Community Characteristics Analysis of Eukaryotic Microplankton via ITS Gene Metabarcoding Based on Environmental DNA in Lower Reaches of Qiantang River, China

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Abstract
Eukaryotic microplankton plays an important role in water biotic community and in maintaining the stability of water ecosystems. Environmental DNA metabarcoding provides the opportunity to integrate traditional and emerging approaches to discover more new species, and develop molecular biotic indices that can be more rapidly, frequently, and robustly used in water quality assessments. In order to examine assemblages of eukaryotic microplankton in lower reaches of Qiantang River, ITS gene metabarcoding technology based on environmental DNA was carried out. As a result, various species of phytoplankton, fungi and zooplankton were annotated on. More phylum, classes and species of eukaryotic phytoplankton and zooplankton were found after compared communities taxa based on metabarcoding with that obtained from morphological examination. Nevertheless, Chlorophyceae was the most common assemblage both identified by using these two methods, also Mesocyclops leuckarti and Acanthocyclops bicuspidatus were both found to be the dominant species of Cyclopoida in the river. Additionally, the reads proportions of phytoplankton and zooplankton at the three freshwater sampling sites (Tonglu, Fuyang and Wenyan) decreased as temperature drop. Meanwhile, twenty classes of fungi were annotated on, of which the community characteristic was first researched in the river. There were significant spatial differences in values of Chao1 index for eukaryotic microplankton.

Cluster analysis and Non-metric multidimensional scaling ordination further
confirmed that the community composition of eukaryotic microplankton at class level for Jiashao-September sample had the most dissimilarity with the others.

**Keywords**

Microplankton, eDNA, Metabarcoding, ITS, Diversity, Qiantang River

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### 1. Introduction

Eukaryotic microplankton is a group of plankton with particle sizes less than 20-µm [1]. It plays an important role in water biotic community and in maintaining the stability of water ecosystems due to its important link in energy flow and material circulation [1]. As the basis of the food chain, analyzing its metacommunity structure is very important to assess the status and development tendency of an ecosystem [2].

Traditional practices for biological surveys of inland waters usually center on a common set of ecological indicators or indices/measurements of biodiversity [3], requiring many preparations, such as morphological taxonomic expertise, intact specimens and adequate time [4] [5]. Environmental DNA (eDNA) metabarcoding provides the opportunity to integrate traditional and emerging approaches to discover more new species [6] [7], and develop molecular biotic indices that can be more rapidly, frequently, and robustly used in water quality assessments [6] [8] [9]. So far, metabarcoding technology has been widely used for biodiversity monitoring in biological and environmental samples [10] [11] [12] [13]. Despite some limitations (each marker region might import some biases and the blast sequence database is incomplete), the practices of metabarcoding-based analysis for estimating diversity and relative abundance of taxonomic groups in aquatic systems will likely increase as technology improved [14].

Now, marker genes used for the analysis of plankton communities via eDNA metabarcoding often focuses on ribosomal DNA (rDNA), ribulose bisphosphate carboxylase large subunit gene (rbc L) and cytochrome C oxidase subunit gene 1 (cox1) [8] [15] [16] [17] [18]. Among these, the internal transcribed spacer (ITS), as one gene fragment of rDNA, has been widely used due to its fast evolution and high specificity [17], and its relevant metabarcoding practices for the purpose of monitoring diversity of eukaryon communities mainly focused on soil, plant and marine systems [19] [20] [21] [22].

Here, an ITS metabarcoding assessing assemblages of eukaryotic microplankton were conducted. Specifically, ITS gene sequence analysis was performed on surface water samples collected from four sites in Qiantang River lower reaches (Zhejiang, China), an important freshwater fishing water used for drinking, electricity generation, flood control and recreation, in order to analyze the community diversity of eukaryotic microplankton in the section, to assess the
utility of this approach for monitoring diversity of freshwater eukaryotic microplankton community. As a whole, this study provided a theoretical basis for further study on the function of eukaryotic microplankton in freshwater ecosystems.

2. Materials and Methods

2.1. Water Sample Collection and Physicochemical Analysis

A total of eight water samples were collected in September and November 2019, at four separate sampling sites in Qiantang River lower reaches, including Tonglu, Fuyang, Wenyan and Jiashao sites, hereafter referred to as TL, FY, WY and JS separately (Figure 1). All sampling, filtering, and other equipments were sterilized before use.

