by the following equation:

$$\text{Egg density}\left(\text{ind.m}^{-2}\right) = \frac{n\text{ attached eggs}}{\text{surface area of substrate.}}$$

The percentage of dead eggs attached on the artificial substrate was calculated by the following equation:

$$\text{Egg mortality}\left[\%\right] = \frac{n\text{ dead eggs} \times 100}{\text{total } n \text{ of eggs}}$$

The pairwise comparisons of daily egg density and egg mortality among different artificial substrates and water layers were conducted when the spawning events were observed.

2.6.2. Hatching rate and survival rate

In total, 22, 20 and 26 batches (about 100 eggs per batch) of eggs from PAS, PEN and PEF were used in hatching experiment, respectively. The initial number of eggs in each batch (n_1) were reared until hatch where surviving fry were enumerated to determine egg hatch number (n_2). Initial numbers of fry (n_2) were enumerated after 7 days to determine the 7-day survival rate (n_3). The hatching rate (the probability of eggs hatching successfully), 7-day survival rate (the probability of hatched fry surviving for 7 days) and the total survival rate (the probability of eggs hatching into fry and surviving for 7 days) could be calculated as follow:

$$\text{Hatching rate}\left[\%\right] = \frac{n_2}{n_1} \times 100$$

$$\text{7 - day survival rate}\left[\%\right] = \frac{n_3}{n_2} \times 100$$

$$\text{Total survival rate}\left[\%\right] = \frac{n_3}{n_1} \times 100$$

2.6.3. Fish length of spawning group

Spawning fish were enumerated and measured from DIDSON data analyzed using ARISFish (version 2.6.2, Sound Metrics, 2019) software. Fish observed by the DIDSON were measured and counted from 6:30 AM on April 9 to 6:30 AM on April 10 when the spawning peaks occurred.

2.6.4. Statistics

Statistical analyses were performed using SPSS (version 19.0, Statistical Package for Social Sciences (SPSS), 2010), R (version 3.5.2, R Core Team, 2018), and Origin Pro (version 2017C, Origin Lab Corporation, 2017). For egg density and egg mortality, pairwise comparisons between values of all substrate materials (PAS, PEF, PEN) of same water layers were conducted with the pairwise Mann–Whitney U-test (nonparametric method chosen due to nonnormal distribution of data). The same pairwise comparisons were conducted between values of all water layers (Lower, Middle, Upper) of same substrate materials. For hatching rate and survival rate of eggs, pairwise Mann–Whitney *U*-test were conducted to compare the difference of substrate materials. Paired test was applied to eliminate the effect of different sampling batches (different sampling data and locations in spawning ground). The *p* values of pairwise comparisons were adjusted by Bonferroni correction. The significance level for all statistical analyses within the study was set to $p < 0.05$.

The range of water temperatures during the study period in which fish spawn most vigorously was tested. Average the egg density of all samples and water layers for each material each day, and divide the data into three groups according to the water temperature of the day: < 20°C, 20–25°C, >25°C. Kruskal–Wallis test was conducted to test if there were significant differences between the different temperature groups.

3. Results

3.1. Effects of different materials on egg adhesion and hatching

In terms of the averaged egg density on the three types of substrates, PAS was the most preferred spawning substrate, followed by PEF (not significant with PAS, $p > 0.05$; Figure 4A) and PEN has the lowest egg density (significant with PAS and PEF, $p < 0.05$; Figure 4A). However, the egg mortality of PAS was significantly higher than PEF and PEN ($p < 0.05$; Figure 4C).

In incubation experiments of three different materials, the hatching rate of egg, seven-day survival rate of fry and total survival rate of egg on PEN were significantly lower than PAS and PEF ($p < 0.05$). There was no significant difference between PAS and PEF ($p > 0.05$; Figure 5).

3.2. Influence of water depth and temperature on artificial spawning substrates

Different water layers apparently influence the density of attached eggs on artificial spawning substrates. In all types of artificial spawning grounds, the upper water layer was the most preferred spawning water layer, followed by the middle and lower water layer (all significant between each layer, $p < 0.05$; Figure 4B). However, there was no significant difference in egg mortality among the three water layers ($p > 0.05$; Figure 4D).

