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Stochastic Processes Drive the Assembly of Planktonic Ciliates in A Trellised River Network

Chang Chu^{1,2} | Lianwen Zhao^{1,2} | Lele Chen^{1,2} | Lijing Chen³ | Liqing Wang^{1,2} | Chenhong Li^{1,2} | Jiamei Jiang^{1,2} | Hongbo Pan^{1,2} 

¹Shanghai Universities Key Laboratory of Marine Animal Taxonomy and Evolution, Shanghai Ocean University, Shanghai, China | ²Engineering Research Center of Environmental DNA and Ecological Water Health Assessment, Shanghai Ocean University, Shanghai, China | ³Shanghai Vocational College of Agriculture and Forestry, Shanghai, China

Correspondence: Jiamei Jiang (jm-jiang@shou.edu.cn) | Hongbo Pan (hbpan@shou.edu.cn)

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ABSTRACT

The topology and connectivity of rivers can be modified by the water project operation for agriculture and civilization, as exemplified by the trellised river system in Chongming Island. Ciliates, as an exceptional indicator, are often employed for monitoring the health of aquatic ecosystems. However, the assembly and structure of planktonic microeukaryote (e.g., ciliates) communities, especially in complex river networks, are rarely investigated. Here, we investigated the ciliate community structure and assembly mechanism among seasons in Chongming Island's trellised river network using both metabarcoding and quantitative protargol stain (QPS) approaches. Both methods showed that ciliates exhibited similar community composition at the class level and distinct seasonal succession, which are attributed to variations in nutrients, dissolved oxygen and water temperature. The stochastic process was the main factor in explaining the assembly of the ciliate community in all seasons, which is probably attributed to the high connectivity of the river network and the frequent opening of sluices for the water project operation. Collectively, unique ciliate community structure, seasonal variation, and specific community assembly mechanism driven by hydrology were reported in this study. Furthermore, methodological differences also should be fully considered when conducting an ecological study in complex hydrographic waters.

1 | Introduction

Over recent decades, due to increased industrialization and population growth, anthropogenic activities have had a significant impact on freshwater river ecosystems (Liang et al. 2018; Wu et al. 2019). With the construction of river channels and the implementation of sluice gates, river connectivity is significantly transformed and the water flow velocity is modified (McCluney et al. 2014), which impacts the stability of nutrient status and the pattern of aquatic biodiversity. These changes can influence

the composition and assembly processes of microbial communities in rivers and may result in biotic homogenization (Poff et al. 2007).

Ciliates are crucial in aquatic ecosystems as consumers and prey within the microbial loop (Foissner et al. 2007; Weisse 2007; Fenchel 2008). Their high sensitivity to environmental factors and the short generation time make them good indicators of water quality (Ali et al. 2024; Ramírez-Ballesteros et al. 2024). The hydrology of water columns also

manages the dispersal of ciliates due to their tiny size (Custer et al. 2022). Therefore, ciliates are usually used for reflecting water quality and hydrological conditions in ecosystems, such as activated sludge, marine coastal lines, rivers, and aquaculture ponds (Lee et al. 2004; Xu et al. 2014; Debastiani et al. 2016; Zheng et al. 2023).

Traditionally in the morphology-based method, the quantitative protargol stain (QPS) is mainly employed for the accurate identification and quantification of ciliates to reveal the biogeography and biodiversity patterns in various habitats (Skkibe 1994; Scherwass and Arndt 2005; Zhao et al. 2012; Van Wichelen et al. 2013; Weisse and Montagnes 2022). However, this method is time-consuming and requires a high level of expertise in taxonomy, hampering its wide application. In the past 20 years, the metabarcoding method based on environmental DNA was developed, which can overcome these problems (Caron et al. 1999; Pawlowski et al. 2016). Particularly, it offers a significant advantage in uncovering cryptic and rare species that are likely to be overlooked under the microscope and has been used as an efficient method for investigating biodiversity and monitoring water quality (Ewers et al. 2023; Brinkmann et al. 2023; Zheng et al. 2023; Langlois 2024).

Community assembly describes the mechanisms that control community diversity, functions, succession, and biogeography, which is central but poorly understood in microbial ecology, particularly in the lotic ecosystems (Hanson et al. 2012; Zhou and Ning 2017; Chen et al. 2019). Niche theory posits communities are shaped by deterministic processes, including biotic and abiotic environmental selection and niche partitioning, according to the habitat preference and fitness of species (Cadotte 2004; Bahram et al. 2016). The neutral theory asserts that community structure was governed by stochastic processes of birth, death, colonization, extinction, and speciation (Hubbell 2001). Both theories are important and complementary in understanding community assembly mechanisms (Sloan et al. 2006; Lima-Mendez et al. 2015).

With the metabarcoding method prevailing, some investigations find the assembly of the ciliate community is dominated by deterministic processes (Debastiani et al. 2016; Kulaš et al. 2021), which means environmental filtering likely plays an important role in community assembly. But some studies noted that increased connectivity might lead to a domination of stochastic processes, which supports Chase's hypothesis that communities become more similar to each other as increasing harshness reduces the size of the potential colonizer pool so that stochastic processes operate under such conditions (Chase 2007; Lepori and Malmqvist 2009). For example, in the subtropical Tingjiang River, the stochastic processes predominantly shaped microeukaryotic communities because of the high river connectivity, especially in the wet season (Chen et al. 2019).

Hydraulic projects, such as the construction of river channels and ditches, serve to enhance the complexity and connectivity of river networks (McCluney et al. 2014). Within the complex river systems, the transportation and retention of eDNA are largely governed by hydrological features like water velocity and

channel complexity, which in turn can impact the richness of target species present (Shogren et al. 2018; Fremier et al. 2019). Hence, it is imperative to investigate whether the molecular approach is also suitable for complex hydrological environments.