2 L of surface water was collected at a set time by boat from each site. Simultaneously, five environmental variables, such as transparency, salinity, water temperature (WT), pH and dissolved oxygen (DO), were measured in situ by using a secchi disk and a portable water quality detector (Hach, USA). For each

Figure 1. Map shows the study site. (a) The location of Qiantang River in Zhejiang province of China; (b) Sampling sites in Qiantang River lower reaches. The red dot represents the water sampling site, the black dot represents the local government seat.
water sample, a 1.5 L water subsample was used to test six environmental variables immediately after transported to the laboratory within 24 hours at 4°C, including Chemical oxygen demand (COD), Chlorophyll a (Chla), total nitrogen (TN), total phosphorus (TP), ammonium (NH₄-N) and nitrate-nitrite (NO₃-N), the recording of which were following the scheme of [23]. All environmental variables were measured in triplicate.

2.2. DNA Sample Processing and High-Throughput Sequencing (HTS)

For each water sample, a residual 500-mL water subsample was then filtered through a 0.22-μm cellulose acetate filter paper using a peristaltic pump in the field. Then, each paper was placed inside a commercial sterile centrifuge tube and stored in a container filled with liquid nitrogen until subsequent manipulations were performed. DNA was extracted from filters using EZNA water DNA kit (Omega, USA) following the manufacturer’s protocol. The concentration and purity of DNA were determined using NanoDrop 2000c spectrophotometer (Thermo, USA), then followed by multiplex PCR using the universal primers for ITS belonging to eukaryotic mitochondrial DNA fragments, ITS-F (5’-GTGATCATCGARTC-3), ITS-R (5’-TCCTCCGCTTATTGAT-3’) [22]. Each eDNA sample was subjected to PCRs in triplicate [17.35 μL dH₂O, 2.5 μL 10 × PCR buffer, 0.15 μL 5 U/μL Thermo scientific Taq DNA polymerase, 2 μL dNTPs mix (2.5 mM), 1 μL of each primer (10 mM), and 1 μL sample eDNA in a total volume of 25 μL]. PCR cycling parameters were as follows: 96˚C for 3 min, followed by 35 cycles of 96˚C for 30 s, 50˚C for 30 s, and 72˚C for 90 s, with a final elongation step at 72˚C continued for 7 min. Negative control was conducted simultaneously. After subjecting the PCR products to 1% polyacrylamide gels (see Figure S1), the quantified, size-selected libraries were constructed and continuously diluted to a concentration suitable for sequencing. The libraries were finally sequenced on the Illumina MiSeq 2000 platform by following the manufacturer’s protocols step by step.

2.3. Phytoplankton Samples Collection and Treatment

Phytoplankton samples were also simultaneously collected at the four sites. For phytoplankton counts, 1.0 L of water samples were sampled each time and preserved with 1% Lugol’s iodine solution. Phytoplankton samples were concentrated to a final volume of 30 ml after sedimentation for 48 h. Thereafter, the taxa were verified and counted under 200× and 400× magnifications for at least 500 specimens [23]. The data were made to compare with that collected from ITS gene metabarcoding method.

2.4. Bioinformatics and Sequencing Data Upload

The raw sequencing FASTQ file was transformed to a FASTA file by the Fastx toolkit V0.0.1 [24]. Clean reads were gained after trimming the low quality se-
quences and PCR chimeras by using Fqtrim V0.9.4
(http://ccb.jhu.edu/software/fqtrim/) and Vsearch 2.3.4 [25]. Vsearch 2.3.4 software continued to be utilized to cluster Operational taxonomic units (OTUs) at 97% cutoff of sequence similarity. Representative sequences of OTUs were assigned to taxonomic groups against the NCBI Genbank by using Blast+ 2.6.0 at 80% cutoff of identity thresholds with over 80% matches and expect values less than 1e−5. The sequencing data have been uploaded to NCBI Sequence Read Archive database, with accession numbers vary from SRR10800795 to SRR10800802.

2.5. Date Analysis

Three α-diversity indices, including Chao1 estimators, Simpson index, and Shannon index, were calculated based on data obtained by metabarcoding monitoring. Additionally, Cluster analysis taken by group average method and Non-metric multidimensional scaling (NMDS) was employed to cluster samples in Primer 5.0 environment [26], of which species data were first transformed according to [27]. Additionally, basic data processing, drawing and statistical analyses (e.g. one-way ANOVA) were conducted using Excel 2007 and SPSS 16.0 software.