Changes in water temperature also affect the effectiveness of artificial spawning substrates. The daily water temperature of the artificial spawning grounds in Liangzi Lake were recorded and the corresponding water temperature for the spawning peaks were delineated in Figure 3. According to the results of Kruskal-Wallis test, the egg densities on the three materials in the water temperature range of 20 ~ 25°C were significantly ($p < 0.05$) greater than those in other temperature ranges (< 20°C and > 25°C). In addition, spawning peaks always occurred during periods of temperature increase, and visible declines of water temperature were commonly observed prior to the occurrence of spawning peaks (Figure 3).

3.3. Diurnal rhythm patterns of spawning and population composition of fish in artificial spawning grounds

For diurnal rhythm patterns of spawning, 22 spawning events were recorded by DIDSON during 12:00 on April 8 to 12:00 on

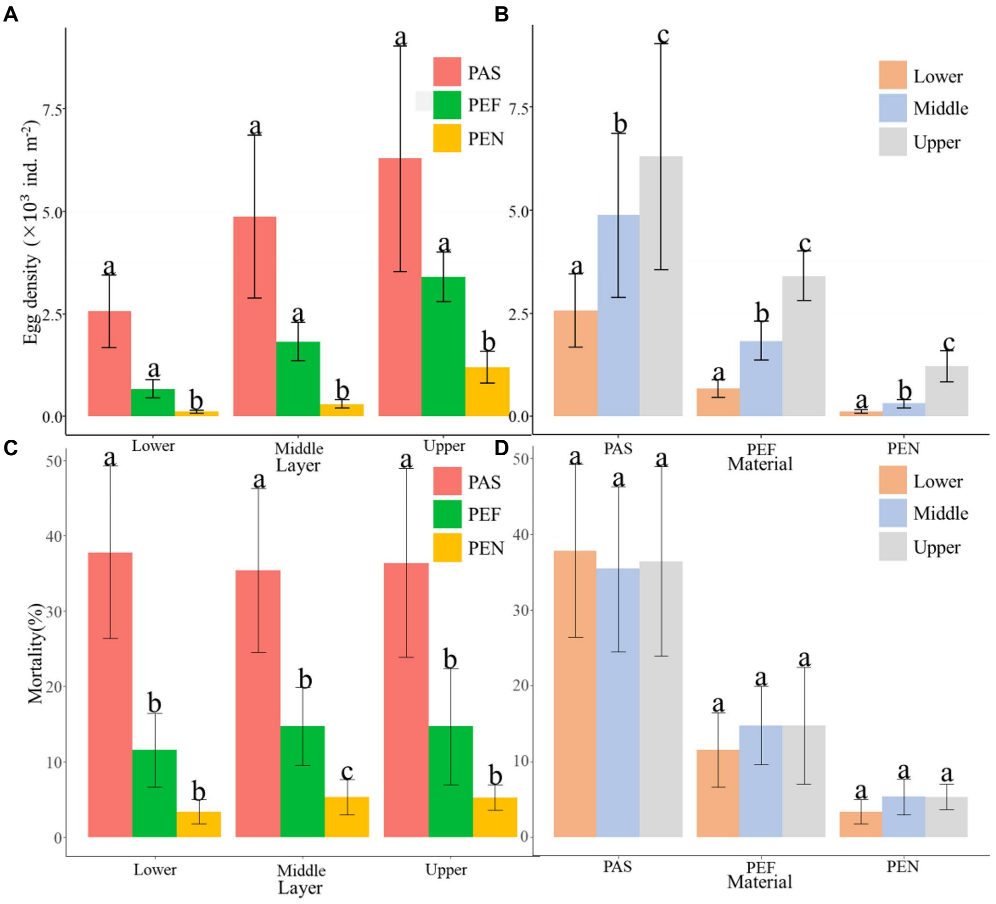

FIGURE 4
The comparison of **(A)** egg density between different materials; **(B)** egg density between different water layers; **(C)** egg mortality between different materials; **(D)** egg mortality between different water layers. PAS, palm sheet; PEF, polyethylene filament; PEN, polyethylene net. Meanwhile, More spawning peaks were observed on PEF (five peaks: March 26~April 1, April 3~April 4, April 8~April 13, April 16~April 20 and April 26~April 30), and followed by PEN and PAS (two peaks: March 26~April 1 and April 8~April 13; Figure 3).