In Chongming Island, lots of channels and sluices were built to support agriculture and civilization, resulting in a more complex river network with higher connectivity than natural dendritic rivers (Le et al. 2009). This distinctive river system presents a valuable opportunity to explore the mechanisms behind ciliate community assembly in complex river networks and determine the applicability of metabarcoding techniques in rivers with such hydrological characteristics. In this study, we employed both the morphology-based QPS method and the 18S rRNA gene-based metabarcoding method to comparatively explore the differences in planktonic ciliate community structure and their assembly mechanisms across seasons in the rivers of Chongming Island. We first posited that the river played host to a distinct seasonal distribution pattern among ciliate communities. Furthermore, given the unique hydrological environment of Chongming Island, it was plausible that the ciliate communities tended to be homogeneous and stochastic processes may play a vital role in community construction. In addition, we suppose that the richness of ciliates in such a complex river network is likely overestimated by the metabarcoding method.

2 | Materials and Methods

2.1 | Sampling Sites and Sample Collection

Chongming Island (121°09'30" – 121°54'00" E, 31°27'00" – 31°51'15" N) is situated at the estuary of the Yangtze River with a flat topography (Figure 1). The island often suffers from typhoons, heavy rainfall, high tides, and floods due to the geographic characteristics. In order to avoid loss from these disasters and to protect and exploit the water resource, a total of 140 sluices have been set up on the island as of the end of the year 2021.

In this study, a total of 23 sampling stations were arranged uniformly in Chongming Island (Figure 1). A total of 92 water samples were collected in four seasons, that is, July 2020 (summer), October 2020 (autumn), January 2021 (winter), and May 2021 (spring). At each sampling site, surface water was collected with a 5-L water sampler at the depth of 0.5 m. The 5-L water volume of each sample was split into two groups. One was 300 mL for the QPS method and was preserved with 15 mL Bouin's fixative (5% final concentration), in which ciliates were identified and enumerated after quantitative protargol staining (Skkibe 1994). The data yielded from the QPS method have been published by Ye et al. (2024). The other was three 1-L parallel water subsamples for the metabarcoding method, which were prefiltered through 200 µm mesh for removing mesozooplanktonic organisms and then were enriched through a 0.2-µm pore polycarbonate membrane (Isopore, Merck) within 1 h after sampling. The filters containing samples were stored in liquid nitrogen until further processing.

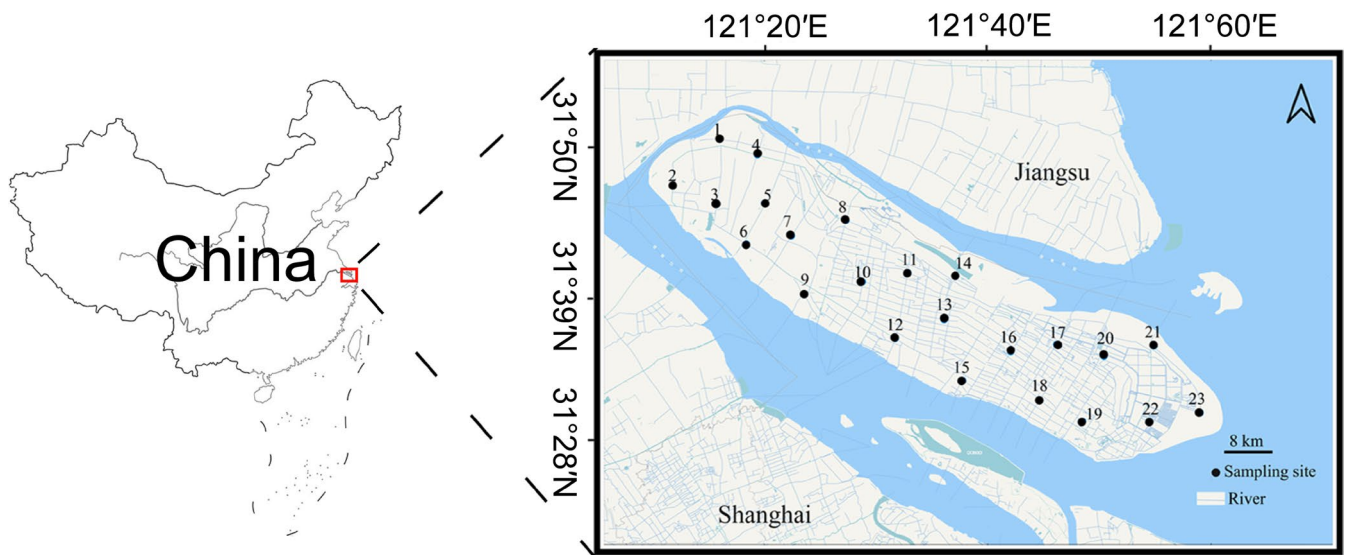


FIGURE 1 | Map of study area in Chongming Island and 23 sampling stations (black dots). Light blue line represents the river system. The map was drawn using Bigemap platform (<http://www.bigemap.com/>).

2.2 | Measurement of Environmental Factors

The abiotic parameters were already reported by Ye et al. (2024). Briefly, salinity (Sal), water temperature (WT), dissolved oxygen (DO), and pH were tested in situ by the multiparameter water quality sonde (YSI Professional Plus, United States). Secchi transparency (SD) was measured with a Secchi disk. Chlorophyll-a (Chl-a) was measured through a FluoroSense Handheld. Additionally, data for the trophic status, that is, total phosphorus (TP), total nitrogen (TN), ammonia nitrogen ($\text{NH}_4\text{-N}$), nitrate nitrogen ($\text{NO}_3\text{-N}$), nitrite nitrogen ($\text{NO}_2\text{-N}$), were determined according to the Environmental Monitoring method standard of the Ministry of Ecology and Environment, China. The data of precipitation was obtained from <https://www.worldweatheronline.com/>.