3. Results

3.1. Environmental Characterization

The results of environmental variables are showed in Table 1. WT showed significant differences between months. TN showed significant negative association with transparency at p < 0.05, with COD at p < 0.01, and significant positive association with TP at p < 0.01. In addition, NH4-N showed a significant positive association with TP and WT at p < 0.05. The values of TN: TP mass ratios were all higher than 7 in all sampling sites, indicating the research area was generally P-limited at the experimental period.

3.2. Sequencing Analysis

ITS gene metabarcoding yielded 67,469 - 129,150 raw reads, of which 55,687 - 112,832 clean reads were obtained after optimization, resulting in effective data rates varying from 64.1% to 88.8% (Table 2). The sequences clustered into a total of 5795 OTUs, varying from 706 to 1911 at an average of 1245 (Table 2). Meanwhile, the rarefaction curves of each sample all showed the observed species number flatted out as sequence increasing, indicating the amount of sequencing data at the 97% similarity threshold was sufficient to satisfy the assessment of species diversity.

3.3. Community Structure Composition

In total, Phytotplankton, fungi, zooplankton and other eukaryotes were annotated on after Blast. Five classes of eukaryotic phytoplankton were annotated on,
Table 1. Values of the eleven environmental variables from sampling sites.

| Sample | DO (mg/L) | pH  | WT(˚C) | Salinity | Transparency (cm) | Chl a (μg/L) | COD (mg/L) | TP (mg/L) | TN (mg/L) | NH4-N (mg/L) | NO3-N (mg/L) |
|--------|-----------|-----|--------|----------|-------------------|-------------|-----------|-----------|-----------|-------------|-------------|
| TL-A   | 6.66      | 7.28| 24.8   | –        | 170               | 1.77        | 17.00     | 0.05      | 2.11      | 0.33        | 1.25        |
| TL-B   | 7.21      | 7.38| 18.0   | –        | 300               | 0.40        | 14.00     | 0.06      | 1.73      | 0.11        | 1.51        |
| FY-A   | 6.53      | 7.37| 26.3   | –        | 75                | 3.21        | 15.00     | 0.05      | 1.93      | 0.32        | 1.26        |
| FY-B   | 7.83      | 7.35| 18.3   | –        | 250               | 0.95        | 14.00     | 0.06      | 1.51      | 0.12        | 1.32        |
| WY-A   | 8.20      | 7.29| 27.5   | –        | 65                | 6.97        | 17.00     | 0.06      | 2.00      | 0.32        | 1.36        |
| WY-B   | 8.25      | 7.48| 18.7   | –        | 110               | 5.52        | 8.00      | 0.04      | 1.88      | 0.42        | 1.28        |
| JS-A   | 7.29      | 7.79| 30.3   | +        | 5                 | 3.82        | 2.02      | 0.28      | 3.77      | 0.89        | 1.68        |
| JS-B   | 9.33      | 7.95| 18.5   | +        | 10                | 2.94        | 2.76      | 0.29      | 3.46      | 0.12        | 2.89        |

A: the “September” sample; B: the “November” sample. Hereinafter inclusive; +: salinity measured; −: no salinity measured.

Table 2. Temporal and spatial variation of indices based on ITS gene metabarcoding data.

| Description                     | Sep. | Nov. | Sep. | Nov. | Sep. | Nov. | Sep. | Nov. |
|---------------------------------|------|------|------|------|------|------|------|------|
|                                | TL   | FY   | WY   | JS   |
| Number of raw reads            | 86,859 | 87,871 | 72,481 | 83,729 | 67,469 | 73,525 | 68,724 | 129,150 |
| Number of clean reads          | 55,687 | 75,178 | 60,425 | 73,516 | 55,784 | 65,278 | 59,221 | 112,832 |
| effective data rate (%)        | 64.1 | 85.6 | 83.4 | 87.8 | 82.7 | 88.8 | 86.2 | 706 |
| Number of OTUs                 | 1911 | 1370 | 1485 | 978 | 1641 | 1130 | 706 | 744 |
| Reads abundance for phytoplankton (%) | 21.8 | 4.9 | 40.9 | 3.7 | 45.7 | 2.2 | 3.6 | 27.0 |
| Reads abundance for zooplankton (%) | 0.3 | 0.1 | 0.3 | 0.0 | 0.1 | 0.1 | 0.0 | 0.1 |
| Reads abundance for fungi (%)   | 9.7 | 10.2 | 4.7 | 3.8 | 3.1 | 22.4 | 22.2 | 43.7 |
| Reads abundance for other eukaryotes (%) | 0.7 | 0.1 | 0.1 | 0.0 | 0.2 | 0.1 | 0.0 | 0.2 |
| Reads abundance for unclassified (%) | 89.3 | 89.6 | 94.9 | 96.2 | 96.6 | 77.4 | 77.8 | 56 |
| Shannon index based on metabarcoding  | 7.7 | 5.1 | 7.0 | 4.6 | 7.6 | 6.0 | 3.3 | 6.1 |
| Simpson index based on metabarcoding  | 1.0 | 0.9 | 1.0 | 0.8 | 1.0 | 0.9 | 0.7 | 1.0 |
| Chao1 index based on metabarcoding  | 2063.2 | 1,494.1 | 1666.4 | 1077.2 | 1813.8 | 1288.3 | 810.0 | 684.9 |