April 12, 2018. 9.1, 72.7, 18.2 and 0.0% of spawning events in the artificial spawning grounds occurred during 18:00–00:00 (sunset to midnight), 00:00 ~ 06:00 (midnight to sunrise), 06:00–12:00 (sunrise to midday) and 12:00–18:00 (midday to sunset) respectively (Figure 6).

A total of 13,924 fish were measured by DIDSON during this period (Figure 7). The fish length mainly ranged from 5 to 20 cm accounting for 79.24% of the total measured fish. Fish with a body size of less than 15 cm dominated during 07:30–19:30. In the remaining 12 h, the proportion of fish larger than 15 cm increased. Especially 23:30–05:30, which coincides with the time with the most spawning events in Figure 6. Meanwhile, the number of fish larger than 20 cm and 30 cm were also increased during 19:30 to 06:30, especially in the mid night (Figure 8). When almost all females of crucian carp (*Carassius auratus*) and common carp (*Cyprinus carpio*) reach sexual maturity, their body lengths are 15 cm and 30 cm, respectively, (Liu and He, 1992).

4. Discussion

This study systematically explored artificial spawning substrates as a means of artificially assisting fish proliferation. The advantages and disadvantages of three artificial spawning substrates in the construction of artificial spawning grounds were studied. PAS had the highest egg density and also the highest egg mortality. PEF has intermediate egg density and egg mortality, but has the advantage of consistently attracting fish to spawn. PEN has the lowest egg density due to its simple structure and has a lower hatchability in hatching experiments. The position of artificial spawning substrate in the water column will affect the density of attached eggs but has no effect on the mortality of eggs. There is an optimum temperature for fish to use artificial spawning grounds, and the time for constructing spawning grounds should follow this basis. Monitoring of fish spawning assemblage and behavior determined the likely size range of spawning individuals and the timing of spawning behavior.

4.1. Explore effective materials and structure for constructing artificial spawning grounds

In recent decades, rehabilitation of degraded aquatic habitats has been one of the important approaches for fisheries management and biodiversity conservation (Slagle and Allen, 2018; Whiterod et al., 2021). Artificial spawning substrates may be a promising tool for habitat enhancement in degraded ecosystems such as lakes (Knaepkens et al., 2004; Malinovskyi et al., 2018). Therefore, the effectiveness of materials and structures of artificial spawning substrates has been a topic of discussion by numerous researchers (Gillet and Dubois, 1995; Nash et al., 1999; Lehtonen et al., 2006; Čech et al., 2012; Crane and Farrell, 2013; Malinovskyi et al., 2018). Dense and complex three-dimensional structures are more attractive for fish spawning than loose and simple two-dimensional structures, and differences in structure appear to be more important than the material itself (Gillet and Dubois, 1995; Čech et al., 2012; Crane and Farrell, 2013). The results of present study are consistent with this theory. PAS attracts more fish to spawn because it has the densest structure among the three selected substrates. On the other hand, although PEF and PEN were made of same materials, the complex three-dimensional structure of PEF formed in water is significantly more attractive to fish spawning than the simple two-dimensional network structure of PEN.