2.3 | DNA Extraction, Amplification and Sequencing

DNA was successfully extracted from 87 samples using the DNeasy PowerSoil Kit (Qiagen, Germany). A nested-PCR was conducted here to do the inventory for the ciliate community (Lara et al. 2007; Alexandra et al. 2013) and Phanta HS Super-Fidelity DNA Polymerase (Vazyme Inc., Cat. No.: P502-d1) was used in both rounds of PCR reactions. For the first PCR reaction, a ciliate-specific primer set was performed for filtering the ciliate SSU rDNA (Lara et al. 2007; Alexandra et al. 2013). This PCR reaction procedure is as follows: the 3-min predenatured process at 95°C, followed by 15 cycles of 10s at 95°C, 30s at 54.6°C, 20s at 72°C, and a final 7-min extension at 72°C. Subsequently, products from the first reaction were subjected as templates to a second PCR, which employed eukaryote-specific primers for the amplification of the hypervariable V4 region (Stoeck et al. 2010; Alexandra et al. 2013). The second PCR reaction procedure details are as follows: 3-min predenatured process at 95°C, followed by 25 cycles of 10s at 95°C, 30s at 58°C, 20s at 72°C, and a final 7-min extension at 72°C. All PCR products were assessed with agarose gel electrophoresis on a 1% agarose gel. Subsequently,

the total qualified PCR products (ca. 418 bp) of three parallels in the second PCR reaction of each sample were pooled and sent to paired-end (2×250 bp) sequencing on an Illumina Miseq platform by the sequencing company Majorbio (Shanghai, China).

2.4 | Sequencing Data Analysis

Fastp (Chen et al. 2018) and FLASH (Magoč and Salzberg 2011) were used for clearing raw sequence data, including quality checking and filtering, demultiplexing, and data assembly. In this study, VSEARCH was used for merging, quality filtering, dereplicating, clustering based on 97% similarity, removing chimeric sequences by comparing the tag sequences to the Protist Ribosomal Reference database v5.0.1 (PR2) and creating an OTU (operational taxonomy unit) table (Rognes et al. 2016; Vaultot et al. 2022). OTUs derived from only 10 or fewer reads were excluded as potential errors. Representative sequences were also taxonomically assigned against the PR2 v5.0.1 database in Qiime2, and only OTUs that were annotated as ciliates were retained, of which OTUs with ambiguous comparison results were further corrected by referring to the literature and BLAST (Rajter and Dunthorn 2021). The OTU table was rarefied to the same sequencing depth of 19,627 reads per sample before manually removing the nonciliate OTUs by randomly re-sampling for the subsequent statistical analysis. To compare the eDNA data with the QPS data, only samples with data from both methodological approaches were retained. Eventually, owing to the loss of some QPS samples and the insufficient DNA concentrations in certain eDNA samples for successful amplification, only data from 82 samples could be retained for this study. Fortunately, the lost samples were random, thereby introducing minimal bias (Table S1).

2.5 | Statistical Analysis

All analyses were performed with software R version 4.3.2. A Venn diagram was used to visualize the shared and unique

classes detected by eDNA and QPS methods using the package “VennDiagram.” Shannon index was calculated with the “vegan” and “picante” packages, and the subsequent analysis of differences between four seasons was conducted by multiple comparisons based on Kruskal–Wallis test and ANOVA test in two methods, respectively. β -diversity depicted the ciliate community differences between the different habitats. For the eDNA data, β -diversity was calculated by unweighted uniFrac with the “GuniFrac” package, where phylogenetic distances were obtained by Qiime2 v2021.8 and visualized on nonmetric multidimensional scaling (NMDS). For the QPS data (transformed by Hellinger), Bray–Curtis was calculated to indicate β -diversity and visualized on Principal Coordinates Analysis (PCoA). The “envfit” function was used to choose the fitted environmental factors with significant influence ($p < 0.05$) in both ordination analyses and “ggplot2” package was mainly utilized for visualization. PERMANOVA was calculated to estimate global and pairwise differences of the ciliate community among four seasons with the “vegan” package. In order to explore whether environmental factors play a more significant role in a certain season, partial least squares path modeling (PLS-PM) was implemented using the PLS-PM package (Sanchez 2013a). We divided the environmental factors into four blocks (latent variables), water temperature, physical (Sal, EC, SD, DO, pH), nutrients (TP, TN, NO₃-N, NO₂-N, NH₄-N) and Chl-a. Diversity was described by the Shannon index, and the community was represented by the first two axes of the ordination (NMDS for eDNA data, PCoA for QPS data). Factors with loading < 0.7 were removed after initial permutation (Sanchez 2013b).

The assembly process of ciliate communities in different seasons was calculated based on the β -Nearest Taxon Index (β NTI) (Stegen et al. 2012). The weighted β -mean nearest taxon distance (β MNTD) was calculated first, which quantifies the mean phylogenetic distance between the evolutionary closest OTUs in two communities. Then the null model was conducted (1000 randomizations) to calculate the deviation between the mean distances. β NTI is the difference between the observed β MNTD and the mean β MNTD of the null distribution. When β NTI values exceed 2, they indicate variable selection, whereas values below -2 suggest homogeneous selection. Both selection processes are deterministic processes structuring the community. On the other hand, values between -2 and 2 signify a stochastic process (Stegen et al. 2012). Then, the turnover of OTUs was quantified to assess the impact of dispersal and drift on community composition, as measured by the Raup–Crick distance (Rcbray) between samples (Chase et al. 2011; Stegen et al. 2012).

