Seasonal Diversity of Microeukaryotes in the Han River, Korea Through 18S rRNA Gene Metabarcoding

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ABSTRACT: Freshwater ecosystems contain a large diversity of microeukaryotes that play important roles in maintaining their structure. Microeukaryote communities vary in composition and abundance on the basis of temporal and environmental variables and may serve as useful bioindicators of environmental changes. In the present study, 18S rRNA metabarcoding was employed to investigate the seasonal diversity of microeukaryote communities during four seasons in the Han River, Korea. In total, 882 unique operational taxonomic units (OTUs) were detected, including various diatoms, metazoans (e.g., arthropods and rotifers), chlorophytes, and fungi. Although alpha diversity revealed insignificant differences based on seasons, beta diversity exhibited a prominent variation in the community composition as per seasons. The analysis revealed that the diversity of microeukaryotes was primarily driven by seasonal changes in the prevailing conditions of environmental water temperature and dissolved oxygen. Moreover, potential indicator OTUs belonging to diatoms and chlorophytes were associated with seasonal and environmental factors. This analysis was a preliminary study that established a continuous monitoring system using metabarcoding. This approach could be an effective tool to manage the Han River along with other freshwater ecosystems in Korea.

KEYWORDS: Seasonal diversity, microeukaryote, 18S rRNA gene metabarcoding, environmental factors, Han River

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Introduction

Freshwater ecosystems harbor various microeukaryotes, including diatoms, metazoans, chlorophytes, and fungi. Microeukaryotes play essential roles in water biotic communities by managing the stability of water ecosystems owing to their crucial link to energy flow and material circulation.[1] They also serve as the bioindicators of water quality in specific environmental conditions.[2] These freshwater microeukaryotic communities have different compositions depending on changes that occur over time, that is, seasonal differences.[3] Changes in microbial eukaryote assemblages have been shown to be associated with seasonal changes in environmental variables.[4,5] For example, zooplankton exhibits fluctuations in abundance, biomass, and community structure with temporal changes.[6,7] Moreover, microeukaryotic communities fluctuate in response to environmental changes in freshwater ecosystems. Most microeukaryotes can be affected by several biological factors (such as predation)[8,9] and physical and chemical factors (including water temperature [WT], dissolved oxygen [DO], pH, and others).[10,11] Thus, exploring microeukaryote community compositions from various perspectives, including temporal as well as biotic and abiotic factors, is vital to understand freshwater ecosystems.

One of the largest river systems in Korea is the Han River. Among the various tributaries of the Han River, its downstream region functions as an essential water resource for nearly half of the Korean population living in the metropolitan region and the neighboring cities. Therefore, the value and interest of the Han River are extremely high, and a safe water quality management is required to use this precious water resource. Since 1960s, with the rapid industrialization of the metropolitan area near the Han River, the inflow of pollutants has risen, leading to water pollution such as green tide. Hence, physical, chemical, geological, and biological studies on the Han River have been continuously conducted.[12,13] By contrast, ecological surveys on the Han River basin were primarily conducted in its middle and upstream regions, and studies focusing on the downstream region are limited.[14,15] The downstream region of this river is a place where the freshwater supplied from the land and seawater is mixed, and physical, chemical, and biological factors at this location interact in a complex manner. In the downstream region of the Han River, studies have mainly focused on phytoplankton; however, such reports were based on microscopic observations,[16] and there are few molecular studies on microeukaryotes based on DNA metabarcoding. Moreover, previous studies to assess the relationship between environmental factors and microeukaryote structure are insufficient to explain community changes. Therefore, it is vital to investigate the diversity of microeukaryotic communities in the downstream region of the Han River across four seasons and to evaluate the relationship between environmental factors and microeukaryotic community.

Biological monitoring generally depends on the identification of taxa via morphological characteristics. However, investigations using morphological identification require considerable time and labor as well as high taxonomic knowledge.[17] Morphological

