

UNVEILING PICOEUKARYOTIC DIVERSITY AND DISTRIBUTION IN THE BAY OF LARGE RESERVOIR SYSTEM

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Abstract. Few studies have reported about the diversity and distribution of picoeukaryotes in the ecosystem of reservoir bay. In this study, Sanger-based clone libraries of 18S rRNA gene were used to determine the picoeukaryotic diversity of three sampling sites; these sites were located across the Xiangxi Bay of Three Gorges Reservoir, China. The sampling sites were monitored across all four seasons in 2013. We identified 13 phyla in Xiangxi Bay. In this experimental survey, we found that picoeukaryotic community was dominated by three main phyla, namely, Cryptophyta, Chlorophyta, and Ciliophora (69.9% of clones in all). Moreover, the remaining 10 phyla showed a relative abundance of less than 6%. In Xiangxi Bay, the seasonal variability of communities overwhelms spatial patterns; upper reaches exhibited highest species diversity, whereas middle reaches exhibited lowest species diversity. The community temporal stability had decreased from upstream to downstream. Primary producers were dominant in all the sample sites, irrespective of the season. In Xiangxi Bay upstream, terrestrial microbes may be integral components of the microbial community assembly. In Xiangxi Bay, the distribution pattern of picoeukaryotes might have been affected by the mainstream of Three Gorges Reservoir.

Keywords: *picoeukaryotes, microbial diversity, 18S rDNA, tributary bay, distribution pattern*

Introduction

Picoeukaryotes are widely distributed in aquatic environments, contributing significantly to biomass and primary production (Li, 1994; Caron et al., 2012). They are core members of aquatic microbial food webs and play a fundamental role in biogeochemical cycling (Massana et al., 2011). This indicates that they play an important role in maintaining the balance of water ecosystems (Biegala et al., 2003; Vaultot et al., 2008). Many studies have reported about the unexpected diversity of picoeukaryotes in Open Ocean, coastal areas, and estuaries (Massana et al., 2011; Wu et al., 2014; Farnelid et al., 2016).

Some studies have determined the diversity of picoeukaryotes in freshwater systems. In oligotrophic systems, picoeukaryotes are very important primary producers of biomass (Stenuite et al., 2009). Moreover, a high level of picoeukaryote diversity was recorded in mesotrophic lakes (Lepère et al., 2008; Lefèvre et al., 2008). Recent studies have reported that picophytoplankton is an important primary producer in eutrophic

estuaries, contributing significantly to the total phytoplankton biomass (Murrell and Lores, 2004; Gaulke et al., 2010). In another study, researchers found that pico-phytoplankton abundance was quite high in rivers; it was almost equivalent to that in lakes and oceans (Contant and Pick, 2013). Currently, molecular techniques are used for surveying the diversity of picoeukaryotes that inhabit rivers (Thomasa et al., 2012), freshwater lakes (Zhao et al., 2011), and shallow aquatic environments (Simon et al., 2015a, b). However, we have little information about the diversity and distribution of picoeukaryotes, which thrive in an ecosystem of reservoir bay.

The Three Gorges Reservoir is located in the lower section of the upper reaches of Yangtze River in China (Han et al., 2015). The Xiangxi River is one of the longest rivers in the Three Gorges Reservoir region. After constructing the Three Gorges Dam, the Xiangxi River was transformed into Xiangxi Bay (Holbach et al., 2015). In this study, we used Sanger-based clone libraries of 18S rRNA gene to determine the picoeukaryote diversity of three sampling sites, which were present along the Xiangxi Bay of the Three Gorges Reservoir; the picoeukaryote diversity was monitored across all the four seasons in 2013. We addressed the following basic questions: what is the picoeukaryotic diversity in the bay of a large reservoir? Does the mainstream reservoir affect the distribution pattern of picoeukaryote in the bay?

Materials and methods

Sample collection and environmental data

Water samples were collected from downstream (XX01, 30°97'32.28"N, 110°75'80.59"E), midstream (XX04, 31°11'06.06"N, 110°77'57.14"E) and upstream (XX07, 31°18'96.64"N, 110°75'33.75"E) of Xiangxi Bay of the Three Gorges Reservoir of China in February (winter), April (spring), August (summer), and October (autumn) of 2013 (Fig. 1). The linear distance between the XX01 and XX07 sample sites was approximately 26 km.