Sep.: September; Nov.: November.

in which Chlorophyceae, Trebouxiophyceae and Cryptophyceae had more reads abundance (Figure 2(a)). Genera Chlamydomonas, Micractinium, Chlorella, Crucigenia, Cryptomonas, Actinastrum, Gonium, Dictyosphaerium and Compactochlorella were the common phytoplankton, most of which belong to phylum Chlorophyta, except Cryptomonas which belongs to phylum Cryptophyta. Meanwhile, three classes of zooplankton, including Ciliatea (Protozoa), Maxillopoda (Arthropoda) and Hydrozoa (Cnidaria), were annotated on, and the first two classes were the common zooplankton assemblages (Figure 2(b)). Further-
more, twenty classes of fungi were annotated on, Dothideomycetes, Sordariomycetes, Eurotiomycetes and Tremellomycetes were the common classes (Figure 2(c)). However, the common classes of fungi in JS-A sample were others, such as Malasseziomycetes, Agaricostilbomycetes, Ustilaginomycetes and Wallemiomycetes.

**Figure 2.** Reads abundance of eukaryotic microplankton (phytoplankton (a), zooplankton (b) and fungi (c)) based on ITS gene metabarcoding in eight samples at class level.
3.4. Seasonal Dynamics of Communities

Overall, the total reads of ITS annotated on eukaryotic microplankton in September was higher than that in November. The dominant assemblage in TL, FY and WY sites in September was phytoplankton, contributing 21.8%, 40.9% and 45.7% of the total reads respectively, followed by fungi (9.7%, 4.70% and 3.1%, respectively) and zooplankton (0.3%, 0.3% and 0.1%, respectively), however, the result in JS site was different, in which fungi was dominant (Table 2; Figure 3). Nevertheless, fungi changed to be the dominant in November in all four sampling sites with 10.2% of total reads in TL, 3.8% in FY, 22.4% in WY and 43.7% in JS site, and phytoplankton became to be the secondary assemblages simultaneously (Table 2; Figure 3). Combined with water temperature and salinity condition, the reads proportions of phytoplankton and zooplankton in the three freshwater sampling sites (TL, FY and WY) decreased as temperature drop, while fungi increased in majority of sites except FY. The reads abundance of phytoplankton and zooplankton at the estuarine site, JS, were fewer than the contemporaneous reads of three freshwater sites, and the reads of fungi were also opposite.

3.5. Diversity Analysis of Eukaryotic Microplankton

Significant spatial differences in values of Chao1 index were deduced (p < 0.05). However, Shannon and Simpson indexes showed no significant spatio-temporal differences. Generally, the three indexes in September at the freshwater sampling sites were all higher than that in November, which was a little different from that in JS sites (Table 2).

Eight samples were divided into two clusters at the 20% level, cluster for JS-A and cluster for the other seven samples, indicating that the microplankton class composition of JS-A had the least similar with that of the other samples (Figure 4(a)), which was also verified by using NMDS ordination method (Figure 4(b)).

![Figure 3. Reads abundance vibration of eukaryotic microplankton based on ITS gene metabarcoding in eight samples.](image-url)
3.6. Data of High-Throughput Sequencing (HTS) and Morphology Comparison

We compared the communities of phytoplankton and zooplankton taxa with results obtained from morphological analysis in order to determine potential biases of the primer set used in our study. Species of three phylum and five classes of eukaryotic phytoplankton were annotated by metabarcoding method, which was different from that identified by microscopic examination. Also, more than 104 phytoplankton species were identified by metabarcoding (193 species vs. 89 species) (Table 3). Chlorophyceae was the most common assemblage, which was consistent with the finding via morphology, accounting for 12.93% - 79.45% of the total eukaryotic phytoplankton reads, among which Chlamydomonas reinhardtii was dominant, contributing 1.22% of the total reads. Meanwhile, genera with higher reads proportion, such as Chlamydomonas, Chlorella, Crucigenia, Cryptomonas, Actinastrum, Gonium and Dictyosphaerium, were widespread in freshwater of Zhejiang province [27] [28] and were also dominant genera that identified via morphology (Table S1).
Table 3. Comparison analysis of eukaryotic phytoplankton data based on metabarcoding technology and morphological examination.