3 | Results

3.1 | Composition and α -Diversity of Ciliate Community in the Riverine Network of Chongming Island

A total of 1,237,143 reads within 1226 OTUs assigned to Ciliophora were obtained after rarefying to the minimum abundance of ciliate OTUs across 82 samples. Totally, 99.3% of reads were annotated into 10 classes (excluding unclassified); Spirotrichea (66.2%) were the most prevalent, followed by Prostomatea (10.8%), Oligohymenophorea (8.4%), Litostomatea (8.2%), Phyllopharyngea (2.6%), Colpodea (1.8%), Heterotrichea (0.6%), Nassophorea (0.6%), Armophorea (0.03%), and Plagiopylea (0.004%). Except for Armophorea and Plagiopylea, all other phyla were detected in results based on the QPS method. Although most phyla from the molecular method were also detected by the QPS method, the composition at the order, family, and genus based on the two methods was quite different (Figure 2).

Both approaches indicated a distinct seasonal succession of ciliate community in Chongming Island, but the patterns were different (Figure 3A,B). For the results from the metabarcoding method, Spirotrichea had its highest relative abundance (77.3%) in winter and its lowest (45.1%) in autumn. On the contrary, QPS results noted that Spirotrichea accounted for as high as 91.6% in autumn but only had 46.4% of the total ciliate abundance in winter. Additionally, Litostomatea, Oligohymenophorea, and Prostomatea showed seasonal variations in different methods. Taking Prostomatea as an example, in the metabarcoding method, its relative abundance was the highest in spring (17.5%) and the lowest in summer (6.6%). In the QPS method, the proportion of Prostomatea was the lowest in autumn, at only 1.4%, and the highest in winter, reaching 48.2%. Neither Armophorea nor Plagiopylea was common in abundance and OTU numbers. Armophorea was only detected in spring and summer, while Plagiopylea was solely detected in summer.

For metabarcoding method, we considered 77 OTUs with relative abundance exceeding 0.001 at more than a quarter of sampling sites to be abundant and exhibited a distinct seasonal variation at the order level (Figure 3C). For QPS method, eight ciliate species with relative abundance greater than 0.01 at more than a quarter of sampling sites were reckoned as abundant and also showed an obvious seasonal change (Figure 3D). Both methods revealed that Tintinnida, Choreotrichida, and

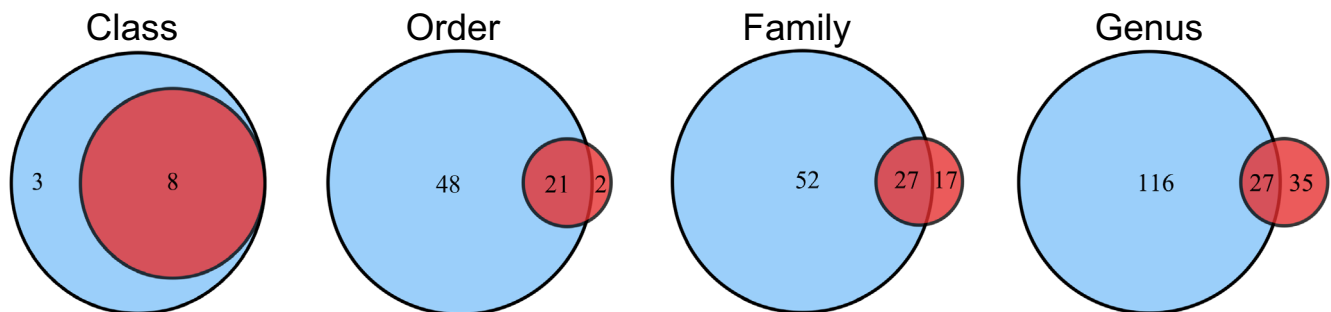


FIGURE 2 | Venn diagrams comparing the ciliates assigned at class, order, family, and genus rank either by the metabarcoding (blue circles) or by the QPS (red circles) approach.