Water samples were collected at a depth of 0.5 m beneath the water surface using a 5-L sampler and pre-filtered through 5 µm pore-size polycarbonate filters (Millipore) followed by filtration onto GF/F filters (0.7 µm, Whatman) for molecular analyses. Samples were immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

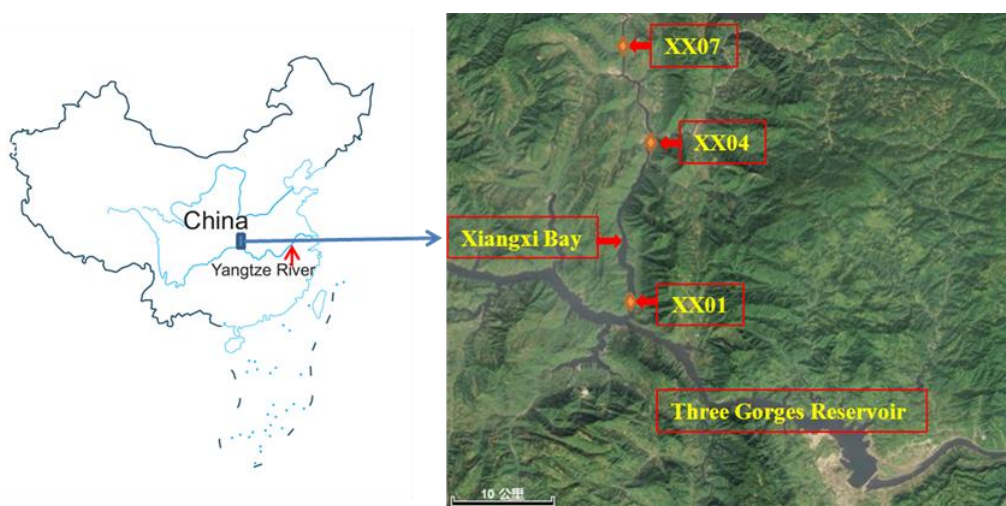


Figure 1. Map of study area and sampling site

Physical and chemical factors, such as water temperature (WT), dissolved oxygen (DO), pH and turbidity, were measured in situ. Concentrations of total nitrogen (TN), orthophosphate (P-PO₄, PO₄³⁻), nitrate (N-NO₃, NO₃⁻), total phosphorus (TP), Chemical Oxygen Demand (COD), Oxidation-Reduction Potential (ORP), water transparency (SD) and ammonium (N-NH₄, NH₄⁺) were measured using standard methods, as described previously (Huang et al., 1999). The trophic states of the Xiangxi Bay in different sample sites and seasons were determined by comprehensive nutritional status index (trophic level index, TLI) using the values of TN, TP and SD (Tian et al., 2017). Trophic states are set as follows: TLI ≤ 30, oligotrophic; 30 < TLI ≤ 50, mesotrophic; 50 < TLI ≤ 60, slightly eutrophic; 60 < TLI ≤ 70, moderately eutrophic; TLI > 70, highly eutrophic (Meng and Zhao, 2007).

DNA extraction and clone library construction

DNA was extracted from filters using a previously described method (Countway et al., 2005). Near full-length 18S rDNA fragments were amplified by polymerase chain reaction (PCR) using the eukaryotic-specific primers Euk328f/Euk329r (Euk328f: 5'-ACCTGGTTGATCCTGCCAG-3', Euk329r: 5'-TGATCCTTCYGCAGGTTTCAC-3') (Moon-van der Staay et al., 2000). PCR was carried out under the following conditions: 35 cycles (denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 2 min) proceeded by 3 min denaturation at 94 °C and followed by 8 min extension at 72 °C. 18S rDNA clone libraries were constructed using the pGEM[®]-T Easy Vector Systems (Promega) following the instructions provided by the manufacturer.

Amplified ribosomal DNA restriction analysis (ARDRA)

Positive clones from each library were screened by re-amplifying the 18S rDNA by PCR as described above, except that 0.1 µl of the culture of *Escherichia coli* containing the insert was used as a template. Positive PCR product was digested with *Hae*III (Fermentas). Gel electronic images were visualized by means of ChemiDoc XRS (Bio-Rad), images were analyzed with Image Lab software v. 3.0 (Bio-Rad). Clones with the same ARDRA pattern were grouped and considered as the same operational taxonomic unit (OTU).

Statistical analyses

Based on the result of ARDRA analysis, a library coverage value was computed according to the literature (Dang et al., 2008). Diversity indexes (Shannon and Pielou evenness) were also calculated. The Shannon diversity index is an index that is commonly used to characterize species diversity in a community. Pielou's evenness refers to how close in numbers each species in an environment are. The Kruskal-Wallis test was used for comparison between multiple groups. Bray-Curtis (temporal) dissimilarity based on relative abundance data OTUs was used as indices of the temporal turnover of community. The community structures were compared with non-metric multidimensional scaling (NMDS) using Bray-Curtis distances. The significance of the observed differences was determined by permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance measurements with 999 permutations. NMDS and the Bray-Curtis dissimilarity between pairwise samples was

calculated on the basis of OTU abundances. All these analyses were done using the R package *vegan*.