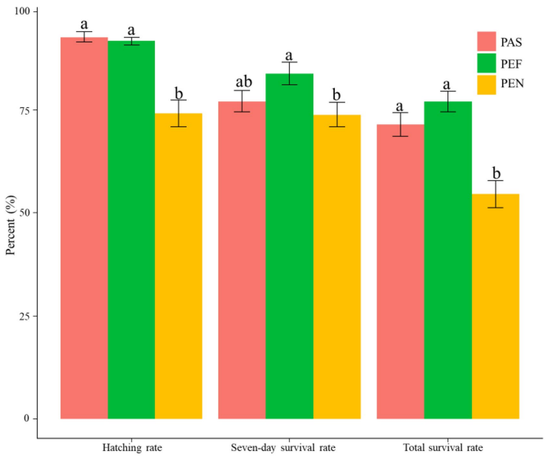

FIGURE 5
The comparison of hatching rate and survival rate among substrate groups. PAS, palm sheet; PEF, polyethylene filament; PEN, polyethylene net.

Egg mortality is another important indicator to assess the efficacy of artificial substrates supporting the recruitment of fish populations (Malinovskyi et al., 2018; von Nordheim et al., 2018). The main reasons for the death of eggs attached to the substrate are the inhibition of oxygen supply and metabolite processing by multi-layered and clustering eggs (Čech et al., 2012; von Nordheim et al., 2018), and the infection of fungi (Malinovskyi et al., 2018; von Nordheim et al., 2018). Both of these above reasons obviously lead to PAS having the highest egg mortality. Excessive density of attached eggs results in hypoxia of eggs on PAS, while degradation of natural materials may generate a suitable medium for fungal infection (Zarski et al., 2015), further increasing the egg mortality. In contrast, the egg mortality of PEF and PEN was significantly lower than that of PAS, which reflects the advantage of artificial synthetic materials in preventing water body from being polluted by organic matter (Malinovskyi et al., 2018). Although not significant, PEF still had a higher egg mortality than PEN due to higher egg density.

PEF exhibited the most enduring attraction to fish, as it had the most spawning peaks and its egg density was at a relative high level from the beginning to the end of the spawning period. In contrast, the egg density on PAS declined rapidly despite having

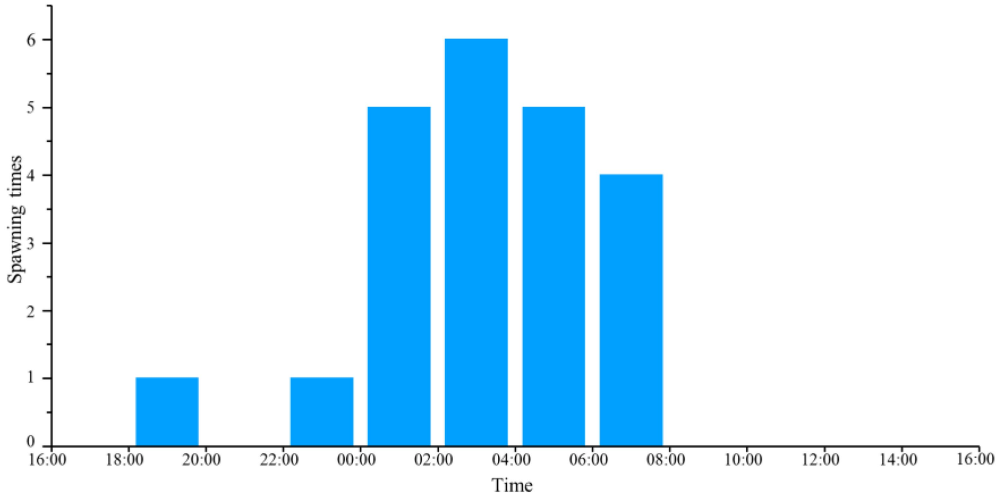

FIGURE 6
The temporal distribution of spawning occurrences in a full day. Spawning times represents the number of fish spawning observed by DIDSON.