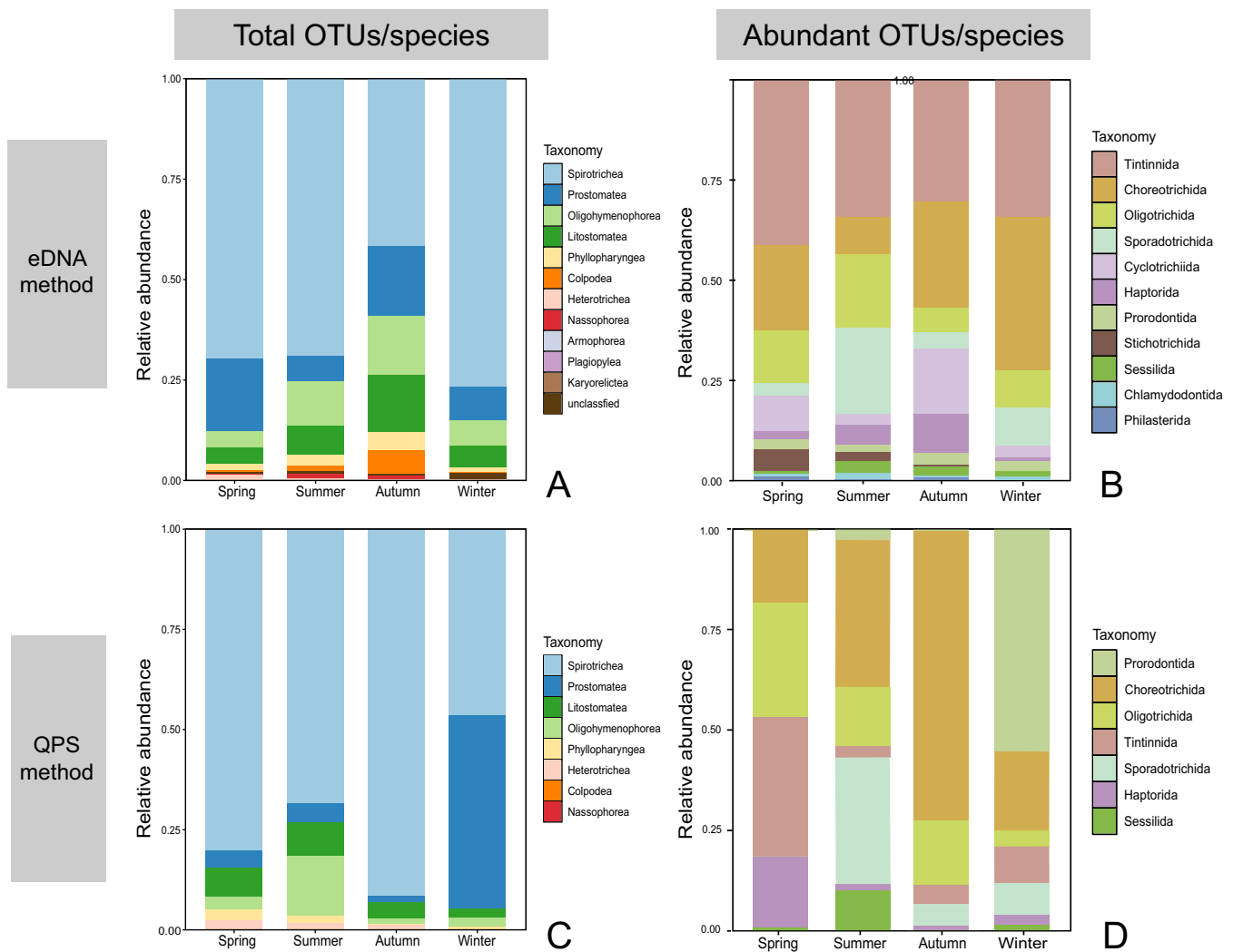


FIGURE 3 | Succession in ciliate community composition during seasons. Abundance and diversity of ciliate OTUs and morphospecies at class level in all (A, B) and order level in abundant (C, D). The sequence of legends in the figures was ranked according to the total abundance proportion of each group.

Oligotrichida were the major abundant taxa of ciliates, which accounted for more than 50% of the relative abundance in each season (except the result in winter by QPS method).

The Shannon–Weiner index was employed in this study to describe differences of α -diversity among different seasons by two approaches. There were significant differences of α -diversity in spring, summer, and winter. The results displayed significant seasonal fluctuations in ciliate α -diversity, with the lowest average of α -diversity and the greatest variation in autumn (Figure 4A,B). In addition, α -diversity was highest in summer and winter for molecular approach and traditional approach, respectively.

3.2 | Seasonal Variation of Ciliate Community Structure

NMDS based on unweighted Unifrac distance was conducted to display the variation of community composition for the metabarcoding method (stress=0.1366) (Figure 4C). PCoA based on Bray–Curtis dissimilarity was used for the same

target in the traditional method, which explained totally 29.6% (Figure 4D). The results of both methods indicated that there was a significant difference in β -diversity among seasons (PERMANOVA, $p < 0.01$). Meanwhile, for results from the molecular approach, β -diversity of the ciliate community was much higher in autumn than in the other three seasons; while for the results from the QPS method, β -diversity in all seasons was higher. After fitting environmental factors onto the ordinations, it was found that the β -diversity of ciliates detected by both methods was influenced by three factors: dissolved oxygen (DO), nitrite ($\text{NO}_2\text{-N}$) and water temperature (WT). For OTUs, DO and pH showed a positive significant ($p < 0.05$) correlation with both axes, whereas $\text{NO}_2\text{-N}$, $\text{NH}_4\text{-N}$, and WT exhibited a negative significant ($p < 0.05$) correlation (Table S3). For QPS, only DO was positively correlated with PCoA1, and it was negatively correlated with PCoA2. Additionally, $\text{NO}_2\text{-N}$ and TP showed a positive significant correlation with PCoA2, WT, and Chl-a showed a negative significant correlation with PCoA2. According to the PLS-PM results (Figures S1 and S2), the effects of environmental factors on ciliate community diversity and community structure in different seasons were inconsistent. Based on the metabarcoding method, the diversity