Analysis based on sequence

One or several representative clones of each ARDRA pattern were partially sequenced (Majorbio, China). Colonies were sequenced using the Euk 528F primer (5'-GCGGTAATTCAGCTCCAA-3') (Elwood et al., 1985). All sequences obtained were edited to exclude the primer sequence using *Lasergene.v7.1* (DNAStar Inc), and checked for chimerical structures using the *CHECK-CHIMERA* program on the Ribosomal Database Project (<http://rdp.cme.msu.edu/html/>). In order to determine the phylogenetic affiliation, all non-chimeric sequences were aligned using the online automatic *SILVA Incremental Aligner SINA v1.2.11* (<http://www.arb-silva.de/aligner/>) to identify known sequences with a high degree of similarity. Phyla that differed significantly ($q < 0.05$) for two groups were identified using White's non-parametric t-test with Storey's multiple testing correction in the *STAMP* software version 2.1 (Parks et al., 2014). Similarity percentage analysis (*SIMPER*) was used to assess which taxa are primarily responsible for an observed difference between groups. *SIMPER* were carried out using *PAST 3.18* (Hammer et al., 2001).

Correlations between picoeukaryotic communities and environmental factors were analyzed using the canonical correlation analysis (*CCA*) using R package *vegan*. *CCA* was performed using the relative abundance data of phyla (except rare phyla) and environmental factors. Significant environmental variables were chosen by the manual forward selection procedure, followed by Monte Carlo permutation tests (1000 iterations).

Nucleotide sequence accession number

The 18S rRNA gene sequences determined have been deposited in the GenBank database under accession numbers KX998709 - KX998893.

Results

Environmental characterization

The physicochemical parameters of water samples were present in *Table A1* in the *Appendix*. Among these parameters, statistically significant difference was observed in only TN values of the three sample sites ($p = 0.036$, Kruskal-Wallis tests). On the whole, the concentration of TN had increased gradually from upstream to downstream. Except for autumn, the TLI of the three sampling sites was greater than 50 through the year (*Table A1*, *Fig. A1*). The median TLI of three sampling sites (XX01, XX04, and XX07) was 51.57, 57.74, and 57.54, respectively (*Fig. A1*).

18S rDNA clone libraries

In total, 12 clone libraries were constructed for the three sample sites. Approximately 85–105 clones were isolated from each clone library; these clones were then subjected to ARDRA analysis. In total, ARDRA analysis was performed on 1116 clones; these clones were clustered into 240 OTUs. In each sample, the number of OTUs ranged from 18 to 59. The coverage of respective libraries ranged from 63% to 91% (*Table 1*). The

distribution of OTUs varied according to seasons or different sample sites. Only 31 OTUs appeared at all sites. Furthermore, only 6 OTUs appeared throughout the year (Fig. A2).

Table 1. Summary of ARDRA analysis of the 12 clone libraries

| Season | Sample sites | No. of RFLP types | Coverage | Shannon-Wiener | Pielou evenness |
|--------|--------------|-------------------|----------|----------------|-----------------|
| Spring | XX01 | 40 | 78.70% | 4.65 | 0.87 |
| | XX04 | 18 | 90.00% | 3.17 | 0.76 |
| | XX07 | 59 | 68.47% | 5.52 | 0.94 |
| Summer | XX01 | 36 | 68.42% | 4.64 | 0.89 |
| | XX04 | 44 | 71.27% | 4.94 | 0.90 |
| | XX07 | 35 | 72.50% | 4.47 | 0.86 |
| Autumn | XX01 | 29 | 90.76% | 3.54 | 0.71 |
| | XX04 | 48 | 68.13% | 5.29 | 0.85 |
| | XX07 | 55 | 63.16% | 5.51 | 0.95 |
| Winter | XX01 | 42 | 71.59% | 4.93 | 0.93 |
| | XX04 | 23 | 80.00% | 4.10 | 0.91 |
| | XX07 | 48 | 78.99% | 5.04 | 0.90 |

The Shannon index and Pielou evenness index of 12 clone libraries were calculated (Table 1). The Shannon index peaked in upstream (XX07 site), and they were least in the middle reaches (XX04 site) of Xiangxi Bay (Fig. A1). No statistically significant difference was observed in the diversity index of the three sample sites; moreover, no statistically significant difference was observed for the four seasons of the year (all $p > 0.09$, Kruskal-Wallis test).

Picoeukaryotic community composition

We obtained 185 high-quality sequences after removing all low-quality, unassembled, and potentially chimeric sequences. We performed phylogenetic analyses on 12 samples; the analyses results indicate that there were 13 phyla in these 12 samples (Fig. 2). Moreover, analyses results indicate that these libraries were dominated by Cryptophyta and Chlorophyta (32.4% and 21.5% of the total 910 clones respectively), followed by Ciliophora (16%) and Bacillariophyta (5.9%), Chrysophyta (3.3%). Telonemia, Pyrrhophyta, and Cercozoa were the three groups of picoeukaryotes, which were present in extremely low proportions. Besides, we also detected a very small amount of Haptophyta (1 sequence, 1 clone). Moreover, some kinds of fungi (Chytridiomycota, Cryptomycota, and Basidiomycota) and fungus-like (Oomycota) taxa were also detected in this study. The relative abundance of these fungi and fungus-like taxa was very low at the three sample sites. In these libraries, a significant number of sequences (37 sequences, 16.5% of the total 910 clones) were not affiliated with any formal taxa.