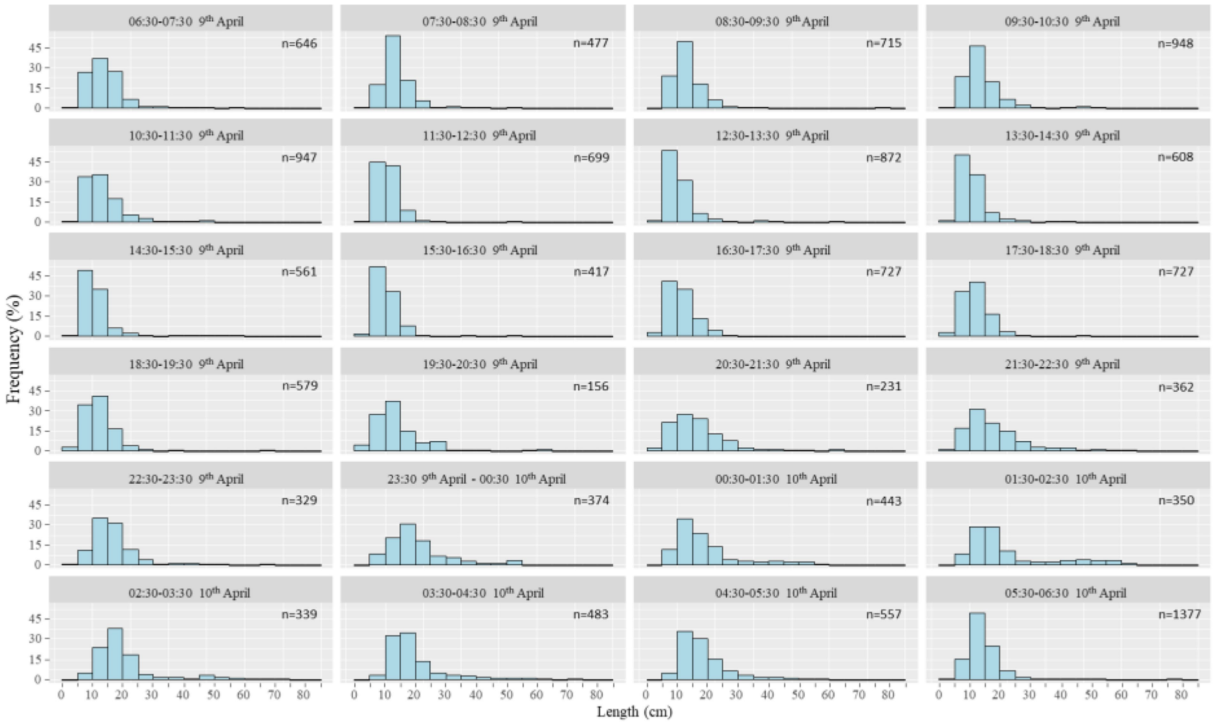

FIGURE 7
The temporal distribution of fish number and body length in a full day. The distribution of body length of spawning fish was depicted by hour in a whole day.

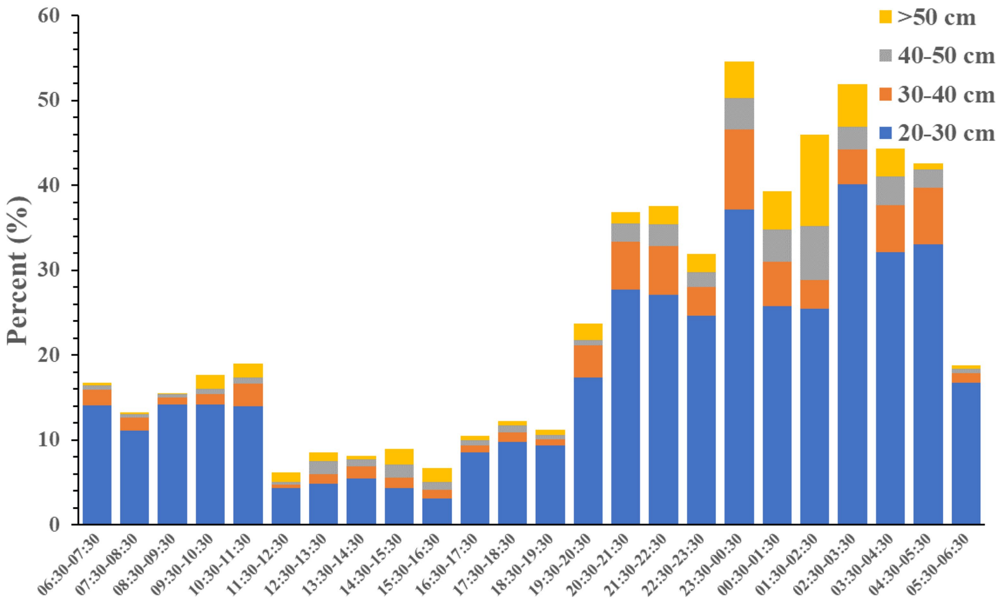

FIGURE 8
The temporal distribution of the percent contribution of the number of different body length group to the total number of fish in a full day.