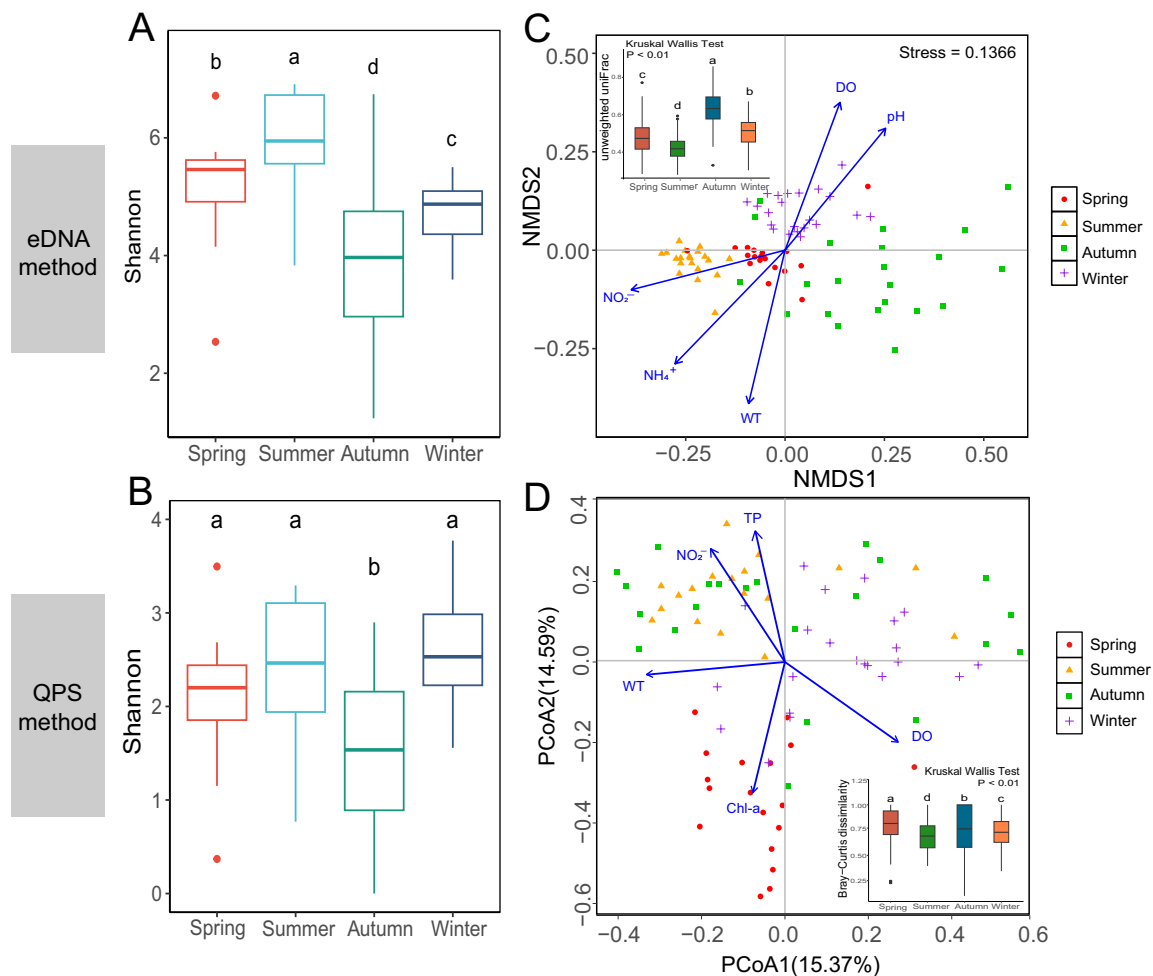


FIGURE 4 | Seasonal α -diversity and β -diversity of ciliate community. (A, B) Box plot of Shannon index of ciliate community at four seasons based on the data obtained by metabarcoding method (A) and QPS method (B), different letters show the significant differences which were analyzed with Kruskal–Wallis test and ANOVA test, respectively. (C) Nonmetric multidimensional distance scaling (NMDS) based on unweighted Unifrac distance of OTUs retrieved from four seasons (PERMANOVA, $p < 0.01$, $R^2 = 0.3014$). (D) Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity of morphospecies at four seasons (PERMANOVA, $p < 0.01$, $R^2 = 0.1962$). Environmental parameters showed in plots were significantly correlation with ciliate community β -diversity ($p < 0.05$). The boxplots in C and D showed the multiple comparisons in β -diversity in both approaches by Kruskal–Wallis test. Chl-a, Chlorophyll-a; DO, dissolved oxygen; NH₄-N, ammonia nitrogen; NO₂-N, nitrite nitrogen; TP, total phosphorus; WT, water temperature.

exhibited the strongest significant correlation with physical factors in spring, while the community was most effectively explained in the summer, with an R^2 value of 0.806. According to the QPS method, nearly all environmental factors (except water temperature) significantly influenced the ciliate diversity in winter with R^2 value of 0.698, while it was most explained in spring with R^2 value of 0.748.

3.3 | Ciliate Community Assembly Mechanism

The community assembly mechanism in different seasons was explored by a null model. Most $|\beta NTI|$ values were less than 2, indicating that the ciliate community was primarily shaped by stochastic processes (Figure 5A). In the context of the stochastic process, homogenizing dispersal was the principal process in all seasons, accounting for 81.05% in spring, 84.21% in summer, 61.04% in autumn, and 83.98% in winter (Figure 5B). Homogenizing dispersal was followed by variable selection, which contributed most in autumn at 20.35% and least in

summer at 5.26%. Drift exhibited a different pattern that accounted largest for 16% in autumn while accounting lowest for 1.30% in winter.

4 | Discussion

4.1 | Seasonal Dynamics of Ciliate Diversity and Community Structure

Ciliates have been recognized as important bioindicators and their community structure has been extensively studied in rivers and lakes (Kiss et al. 2009; Li et al. 2022). Our results showed that the ciliate community in Chongming Island Rivers was primarily composed of Spirotrichea at the class level, and Tintinnida, Choreotrichida, and Oligotrichida at the order level (Figure 3), which usually dominate in freshwater planktonic ciliate communities (Pitsch et al. 2019; Kulaš et al. 2021; Abdullah et al. 2023). However, different from large rivers where peritrichs were also dominant, rivers of Chongming Island bore few