Among the Cryptophyta, an overwhelming majority belonged to Cryptomonadales (39 sequences); we also identified six sequences that were affiliated to uncultured Kathablepharidae. The Chlorophyta sequences were grouped into four taxa:

Chlorophyceae, Chlorodendrophyceae, Mamiellophyceae, and Trebouxiophyceae. Among them, Chlorophyceae was the most abundant taxon (26 of 35 Chlorophyta sequence). The Ciliophora sequences were grouped into three taxa: uncultured Choreotrichia, Litostomatea, and Prostomatea. Among them, uncultured Choreotrichia was the most abundant taxon (20 of 40 Ciliophora sequence). All the six sequences of Bacillariophyta belonged to the taxon Mediophyceae. Chrysophyta contained different types of sequences: three sequences were affiliated to the taxon Chrysophyceae, while one sequence was affiliated to Nannochloropsis. Telonemia consisted of four sequences, which were affiliated to the taxon Telonema (Shalchian-Tabrizi et al., 2006).

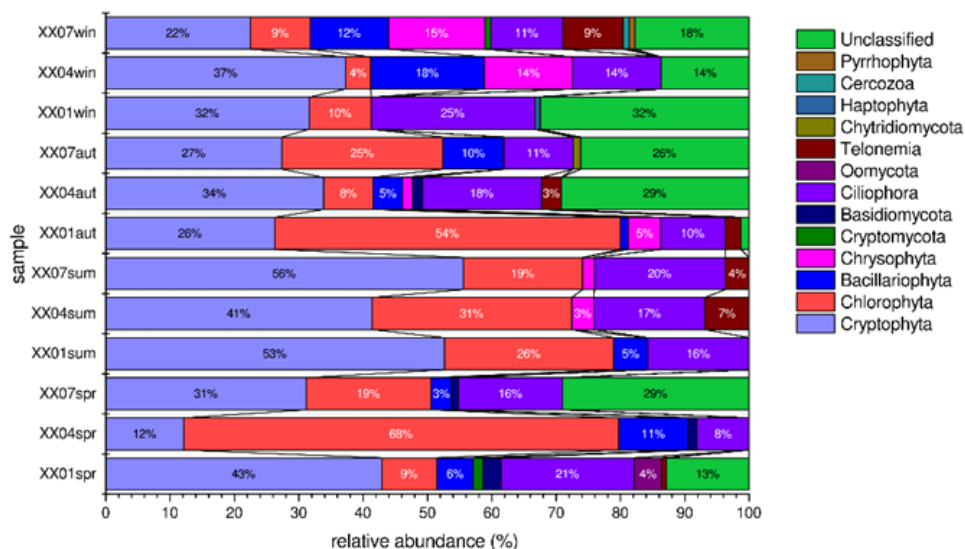


Figure 2. Distribution of the phyla in all samples. Spr: spring, sum: summer, aut: autumn, win: winter

Community structure across space and time

From winter to spring, the structure of picoeukaryotic communities changed slightly; a different picoeukaryotic community structure was observed from summer to autumn, with slight variation in composition. Seasonal patterns were detected by NMDS analyses (Fig. 3). By performing PERMANOVA analyses, we found that the composition of picoeukaryotic communities was significantly different across all the four seasons ($p = 0.0011$); however, there was no significant difference between the composition of picoeukaryotic communities of three sample sites ($p = 0.9937$). By SIMPER analysis, we observed the seasonal differences were mainly attributed to appreciable seasonal fluctuations in the relative abundance of dominant phyla. The seasonal differences were mainly observed in following phyla: Chlorophyta (24.41%), Cryptophyta (20.3%), and Ciliophora (12.25%).

In Xiangxi Bay, picoeukaryotes were unevenly distributed across the three sample sites; moreover, the distribution of picoeukaryotes also changed across the four seasons. By determining the Bray-Curtis dissimilarity matrix of all the samples, we found that the community temporal stability decreased from upstream to downstream (Fig. 4). By STAMP analysis, we found that Basidiomycota has a significantly higher abundance in spring than in summer ($q < 0.01$) (Fig. 5). No significant variations were observed in the composition of the phyla, regardless of the sample site.