| Description     | Metabarcoding monitoring | Morphological monitoring |
|-----------------|--------------------------|--------------------------|
| Number of phylum| 3                        | 5                        |
| Phylum list     | Chlorophyta, Bacillariophyta and Cryptophyta | Chlorophyta, Bacillariophyta, Cryptophyta, Euglenophyta and Dinoflagellate |
| Number of classes | 5                        | 6                        |
| Classes list    | Pedinophyceae, Chlorophyceae, Trebouxiiophyceae, Centricae and Cryptophyceae | Chlorophyceae, Centricae, Pennatae, Euglenophyceae, and Dinophyceae |
| Number of genus | 99                       | 50                       |
| Number of species | 193                     | 89                       |

For the zooplankton, the comparisons were made with data reported by [29] and [30]. As a widely distributed taxa in Qiantang River, Cyclopoida was identified in this study, and the dominant species of Cyclopoida annotated on were *Mesocyclops leuckarti* and *Acanthocyclops bicuspidatus*, which was consistent with the discovery of [29]. However, as another dominant assemblage [30], rotifers were not annotated here.

4. Discussion

4.1. The Feasibility of Microplankton Community Analysis Based on ITS Gene Metabarcoding

In this study, we selected ITS with fast evolution and high specificity as the amplicon to describe the community structure and its dynamics of eukaryotic microplankton in Qiantang River lower reaches. Here, the community characteristics of fungi in the Qiantang River were first researched, it’s found that Dothideomycetes, Sordariomycetes, Eurotiomycetes and Tremellomycetes were the most common groups, which were different from that in the Yellow Sea concluded by using the same amplicon [22].

After compared the communities of phytoplankton and zooplankton taxa with results obtained from metabarcoding and morphological analysis, we revealed the same most common assemblage, and discovered 104 more species via metabarcoding. Compared with previous studies [28], it’s speculated that the phytoplankton community had changed somewhat since then, but some dominant specieses maintained unchanged, genera *Chlamydomonas* and *Cryptophyllum* were still common dominant groups. Meanwhile, Anthoathecata, a zooplankton taxa that hasn’t been identified by microscopic examination, was annotated on JS samples. Anthoathecata is an order of class Hydrozoa and distributes on the east and south coast of China. Here, JS sites are located in the estuary area of Qiantang River, indicating a possibility that species of Anthoa-
thecata distribution. However, rotifers were not detected by metabarcoding. The predatory relationship between species may be a considerable reason. Generally, the DNA of the prey does not get separated out during the blast, especially if the prey items belong to the same genus as the predator. In addition, the preference of primer and differences in rRNA gene copy numbers may also explain some of these differences.

Overall, these findings demonstrated that metabarcoding could yield comparable results to conventional methods for several abundant eukaryotic taxa, but that each method has different limitations as far as accurately describing the eukaryotic composition in this river.

4.2. Community Diversity Characteristic of Eukaryotic Microplankton

Here, geographically, the sampling sites from top to bottom are, in order, Tonglu, Fuyang, Wenyan and Jiashao. There is no hydraulic dam between the four sampling sites, and all of them are in the tidal reach of the river, resulting in a relatively frequent water exchange caused by the flow of tide. Compared with other sites, JS is near estuarine region and more affected by seawater. Correlation analysis showed that there were significant spatial differences in values of Chao1 index (p < 0.05), indicating the species richness had obvious dissimilarity between sampling sites. Cluster analysis and NMDS ordination further confirmed the microplankton class composition of JS-A had the most dissimilarity with that of the other samples, the content of salinity might be one of the reasons [31]. Studies have shown that some spatial differences in eukaryotic plankton α-diversity is more the result of selection by local environmental conditions than dispersal [32], the feasibility of α-diversity based on ITS rRNA gene metabarcoding might be a useful indicator for discriminating ecological condition.