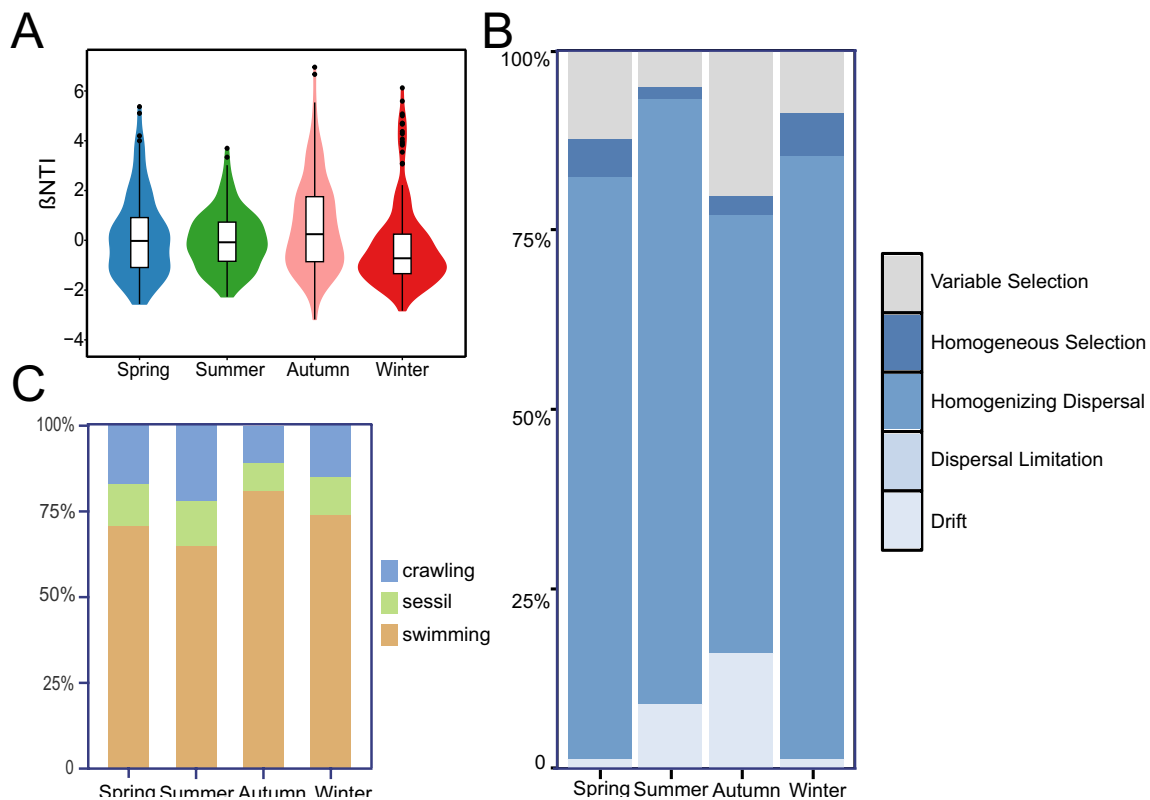


FIGURE 5 | Community assembly mechanism of ciliate community. (A, B) β NTI (A) and ciliate community assembly process (B) in different seasons. (C) Proportion of ciliate OTUs with distinct locomotion patterns at four seasons, ciliate categories were determined through matching with PR2 database and BLAST.

peritrichs, in terms of both abundance and richness (Scherwass and Arndt 2005; Kiss et al. 2009). This phenomenon could be attributed to the swift current velocity of the water in large rivers, which increases turbidity and suspends matter, providing habitats for peritrichs (Table S2). Alternatively, the sessile ciliates may have been stirred up, leading to the highest detection of peritrichs. This can also explain why Sessilida had the highest detection rate in summer in the rivers of Chongming Island.

The ciliate communities often exhibit a distinct seasonal succession (John and Thomas 1989; Biserka et al. 1996; Pitsch et al. 2019; Şahin Doğan and Kocabaş 2023). We also observed a seasonal variation of the ciliate community in Chongming Island. The α -diversity was relatively high in spring, and Prostomatea displayed a marked change in relative abundance, peaking in spring as determined by molecular method. The ample supply of phytoplankton for algivorous prostomatids, as evidenced by the concentration of Chl-a in Table S2, can afford the high abundance of prostomateans in the spring. In the summer, Chongming Island received heavy rainfalls, which brought ciliates from terrestrial habitats into the river, together with the opening of the diversion gates to balance the water level with that of the Yangtze River, leading to an increase in the abundance and diversity of ciliates. Furthermore, frequent precipitation stirred up the sediment, making DO, SD, and Chl-a lower in the water column and elevating concentrations of nitrogen and phosphorus. Meanwhile, ciliates living at the anaerobic bottom of rivers, such as *Tropidoatractus spinosus* and *Trimyema compressum*, were also stirred up, which contributed to the abundance and diversity of the ciliate community (Yamada

et al. 1997; Rotterová et al. 2018; Chen et al. 2019). The results of the QPS method revealed that the abundance and diversity of planktonic ciliates in the rivers of Chongming Island during winter were even higher than those in summer (Figure 4A,B), which is similar to an investigation of a freshwater tidal estuary (Koenraad et al. 2000). It was notable that Chl-a was relatively high in the winter, indicating that the primary production in the water column could provide sufficient food sources for ciliates (Figure S2). Therefore, the abundance of *Balanion planctonicum* and *Rimostrombidium hyalinum* increased dramatically (Ye et al. 2024). In the meantime, zooplankton was limited to a few sites and mainly consisted of algivorous species (Xu and Chen, unpubl. data), resulting in a minimal predation pressure on ciliates (Sommer et al. 2012). As a result, the abundance and diversity of ciliates were high in the winter.

Moreover, our results supported the conclusions predicted by the model that intermediate rates of dispersal often result in higher levels of α -diversity and higher rates of dispersal result in lower β -diversity (Figure 4A,C), especially in molecular results (Nicolas and Michel 2002, 2003). Ciliate communities were significantly different among seasons both in α - and β -diversity, and a significantly higher β -diversity in the autumn was detected (Figure 4C,D). Flow velocity was likely slow in autumn since both the precipitation was low and the water dispatching was less frequent. Therefore, the planktonic ciliates were not as evenly mixed as in other seasons. Additionally, ciliate communities were associated with local environmental variables, as indicated by the significant correlations observed that DO, WT, and $\text{NO}_2\text{-N}$ were shown in both methods. It was

undoubted that DO and WT emerged as crucial environmental elements significantly impacting the ciliate communities (Weisse and Montagnes 1998; Fenchel and Finlay 2008; Finlay and Esteban 2009; Babko et al. 2020). Ciliate communities were also significantly influenced by nitrogen ($\text{NO}_2\text{-N}$, $\text{NH}_4\text{-N}$) and phosphorus (TP), with particularly pronounced correlations observed during the summer months when water quality was degraded (Table S2; Figure S1). These findings were consistent with previous reports and further underscore the potential of ciliates as sensitive bioindicators of environmental quality (Yang et al. 2023).