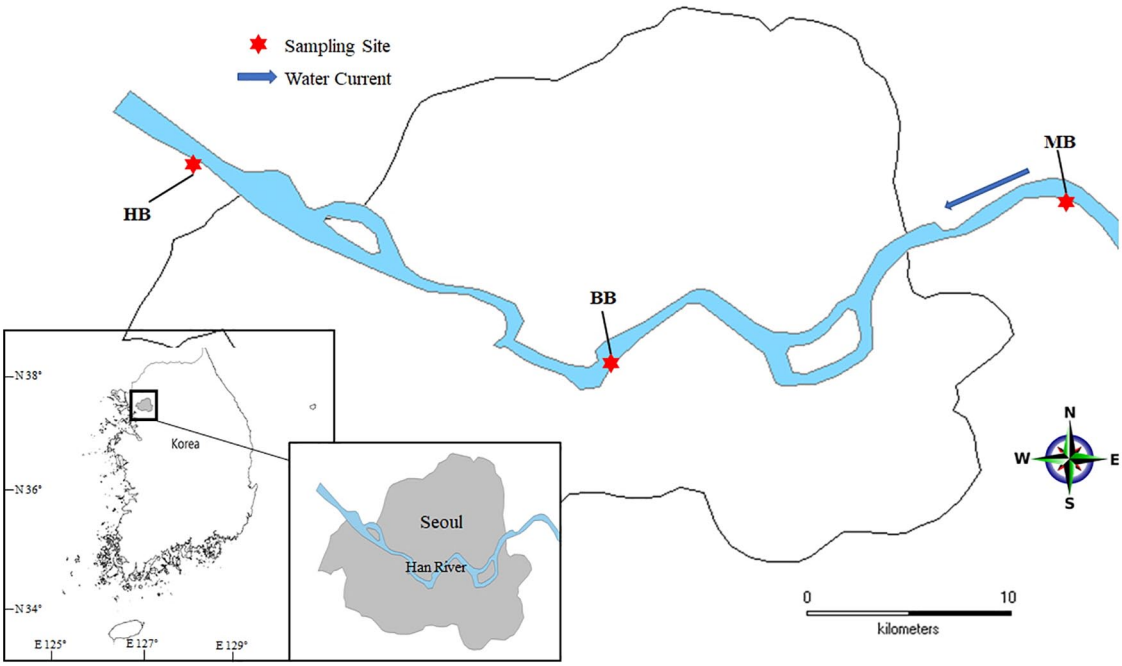

Figure 1. Sampling sites in the Han River, South Korea. MB: Misa Bridge (37°34'41.3" N, 127°12'13.4" E), BB: Banpo Bridge (37°30'46.0" N, 126°59'53.7" E), HB: Haengju Bridge (37°35'47.7" N, 126°48'27.9" E).

identification can be effective for monitoring, but the absence of diagnostic morphological characteristics leads to the laborious and incorrect identification of taxonomic diversity among closely related organisms.[18,19] DNA metabarcoding enables researchers to quickly and economically obtain the comprehensive profiles of aquatic communities while lowering their dependence on taxonomic expertise.[20,21] This approach has been used to investigate various microeukaryotic communities, including diatoms, arthropods, chlorophytes, and fungi.[22,23] Moreover, metabarcoding analysis has provided compositional data from samples containing organisms that are challenging to identify because of their smaller sizes or owing to the use of classical identification methods.[24]

For microeukaryotes, 18S rRNA gene sequences have been used in various studies to investigate different groups, including diatoms,[25] arthropods,[26] and fungi.[27] These studies have shown the possibility for high-throughput analysis based on 18S rRNA gene sequences to assess microeukaryote biodiversity in freshwater ecosystems. Although there are many compositional data regarding microeukaryotes in the Han River based on morphological observations,[28,29] studies using molecular biological methods in environmental samples are limited. Further, nearly all previous ecological surveys conducted in the Han River water system mainly focused on investigating the structure and diversity of bacterial communities based on 16S rRNA gene sequences,[30] whereas only a limited number of such surveys targeted the microeukaryote communities based on these sequences.[31] Finally, only few studies have used DNA metabarcoding to monitor changes in the structure of microeukaryotes as per changes in environmental factors and changes over time.

In the present study, a DNA metabarcoding method based on 18S rRNA gene sequences was used to assess the seasonal diversity of microeukaryotic communities in the downstream region of the Han River (Seoul and Gyeonggi-do, Korea) over four seasons and to correlate it with environmental factors. The diversity of microeukaryotic communities was expected to vary by seasons, and the diversity may be affected by the interaction of complex environmental factors. Although the results contained various diatoms, metazoans, chlorophytes, and fungi, this study mainly focused on taxa with high relative abundance (sequences comprising >10% of all sequences), including diatoms, arthropods, rotifers, chlorophytes, and fungi. This was a preliminary study, and its results could be used as reference data for long-term monitoring by sampling in various locations and seasons in the future.

Materials and Methods

Sampling and environmental data collection

Over a period of 1 year, water samples were collected from three locations (Misa Bridge [MB], Banpo Bridge [BB], and Haengju Bridge [HB]) in the downstream region of the Han River flowing in Seoul and the Gyeong-gi province (Figure 1). The sample collection site that was downstream of the Han River was selected as the upstream direction MB and the downstream direction HB by calculating the average distance based on BB, which is the metropolitan area. Samples were collected once during each season from June 2019 to April 2020. To investigate the seasonal diversity of microeukaryote communities, samples were collected in June, September, and December 2019 as well as in March and April 2020, representing the four seasons. Ten liters from the top ~2 m of water was

collected using a 10-L bucket. Water sampling was performed in triplicate at each sampling location at 50-m intervals, and the collected water samples were pooled. A total of 12 water samples were collected, with 3 samples per season. Each sample containing 30 L water was prefiltered to remove large-sized organisms using 100-µm-pore-sized mesh, and microeukaryotes were retained; bacteria were removed using a 0.8-µm-pore-sized, 90 mm-diameter cellulose acetate membrane (Hyundai-Micro, Korea) with MilliporeSigma™ Pellicon™ Easy-Load Peristaltic Pump (Millipore, USA). The membranes were transferred to sterile 50-mL conical tubes and stored at −80°C until further processing.