5. Conclusion

Our data were generated using a primer set that targets the ITS region of ribosomal RNA gene, a region that has been widely used in biodiversity assessments in phytoplankton, fungi, zooplankton, etc. As a result, various species of phytoplankton, fungi and zooplankton were annotated. We identified several groups of eukaryotic phytoplankton and zooplankton that were not described by morphological analysis, and increased research on fungi in Qiantang River that never had been studied before. Chlorophyceae was the most common assemblage both identified by using ITS gene metabarcoding and morphological examination methods, also Mesocyclops leuckarti and Acanthocyclops bicuspidatus were both found to be the dominant species of Cyclopoida in the river. The reads proportions of phytoplankton and zooplankton at the three freshwater sampling sites (Tonglu, Fuyang and Wenyan) decreased as temperature drop. In addition, there were significant spatial differences in values of Chao1 index for eukaryotic microplankton. Finally, it’s confirmed that the Metabarcoding-based approach
herein described can be used in analyzing community characteristics of eukaryotic microplankton to some extent and will significantly be complete as technology improved.

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Conflict of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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**Supplementary Information**

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**Additional file 1: Figure S1.** Results of eight water DNA samples amplified by the ITS primer. **Table S1.** Species of eukaryotic phytoplankton indentified by using ITS gene metabarcoding technology and morphological examination.

![Figure S1](image_url). Results of eight water DNA samples amplified by the ITS primer. Lane M: Takara DL2000 DNA Marker; Lane 1 to 8: PCR products of JS-A, WY-A, FY-A, TL-A, JS-B, WY-B, FY-B and TL-B; Lane NTC: Negative control.

**Table S1.** Species of eukaryotic phytoplankton indentified by using ITS gene metabarcoding technology and morphological method.