4.2 | Assembly Mechanism of Planktonic Ciliate Community

In recent years, with the development and widespread application of high-throughput sequencing technologies, many studies have coupled spatiotemporal data on microbial communities and used null models of sequencing data to quantify the relative importance of deterministic and stochastic processes in microbial communities (Stegen et al. 2012; Fang et al. 2023; Zhao et al. 2023; Li et al. 2024; Niu et al. 2024). However, there are few studies on the community assembly mechanisms of eukaryotic microorganisms in rivers. Investigations on Jiulong River and Yarlung Zangbu River unveiled that the eukaryotic microbial community was driven by deterministic processes; however, a study on the Tingjiang River revealed that the microeukaryotic community was dominated by stochastic processes because of the rise of river connectivity (Isabwe et al. 2018; Chen et al. 2019; Yang et al. 2023). In Chongming Island, the assembly of the riverine ciliate community was primarily driven by homogenizing dispersal of stochastic process (Figure 5A,B). Dispersal was defined as the movement of organisms across space, and this process was conducted by immigration or emigration (Vellend 2010; McFadden et al. 2023). In rivers, the motility of microorganisms mainly relies upon the diffusibility of the system, and the community assembly of microbial communities is often driven by hydrology (Nino-Garcia et al. 2016; Isabwe et al. 2018). A high rate of dispersal can make the local community homogenized (Custer et al. 2022). The river system in Chongming Island is crisscrossed and has a higher degree of connectivity than dendritic rivers (Isabwe et al. 2018; Chen et al. 2019). There were also 140 sluices on the island, and of them, 23 sluices were constructed for balancing the water levels inside and outside the island (Chen, pers. commu). Normally, water from the Yangtze River is brought in by sluices in the south. After running through the whole island, water is drained out to the East China Sea through the northern sluices. Such kinds of exchanges take place frequently in all seasons frequently which supports the ciliate community in rivers becoming homogeneous. Therefore, we suppose that it is the hydrological conditions of Chongming Island that led to the homogenizing dispersal of the ciliate community assembly process.

Although ciliate community assembly was dominated by homogenizing dispersal, there were still differences in the proportion of each process among seasons (Figure 5B). Heavy rainfall events carried abundant nutrients from the Yangtze River and land, which could weaken niche selection by providing excessive resources and reduce survival stress for ciliates and other

microorganisms and further resulted in a decrease of homogeneous selection (Zhao et al. 2023). Substantial nutrient input can create additional niches to stimulate the growth of microorganisms, which enhance the stochastic process by increasing birth and death (ecological drift) (Vellend 2010; Zhao et al. 2023). Furthermore, water project operation plays an important role in ciliate community assembly as well. To avoid rivers flooding in Chongming Island, sluices are used to control the connections between river networks and outer waters, that is, the Yangtze River. Thus, in the wet season, particularly in the days of heavy rains, sluices were opened more frequently (Chen, pers. commu). This operation enhanced the water mixing and resulted in a high dispersal of microorganisms (Custer et al. 2022). Similarly, autumn is the dry season of Chongming Island; the lower current velocities led to more contribution of homogeneous selection and drift and less effect of dispersal (Chen et al. 2019).

4.3 | Methodological Limitation in Studying Riverine Ciliate Community

The eDNA method has been widely used in ecological studies of ciliates, offering a powerful tool for understanding the distribution and diversity of these microorganisms in various ecosystems (Kulaš et al. 2021; Li et al. 2022, 2024; Wang et al. 2022). Compared to traditional methods, the ciliate community data obtained by the metabarcoding method were independent of the underestimation of the abundance and diversity due to the loss of cells after fixation and storage (Santoferrara et al. 2014; Huang et al. 2020). It has also been confirmed that the metabarcoding method was superior in detecting species in low abundance and usually revealed more evident diversity assemblies compared with traditional morphological methods (Medinger et al. 2010; Santoferrara et al. 2014; Sun et al. 2019).

In the present study, the metabarcoding method uncovered 1226 OTUs which were almost 10 times the species number detected by the QPS method and showed higher values of α -diversity (Figure 4A,B). This discrepancy suggests that the community compositions identified by the two methods do not align precisely. Although rare species can be detected by the metabarcoding method, contaminations and errors during the two-step PCR amplification, and extracellular or relic DNA may contribute to an overestimation of the diversity. To overcome this problem, one-step PCR or target PCR should be considered for minimizing the PCR errors and contaminations (Bohmann et al. 2021; Ni et al. 2023), and propidium monoazide (PMA) can be used to inhibit extracellular or relic DNA amplification (Bairoliya et al. 2022). The copy number of the SSU rDNA greatly varies among different species, and some investigations unveiled that the copy number is likely correlated to the cell size (Gong et al. 2013; Wang et al. 2017; Pitsch et al. 2019), which may explain the inconsistency of the relative abundance data yielded by the QPS and metabarcoding methods in the present study.

Moreover, the insufficient reference database also negatively affects our assessment of ciliates diversity using the metabarcoding method. In the present study, less than 15% of OTUs could be annotated at the species level. It is imperative to intensify efforts to associate the sequences in the reference database, for

example, PR2 database, with a comprehensive and accurate inventory of ciliates (Huang et al. 2020; Chi et al. 2023).

5 | Conclusion

It is the first time to investigate the diversity of ciliates in a complex river network by both metabarcoding and traditional (QPS) methods. Both methods revealed that there was a seasonal succession of the ciliate community and nutrients; water temperature and DO are key environmental factors that drove the succession. Moreover, in the trellised river network of Chongming Island, homogenizing dispersal emerged as the dominant process shaping the ciliate community assembly, and the role of environmental factors was masked by the impact of the river network's topology and water project operations. These results may further benefit in balancing the water operation projects and biodiversity conservation. But due to the lack of water velocity data, the conclusions are merely based on circumstantial evidence, which is needed to be tested by further investigations with detailed hydrologic data.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All raw sequences from this study have been submitted to the Sequence Read Archive database of NCBI under the BioProject number PRJNA 1208360 (<http://www.ncbi.nlm.nih.gov/bioproject/1208360>).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.