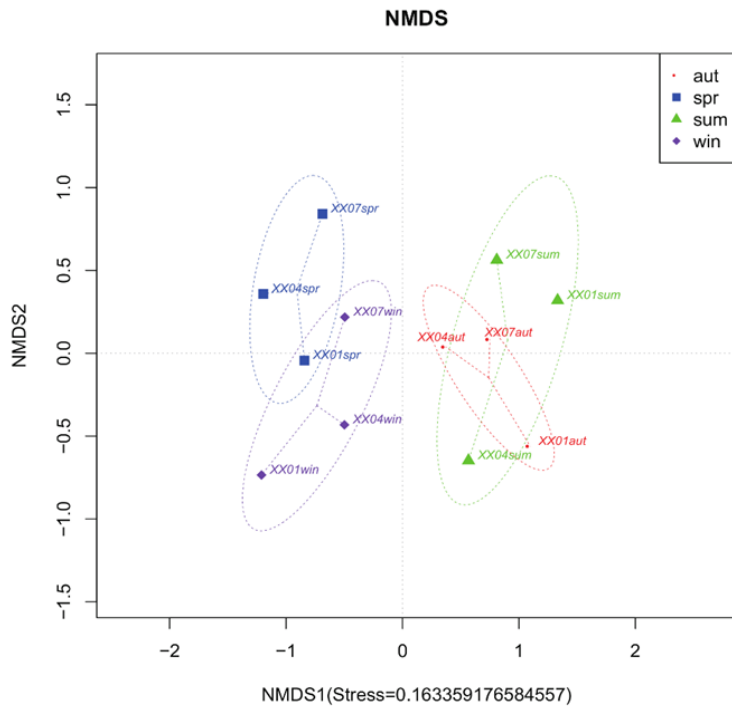


Figure 3. Non-metric MultiDimensional Scaling (NMDS) plot based on Bray-Curtis distances

Picoeukaryotic communities in relation to environmental variables

The results of CCA analysis indicate that abundances of specific taxa depended upon environmental variables (Fig. 6). The first two axes accounted for 35.3% of the variance. The abundances of Chrysophyta and Bacillariophyta were negatively related to pH and turbidity. The distribution of Bacillariophyta was greater in the area with higher concentration of N-NH₄. The distribution of Chrysophyta was concentrated in the least turbid area, which had lower values of N-NH₄.

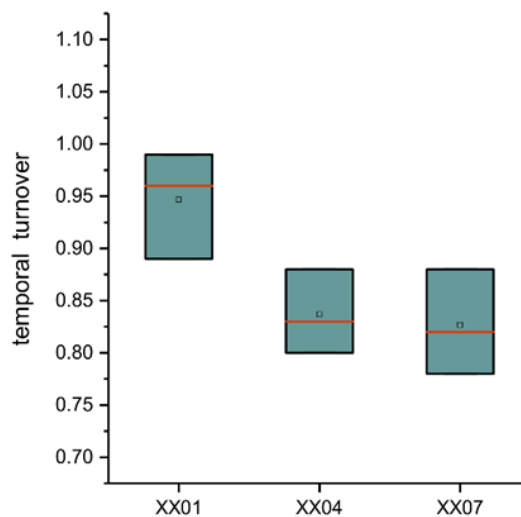


Figure 4. Boxplot of spring-summer, summer-autumn and autumn-winter pairwise dissimilarity (temporal turnover) coefficient of the three sample sites. Median is indicated by the red center lines, arithmetic average is indicated by the black hollow circle

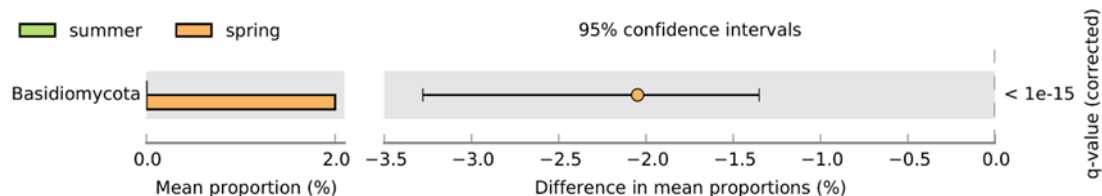


Figure 5. Phyla that was significantly different between two seasons

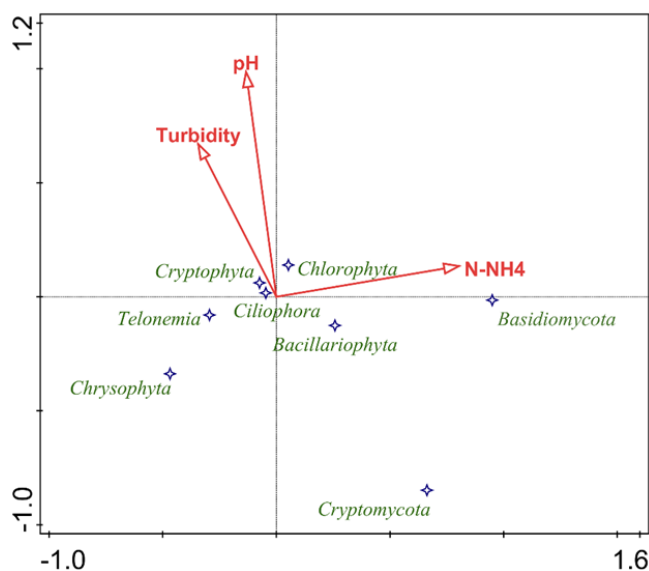


Figure 6. Canonical correspondence analysis (CCA) plot for the environmental parameters and the picoeukaryotic communities. N-NH4: ammonium