the highest level at the beginning of the spawning period. This is because natural materials degrade and produce organic contamination when introduced into the water, which may prevent fish from accessing the substrate (Nash et al., 1999; Malinovskyi et al., 2018). In addition, Nash et al. (1999) proposed that artificial spawning substrates require regular maintenance (cleaning or replacement) to maintain their effectiveness in attracting fish.

The hatching rate profiles among the three substrates were normal compared with previous studies (Peng et al., 2016). However, the hatching rate of PEN was slightly lower than the other two substrates. In hatching experiments, only healthy eggs were selected and incubated under the same conditions along with the substrate. Therefore, only the different attachment patterns of the eggs on the substrate affected the hatchability. Previous studies have shown that eggs were scattered on more structurally complex substrates, while eggs on less complex substrates adjoined side-by-side, which may block oxygen supply and metabolite processing during egg hatching (von Nordheim et al., 2018).

4.2. Explore effective deployment period and water column position of artificial spawning grounds

The recruitment efficiency of artificial spawning substrates for fish depends on the deployment period of artificial spawning grounds (Gillet and Dubois, 1995). Setting too early can make maintenance and cleaning of artificial spawning grounds cumbersome, while setting too late can miss spawning peaks (Nash et al., 1999). The deployment period of the artificial spawning ground needs to be consistent with the spawning time of fish, which depends on the water temperature (Malinovskyi et al., 2018). The condition for triggering fish spawning is that the water temperature threshold is reached, so it is feasible to determine the deployment period of artificial spawning grounds according to the water temperature (Gillet and Dubois, 1995). At the same time, mastering the relationship between spawning peak and water temperature is conducive to the monitoring and maintenance of artificial spawning grounds (Nash et al., 1999).

Under natural conditions, common carp begin to spawn when the water temperature rises to 17°C, generally spawn in large batches at water temperature of 18 ~ 21°C and basically stops when the water temperature is above 26°C. The spawning temperature of crucian carp is very similar to those of common carp (Liu and He, 1992). In this study, the water temperature of spawning peaks on three types of artificial substrates were almost within the range of 20 ~ 25°C. Therefore, this water temperature range is within the suitable range for spawning for both species. In addition, spawning peaks mainly occurred during the rising phases of water temperature after dramatic declines due to strong wind or heavy rainfall. Our results are in line with Malinovskyi et al. (2018) who suggested a strong relationship between fish spawning and water temperature rise under natural condition. Meanwhile, Gillet and Dubois (1995) suggested sudden drop in water temperature slows spawning intensity.

Previous studies have found that the choice of artificial spawning substrate by fish is related to its location in the water column (Gillet and Dubois, 1995; Knaepkens et al., 2004; Čech et al., 2012). There are differences in the selection of different species of fish. For example, in Europe, where artificial spawning substrates are widely used, perch and pike spawn on the bottom of the water, and the roach spawns near the surface (Gillet and Dubois, 1995). Common carp and crucian carp are phytophilic spawner, and they choose submerged plants for spawning under natural conditions (Liu and He, 1992). Santos et al. (2008) pointed out that the position of the artificial substrate in the water column may influence fish selection more than the structure of the artificial substrate. In our study, the spawning substrate in the upper layer of the water column (200 mm from the water surface) was more attractive for fish to spawn. Determining the optimal water column location for artificial spawning substrates is critical to the effectiveness of spawning ground arrangements.