| Number | Metabarcoding monitoring | Morphological monitoring |
|--------|--------------------------|--------------------------|
|        | Classes                  | Species                  | Classes | Species                  |
| 1      | Chlorophyta              | *Pedinomonas sp.*        | Chlorophyta | *Cosmarium sp.*          |
| 2      | *Acutodesmus bajaracalifornicus* |          |          | *Cosmarium abbreviatum*  |
| 3      | *Acutodesmus deserticola* |          |          | *Cosmarium circulare*   |
| 4      | *Acutodesmus sp. KNUA038* |          |          | *Cosmarium abbreviatum*  |
| 5      | *Coelastrella rubescens*  |          |          | *Cosmarium absolueum*   |
| 6      | *Coelastrella vacuolata*  |          |          | *Euastrum dubium*        |
| 7      | *Coelastrella sp.*        |          |          | *Penium Cruciferum*      |
| 8      | *Coronastrum ellipsoideum*|          |          | *Closterium sp.*         |
| 9      | *Nephrochlamys subsolitaria* |          |          | *Pleodorina sp.*         |
| 10     | *Planktosphaeria gelatinosa* |          |          | *Tetraedron minimum*     |
| 11     | *Desmodesmus armatus*     |          |          | *Tetraspora sp.*         |
| 12     | *Desmodesmus asymmetricus*|          |          | *Raphidonema sp.*        |
| 13     | *Desmodesmus bicuscularis* |          |          | *Chlamydomonas sp.*      |
| 14     | *Desmodesmus brasiensis*  |          |          | *Platymonas elliptica*   |
| 15     | *Desmodesmus communis*    |          |          | *Coelastrium microporum* |
| 16     | *Desmodesmus costato-granulatus* |       |          | *Eudorina sp.*           |
|   | Species                          | Species                        |
|---|----------------------------------|--------------------------------|
| 17| Desmodesmus denticulatus         | Pandorina sp.                  |
| 18| Desmodesmus insignis             | Schroederia spiralis           |
| 19| Desmodesmus intermedius          | Chlorella sp.                  |
| 20| Desmodesmus maximus              | Actinastrum sp.                |
| 21| Desmodesmus opoliensis           | Ankistrodesmus angustus        |
| 22| Desmodesmus pannonicus           | Ankistrodesmus convolutus      |
| 23| Desmodesmus perdollis            | Ankistrodesmus falcatus        |
| 24| Desmodesmus pirkollei            | Ankistrodesmus falcatus var.mirabilis |
| 25| Desmodesmus santosii             | Oocystis lacustris             |
| 26| Desmodesmus sp.                  | Pediastrum simplex            |
| 27| Hariotina reticulata             | Pediastrum tetras              |
| 28| Pectinodesmus pectinatus          | Crucigenia quadrata            |
| 29| Pectinodesmus regularis          | Crucigenia apiculata           |
| 30| Sphaeroplea robusta              | Scenedesmus sp.                |
| 31| Rotundella rotunda               | Scenedesmus bijuga             |
| 32| Monoraphidium griffithii         | Scenedesmus quadricauda        |
| 33| Tetraedrales acuminatas          | Bacillariophyta                |
| 34| Tetraedrales bernardii           | Cyclotella sp.                 |
| 35| Tetraedrales distendus           | Cyclotella meneghiniana        |
| 36| Tetraedrales incrassatulans      | Cyclotella aslerocastata       |
| 37| Tetraedrales nygaardii           | Melosira granulata             |
| 38| Tetraedrales obliquus            | Melosira granulata var. angustissima |
| 39| Tetraedrales reginiae            | Melosira varians               |
| 40| Verrucodesmus parvus             | Coscinodiscus sp.              |
| 41| Neochloris conjuncta             | Coscinodiscus subtilis         |
| 42| Neochloris vigensis              | Coscinodiscus oculusiridis     |
| 43| Neochloris sp. AY2               | Skeletonema costatum           |
| 44| Stauridium privum                | Synedra acus                   |
| 45| Stauridium tetras                | Synedra ulna                   |
| 46| Lacunastrium gracillimum         | Synedra ulna var. impressa     |
| 47| Monactinus sturmii               | Fragilaria sp.                 |
| 48| Hydrodictyon reticulatum         | Fragilaria capucina            |
| 49| Pseudopediastrum sp. KNUA039     | Fragilaria biceps              |
| 50| Chlamydomonas planula            | Gomphonema sp.                 |
| 51| Chlamydomonas asymmetrica        | Gomphonema subclavatum         |
|   | Species                                | Species               |
|---|----------------------------------------|-----------------------|
|52 | Chlamydomonas bacca                    | Gomphonema simus      |
|53 | Chlamydomonas debaryana                | Gomphonema parvulum   |
|54 | Chlamydomonas dorsoventralis           | Cocconeis placentula |
|55 | Chlamydomonas globosa                  | Nitzschia sp.         |
|56 | Chlamydomonas hedleyi                  | Nitzschia acula       |
|57 | Chlamydomonas inflexa                  | Nitzschia pala        |
|58 | Chlamydomonas leiostraca               | Nitzschia acicularis  |
|59 | Chlamydomonas mexicana                 | Surirella ovata       |
|60 | Chlamydomonas proboscigera             | Surirella robusta     |
|61 | Chlamydomonas rapa                     | Gyrosigma acuminatum  |
|62 | Chlamydomonas reinhardtii              | Didymosphenia geminata|
|63 | Chlamydomonas sphagnophila             | Amphoraovalis         |
|64 | Chlamydomonas splendidida              | Frustulia vulgaris    |
|65 | Chlamydomonas sp.                      | Navicula sp.          |
|66 | Gloeomonas anomalipyrenoides           | Navicula rhynchocephala|
|67 | Lobochlamys segnis                     | Navicula cuspidata    |
|68 | Chloromonas perforata                  | Cymbella sp.          |
|69 | Carteria eugametos                    | Cymbella lanceolata   |
|70 | Carteria incisa                        | Cymbellaturgidula     |
|71 | Tetraselmis suecica                    | Diatoma vulgaris      |
|72 | Vitreochlamys nekrassovii              | Eunotia sp.           |
|73 | Colemanosphaera charkwioniensis        | Pinnularia sp.        |
|74 | Volvulina compacta                     | Cymatopleura sp.      |
|75 | Gonium pectorale                       | Cymatopleura elliptica|
|76 | Coelastrum astroideum                 | Leptocylindrus danicus|
|77 | Coelastrum microporum                 | Trioreatum favm       |
|78 | Coelastrum pseudomicroporum            | Cryptophyta           |
|    | Eudorina cylindrica                    | Cryptomonas ovata     |
|79 | Eudorina elegans                       | Cryptomonas erosa     |
|80 | Eudorina sp. KMMCC 1278                | Chroomonas caudata    |
|81 | Eudorina sp.                           | Euglenophyta          |
|    |'Euglena geniculata                     | Euglena sp.           |
|82 | Eudorina unicocca                      | Trachelomonas curta   |
|83 | Pandorina colemaniae                   | Strombomonas fluviatilis|
|84 | Pandorina morum                        | Pyrrophyta            |
|    | Volvox carteri                         | Peridinium sp.        |
|85 | Dunaliella parva                       | Peridinium pusillum    |
Continued