Discussion

In this study, we found that TLI was the highest in middle reaches (XX04 site) and least in the downstream (XX01 site) of Xiangxi Bay (Fig. A1). There is water exchange between the Three Gorges Reservoir mainstream and its tributaries (Liu et al., 2012; Huang et al., 2014). Owing to water exchange, nutrients are supplied from the Three Gorges Reservoir mainstream to the Xiangxi Bay (Yang et al., 2015). Water exchange occurs frequently at the river mouth; however, the time of water retention is short (Liu et al., 2012). Consequently, the middle reaches of Xiangxi Bay were replenished with several nutrients (Ji et al., 2010). Therefore, middle reaches of Xiangxi Bay have the highest TLI.

Previous studies have reported that the diversity of species decreases with eutrophication (Baho et al., 2017; Cook et al., 2018). Therefore, the diversity index was lowest in middle reaches of Xiangxi Bay (Fig. A1). Surprisingly, the diversity index was highest at the upstream (XX07 site) of Xiangxi Bay, but its trophic level was higher than the downstream of Xiangxi Bay. With the flows of water, soil, litter, and anthropogenic materials were transported in and around rivers. These materials got easily mixed with the resident microbial communities (Mansour et al., 2018). Recently, several studies have been carried out on different water ecosystems; these studies have reported that terrestrial microbes show significant abundance in the microbial communities of headwater ecosystems (Crump et al., 2012; Besemer et al., 2013; Ruiz-

González et al., 2015). In the upstream sampling site, microbial diversity could be structured by inoculating the microbes from terrestrial sources; these microbes would contribute to community assembly. This may be the reason for the differences in the diversity of picoeukaryotic communities, which are found in the down and upper reaches of Xiangxi Bay.

The upstream of Xiangxi Bay had the highest diversity index, while the midstream of Xiangxi Bay had the lowest diversity index (*Fig. A1*). Before the construction of the Three Gorges Reservoir, the diversity index of Xiangxi River was lower down-stream (Kuang et al., 2004). This implies that the distribution pattern of plankton diversity has changed to some extent after the construction of the Three Gorges Reservoir. Furthermore, our results indicate that diversity indices of summer and winter were greater than those of spring and autumn (*Fig. A3*). Similar results have been presented by researchers in previous studies (Zhang et al., 2017). Previous studies have proved that the mainstream of Three Gorges Reservoir significantly influences its tributaries in spring and autumn seasons: this is because water is discharged from the mainstream of Three Gorges Reservoir during spring season; moreover, water is impounded into the same reservoir during autumn (Li et al., 2012). After analyzing the diversity indices and temporal turnover of picoeukaryotic communities, we inferred that the Three Gorges Reservoir does impact plankton diversity of its tributaries.

The concentration of N-NO₃ is much higher than that of N-NH₄ in Xiangxi Bay (*Table A1*). The results of ordination analysis prove that Bacillariophyta had a positive relationship with N-NH₄. In the presence of sufficient nutrients, diatoms are generally N-NO₃ opportunists (Donald et al., 2011; Glibert and Wilkerson, 2014). Some studies have reported that diatoms are distributed in the area, which has higher concentration of N-NH₄ values (Zhao et al., 2013). Therefore, further studies must be conducted to determine whether elevated levels of N-NH₄ favor the growth of pico-diatoms in Xiangxi Bay. Chrysophyta often grows in highly transparency water, which has low organic content (Lin et al., 2003). In this survey, Chrysophyta appear in less turbid areas, which have comparatively lower concentration of N-NH₄. The relative abundance of Chrysophyta was much greater in oligotrophic lakes than in Xiangxi Bay. This indicates that eutrophication affects the composition of plankton communities in different water bodies.

In summary, water exchange between Xiangxi Bay and Three Gorges Reservoir mainstream may affected the distribution pattern of biodiversity and nutritional state of Xiangxi Bay. So, middle reaches of Xiangxi Bay have the highest TLI. Furthermore, upper reaches exhibited highest species diversity, whereas middle reaches exhibited lowest species diversity. In Xiangxi Bay, the seasonal variability of communities overwhelms spatial patterns. The community temporal stability had decreased from upstream to downstream. In the upstream sampling site, microbial diversity could be structured by inoculating the microbes from terrestrial sources.