4.3. Explore effective management and protection strategies on spawning population of artificial spawning grounds

To formulate management strategies for efficient operation of artificial spawning grounds, it is necessary to study the behavior of spawning population. The artificial spawning substrate is an ideal material for studying spawning behavior (Gillet and Dubois, 1995). The observation of fish spawning activity in the artificial spawning grounds has been subject to difficulties that cannot be solved by traditional methods such as diving observation, underwater video and passive acoustic because these methods are either affected by turbidity in lakes or do not enable continuous 24-h monitoring, and therefore all have significant deficiencies (Zhadan et al., 2018; Grabowski et al., 2020; Rastoin-Laplane et al., 2020). In this study, DIDSON enables full day observation (especially at night) of fish spawning and diminishes anthropogenic disturbances to fish spawning. It provides a new attempt to observe spawning behaviors in artificial structures (Pavlov et al., 2009; Langkau et al., 2016; Smith et al., 2021). Mastering the circadian rhythm of spawning and the composition of spawning populations is conducive to formulating protection and management strategies for artificial spawning grounds.

DIDSON monitoring results revealed that spawning was focused between 18.00 and 08.00 h, especially between 00.00 and 08.00 h in Liangzi Lake. These findings are consistent with previous studies that spawning usually occurs at night and early morning (Schlumberger and Proteau, 1996; Malinovskyi et al., 2018). And this has also been confirmed in previous observations using DIDSON (Pavlov et al., 2009; Langkau et al., 2016). In respect to the spawning aggregations, small-sized fish (<20 cm) consistently predominated in the artificial spawning grounds, which may be related to the miniaturization of fish due to overfishing in Liangzi Lake (Xie et al., 2001; Ye et al., 2006; Wang et al., 2019). Large-sized fish (> 20 cm) were found mainly from

19.30 to 06.30 h. These findings are generally in line with previous study that large fish utilize artificial structures only after sunset (Santos et al., 2008).

The present research is of great significance to the management of artificial spawning grounds and the protection of spawning populations. Human disturbance to spawning grounds should be reduced during the spawning time of fish, especially to prevent poaching in artificial spawning grounds. Targeted protection on larger individuals of spawning aggregations can maintain adequate numbers of parents in artificial spawning grounds. In our observations, small individual fish always aggregated around the substrate after spawning behavior occurred. Although it cannot be concluded from DIDSON observations, but some species of small individuals in the lake have the potential to feed on fish eggs (Jin et al., 1996). Therefore, the selective removal of these small individual species can also have a protective effect on artificial spawning grounds.

4.4. Exploration in the application of artificial spawning substrate

Despite these promising results, applications in artificial spawning substrates remain explorable. The contradiction between multi-layered eggs caused by high egg density attached on substrates and high egg mortality is obvious in the present study. This may be due to the insufficient number and size of artificial spawning substrates in this experiment to support the recruitment of lacustrine fish (Malinovskyi et al., 2018). Hence, further research should be undertaken to determine the minimum scale of artificial spawning grounds. Meanwhile, artificial spawning grounds also require regular maintenance and cleaning (Nash et al., 1999). While these issues may be mitigated by setting the optimum deployment period of artificial spawning grounds based on water temperature, a range of management deadlines still needs to be explored in practice. Structure-specific spawning substrates could be selectively used by specific taxa group of fish (Santos et al., 2008; Rastoin-Laplane et al., 2020). Therefore, based on the principle of mimicking natural spawning grounds (Winfield, 1986), there is abundant room for further development of artificial spawning substrates that support the recruitment of more species.

Despite our use of DIDSON, there are still limitations in observing fish behavior in artificial spawning grounds. In the present study, DIDSON observations were specific to PAS only, as this substrate is most attractive to fish spawners. However, differences in fish spawning behavior between all materials were not investigated. DIDSON does not directly identify species. It is even more impossible to distinguish whether the individual in the field of view has been observed, so repeated observations may be unavoidable (Foote, 2009).