|   | Species                                      |
|---|---------------------------------------------|
| 87| *Dunaliella pseudosalina*                   |
| 88| *Peridinium umbonatum*                      |
| 89| *Characium oviforme*                        |
| 90| *Ceratium hirundinella*                     |
| 91| *Schroederia setigera*                      |
| 92| *Gymnodinium sp.*                           |
| 93| *Closteriopsis acicularis*                  |
| 94| *Didymogenes anomala*                       |
| 95| *Didymogenes palatina*                      |
| 96| *Kalenjinia gelatinosa*                     |
| 97| *Marasphaerium gattermannii*                |
| 98| *Marvania geminata*                         |
| 99| *Masaia oligia*                              |
|100| *Meyerella planktonica*                     |
|101| *Graesiella emersonii*                      |
|102| *Pseudochlorella signiensis*                |
|103| *Nannochloris sp. AICB 424*                 |
|104| *Compactochlorella dohrmannii*              |
|105| *Compactochlorella kochii*                  |
|106| *Chlorella miniata*                         |
|107| *Chlorella pulchelloides*                   |
|108| *Chlorella sorokiniana*                     |
|109| *Chlorella sp.*                             |
|110| *Actinastrum hantzschii*                    |
|111| *Selenastrum capricornatum*                 |
|112| *Kirchneriella aperta*                      |
|113| *Kirchneriella lunaris*                     |
|114| *Ankistrodesmus falcatus*                   |
|115| *Ankya judayi*                              |
|116| *Chlorococcum oleofaciens*                  |
|117| *Chlorococcum sp. CCAP 11/52*               |
|118| *Micractinium belenophorum*                 |
|119| *Micractinium inermum*                      |
|120| *Micractinium reisseri*                     |
|121| *Micractinium sp.*                          |
|122| *Neospongiococcum sp. SAG 2474*             |
|123| *Coenoehloris sp. KR 2006/325*              |
|124| *Franceia amphitricha*                      |
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Continued

122  Oocystella oogama
123  Oocystis sp. KMMCC 251
124  Pediastrum duplex
125  Pedinomonas minor
126  Crucigenia lauterbornii
127  Westella botryoides
128  Scenedesmus armatus
129  Scenedesmus bijugas
130  Scenedesmus quadricauda
131  Scenedesmus sp.
132  Dictyosphaerium ehrenbergianum
133  Dictyosphaerium lacustre
134  Dictyosphaerium libertatis
135  Dictyosphaerium sp.
136  Sorastrum pediatricsforme
137  Oedogonium angustistomum
138  Oedogonium cardiatum
139  Oedogonium cylindrosorum
140  Oedogonium donnellii
141  Oedogonium subdissimile
142  Oedogonium tenerum
143  Oedogonium undulatum
144  Oedogonium sp.
145  Gloeotilopsis planctonica
146  Chamaetrichon basiliensis
147  Uronema sp. AF-2012
148  Uronema sp. CCAP 334/1
149  Uronema sp. CCAP 335/1B
150  Urospora neglecta
151  Chaetopeltis orbicularis
152  Chaetophora sp. BEA 0173B
153  Hormotilopsis gelatinosa
154  Hormotilopsis tetravacuolaris
155  Draparnaldia plumosa
156  Stigeoclonium helveticum
157  Schizomeris leibleinii
Continued

158  Aphanochaete magnum
159  Cladophora sp. ZZ-2016
160  Rhizoclonium pachydermum
161  Tetracystis excentrica
162  Tetracystis intermedia
163  Tetracystis pulchra
164  Tetracystis sarcinalis
165  Tetracystis sp. 14601-7.1
166  Tetracystis tetraspora
167  Tetracystis texensis
168  uncultured Chlorophyta
169  Pleurastrum sp. CCCryo 194-04
170  Deasonia sp. 14615-7
171  uncultured Desmodesmus
172  Spermatozopsis exultans
173  Spermatozopsis similis
174  Chlamydocapsa ampla
175  Chlamydodopodium starrii
176  Scherffelia dubia
177  Chlorosarcinopsis sp. WJT16-VFN5
178  Desmochloris halophila
179  Dicloster acuatus
180  Hindakia fallax
181  Elliptochloris marina
182  uncultured Trebouxiophyceae
183  Heterochorella luteoviridis
184  Chloroidium saccharophilum
185  Choricystis parasitica
186  Chlorophyta sp. MCWWS13
187  Chlorophyta sp. SP2-3
188  Bacillariophyta  uncultured Thalassiosirales
189  Cyclotella cf. scaldensis G18W42
190  Cyclotella megniniana
191  Cryptophyta  uncultured Cryptophyta
192  Rhinomonas nottbecki
193  Rhodomonas sp. CCMP740