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APPENDIX

Table A1. Physical and environmental characteristics of the sampling stations. Spr: spring, sum: summer, aut: autumn, win: winter. WT: water temperature, DO: dissolved oxygen, TN: total nitrogen, P-PO₄: orthophosphate, N-NO₃: nitrate, TP: total phosphorus, N-NH₄: ammonium, COD: chemical oxygen demand, ORP: oxidation-reduction potential, TLI: trophic level index

| | XX01spr | XX04spr | XX07spr | XX01sum | XX04sum | XX07sum | XX01aut | XX04aut | XX07aut | XX01win | XX04win | XX07win |
|--------------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| TP(mg/L) | 0.20 | 0.24 | 0.41 | 0.08 | 0.15 | 0.26 | 0.07 | 0.08 | 0.05 | 0.15 | 0.17 | 0.20 |
| P-PO ₄ (mg/L) | 0.16 | 0.14 | 0.27 | 0.10 | 0.01 | 0.05 | 0.07 | 0.06 | 0.04 | 0.13 | 0.17 | 0.19 |
| N-NH ₄ (mg/L) | 0.12 | 0.14 | 0.04 | 0.02 | 0.05 | 0.07 | 0.03 | 0.03 | 0.03 | 0 | 0 | 0.02 |
| N-NO ₃ (mg/L) | 1.54 | 0.80 | 0.11 | 1.00 | 0.84 | 0.12 | 1.47 | 1.22 | 1.11 | 1.73 | 1.39 | 1.33 |
| TN(mg/L) | 1.77 | 1.24 | 0.91 | 1.6 | 2.17 | 1.34 | 1.55 | 1.47 | 1.19 | 1.84 | 1.47 | 1.45 |
| COD(mg/L) | 1.13 | 1.94 | 3.76 | 2.59 | 3.76 | 3.69 | 1.86 | 1.86 | 2.18 | 1.2 | 1.24 | 1.28 |
| WT(°C) | 17.40 | 18.70 | 21.10 | 29.60 | 28.70 | 29.50 | 24.50 | 24 | 24.10 | 12.80 | 12.20 | 12.4 |
| DO(mg/L) | 7.96 | 12.3 | 18 | 12.5 | 8.16 | 15.6 | 7.7 | 7.3 | 7.81 | 9.18 | 9.74 | 9.01 |
| ORP(mV) | 255 | 211.7 | 192.1 | 61.2 | 61.6 | 48.1 | 104.6 | 95.2 | 88.7 | 304.1 | 295.7 | 298 |
| pH | 8.24 | 8.83 | 9.43 | 9.31 | 9.08 | 9.75 | 8.68 | 8.81 | 8.85 | 8.19 | 8.34 | 8.28 |
| Turbidity(NTU) | 1.30 | 5.80 | 6.10 | 8.20 | 18 | 17.80 | 1.40 | 2.40 | 2.10 | 1.20 | 1.10 | 1.20 |
| TLI | 50.71 | 60.97 | 62.25 | 54.41 | 61.44 | 64.46 | 49.58 | 51.7 | 48.95 | 52.44 | 54.52 | 52.83 |