Although this study proves that the artificial spawning grounds are beneficial to increase fishery resources as a means of assisting fish proliferation, there are still potential risks and deficiencies in their use that need attention. From the perspective of environmental pollution, the selection of artificial spawning substrate materials needs to be cautious. The pollution of freshwater environment by microplastics has become a hot topic (Li et al., 2020), so more consideration should be given to natural and degradable materials. In addition, it is particularly important that artificial spawning grounds can never replace the role of natural aquatic plants in the ecosystem. Although artificial substrates can provide more spawning grounds for fish, they cannot provide other ecological functions of aquatic vegetation, such as water purification, providing nursery and food for fish (Heck et al., 2003; Knaepkens et al., 2004; Churchill et al., 2016). Even this complex artificial structure can cause fish to fall into the trap. Therefore, lake ecosystem managers need to clearly understand that artificial spawning grounds are a compromise to increase fishery resources in the case of natural habitat loss. To truly restore fish resources and ecological environment, it is fundamental to protect and restore natural habitats.

5. Conclusion

In the context of ongoing global degradation of submerged vegetation in littoral zone in lakes, the restoration of aquatic habitats such as spawning grounds is imperative. The results of present study indicate that the artificial substrates with complex and dense three-dimensional structure which have enough space to enable material exchange and oxygen circulation could be used to enhance fish recruitment in lakes. Meanwhile, this study also provides guidance on the restoration engineering parameters including the material and structure of artificial spawning substrates, deployment time and water column position of artificial spawning grounds, management and protection strategies on spawning population in lakes. In general, the results confirm the effectiveness of artificial substrates as suitable spawning substrates for fish. Therefore, artificial substrates may be an important tool in fisheries enhancement in lakes. However, this study proposes that the artificial spawning substrate is only a compromise of artificially assisted fish proliferation, and cannot replace the natural fish habitat.

Data availability statement

Raw data are available upon request to the authors.

Author contributions

KF: methodology, software, investigation, formal analysis, data curation, writing—original draft, writing—review and editing, and visualization. JY: resources. YZ and JQ: investigation. JL, ZL, and

SL: writing—review and editing. QW: conceptualization, methodology, investigation, formal analysis, data curation, writing—review and editing, supervision, project administration, formal analysis, and funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

This work was financially supported by the National Key Research and Development Program of China (no. 2019YFD0900602), National Natural Science Foundation of China (no. 32002396), Youth Innovation Promotion Association CAS (no. 2022344).

Acknowledgments

The contribution of S. S. De Silva from School of Life and Environmental Sciences, Deakin University was made when on a Visiting Professorship under the auspices of the CAS, tenable at the Institute of Hydrobiology, Wuhan.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.1062612/full#supplementary-material

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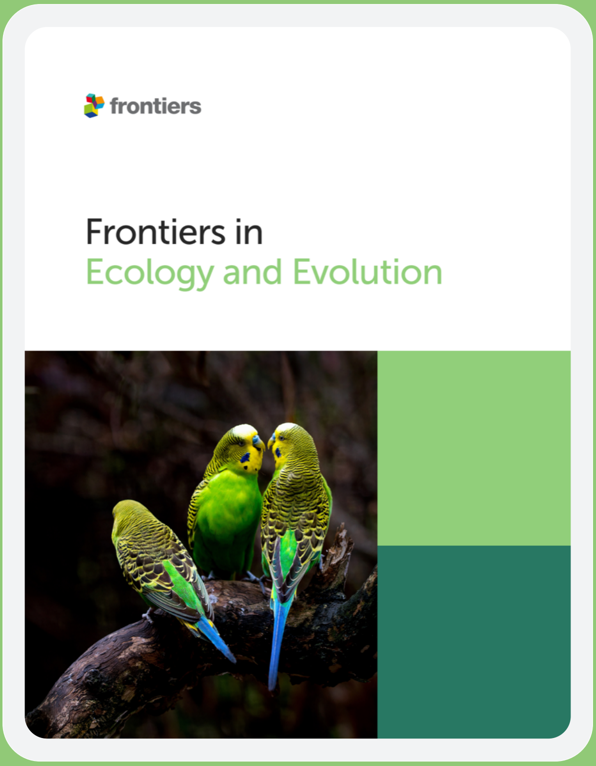

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