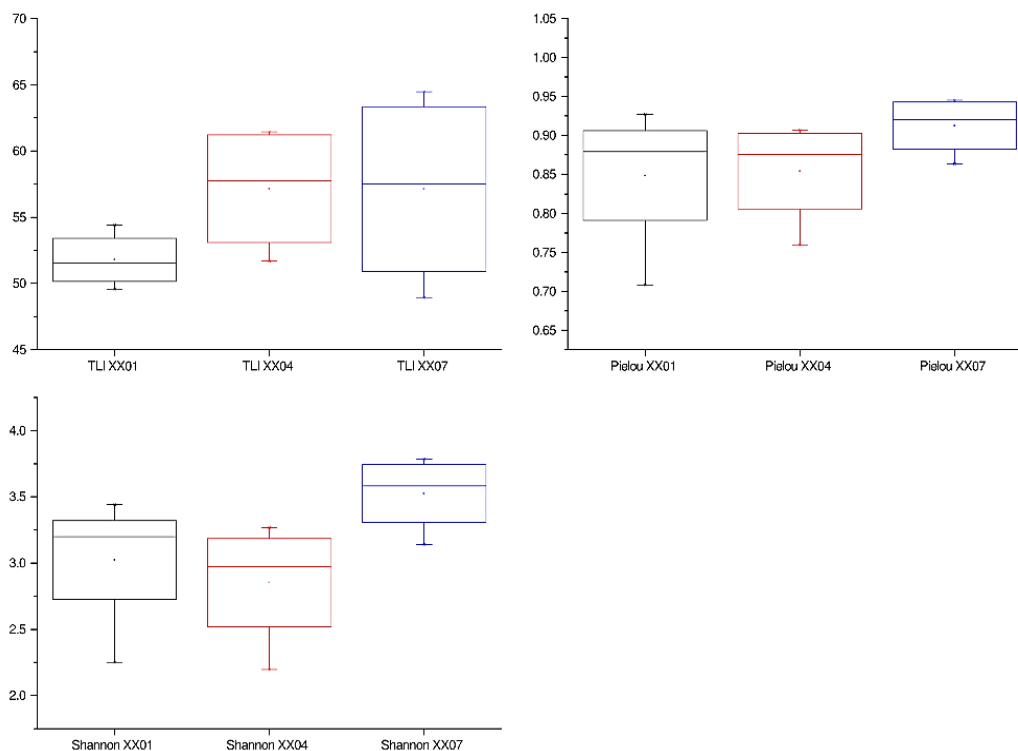


Figure A1. Nutritional status and diversity index plot. TLI: trophic level index. Median is indicated by the center lines, arithmetic average is indicated by the hollow circle

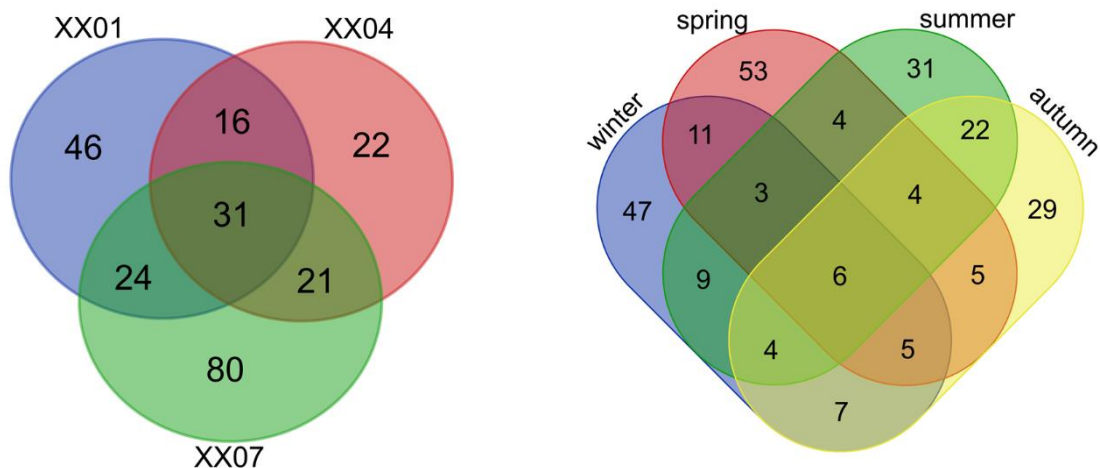


Figure A2. Venn diagrams showing shared and unique season and sample sites OTUs based on clone libraries

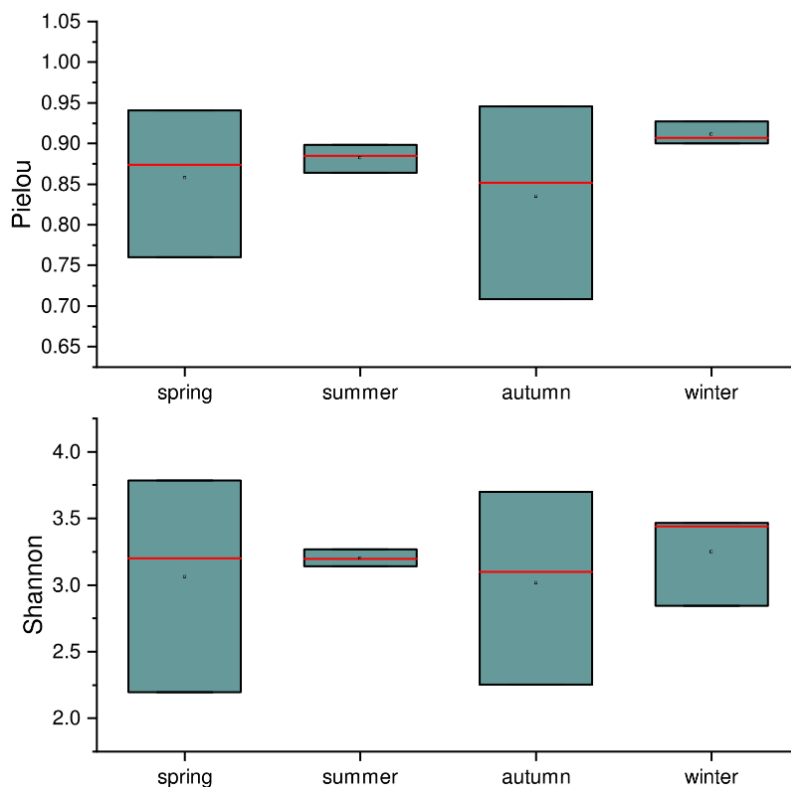


Figure A3. Boxplot of the Shannon and Pielou evenness indexes among the four seasons. Median is indicated by the red center lines, arithmetic average is indicated by the black hollow circle