During sample collection, environmental factors were recorded at each time point to verify the relationships between the microeukaryote diversity and environmental factors. WT, pH, DO, and salinity were measured using a portable meter (YK-2001PHA, LUTRON Co., Taiwan). In addition, to investigate the diversity in microeukaryotic community according to nutrients, total nitrogen (TN) and total phosphorus (TP) levels were obtained at each time point from the Water Environment Information System in the Ministry of Environment (http://water.nier.go.kr/).

DNA extraction, polymerase chain reaction (PCR), and sequencing

Microeukaryote samples for DNA extraction were collected by scraping each filtered membrane using an S/T spoon, which were then placed it in separate 1.5-mL microcentrifuge tubes. Total DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Germany) as per the manufacturer's instructions. A volume of ATL buffer needed to submerge the sample and 20 µL of proteinase *K* were added to the samples, and the samples were lysed overnight at 37°C. Total DNA was stored at −20°C until further processing. For amplification, a primer set to target the V1, V2, and V4 regions of the 18S rRNA gene was chosen. The V1 and V2 regions of the 18S rRNA gene have a broad phylogenetic coverage and an ability to detect metazoan taxa,[32] whereas the V4 region is a hypervariable region in microeukaryotes and is designed to ensure the amplification of diatoms (Bacillariophyta) and chlorophytes (Chlorophyta).[33] The V1 and V2 regions of the 18S rRNA gene were amplified using SSU_F04 (5′-GCTTGTCTCAAAGATTAAGCC-3′) and SSU_R22 (5′-GCCTGCTGCCTTCCTTGGA-3′) primers,[34] and the V4 variable region of the 18S rRNA gene was amplified using the forward (5′-AATTCCAGCTCCA ATAGCGTATAT-3′) and reverse (5′-TTTCAGCCTTGCG ACCATAC-3′) primers.[33] PCR was performed as follows: 5 min denaturing step at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 50°C, 1 minute at 72°C, and a final extension step at 72°C for 5 minutes. Agarose gel electrophoresis was used to confirm the size of the amplification products. The expected size of the PCR products of V1–V2 and V4 region was 450 bp and 500 bp, respectively. The amplified PCR products were then purified using Agencourt AMPure XP beads (Beckman Colter, Brea, CA), and paired-end sequencing was performed at LAS Inc. (Gimpo, Korea) on Illumina MiSeq platform.

Read Processing and Taxonomic Assignment

The 18S rRNA gene sequence data produced by Illumina MiSeq platform were analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) v 2019.4.0.[35] The raw FASTQ data of V1–V2 and V4 region were subsampled so that they had the same read depth, and the libraries of V1–V2 and V4 region were merged into a single library file as per sample. To investigate the diversity of metazoans and diatoms, chlorophytes, and fungi, raw reads containing both the V1–V2 and V4 region libraries were merged using QIIME2.[36] The quality of the paired-end raw data was evaluated using FastQC.[37] Forward and reverse reads were concatenated using VSEARCH with the default parameters.[38] Short (<300 bp), ambiguous base calls and low-quality assembled reads (Q<30) were removed from further bioinformatic analysis. The reads were also filtered using a maximum expected error filtering of 3.0, and suspected chimeras were removed. From the filtered sequences, operational taxonomic units (OTUs) with 97% similarity were clustered using VSEARCH in QIIME2.[38] Taxonomy was assigned to each OTU cluster using the qiime2-feature-classifier classify-sklearn against the SILVA reference database (Silva SSU 132).[39] To obtain an accurate dataset, singleton sequences (OTUs with only one sequence) were filtered and discarded from the analysis as they were suspected to be erroneous sequences. The reads belonging to OTUs related to bacteria were removed from the dataset, whereas reads affiliated to microeukaryotes were included in the final data matrix. Reads that were not assigned to any taxonomic group in comparison with the SILVA 132 database were also removed. The SILVA database that is used to assign taxonomy to the OTU sequences often has taxonomic level differences within sequence taxonomy string.[40] Thus, mislabeled taxa were reannotated based on the National Center for Biotechnology Information (NCBI) taxonomy to obtain missing information from the SILVA database.[41]

Data analysis

For microeukaryote diversity indices, alpha-diversity metrics (observed species [OTUs], Chao1, Shannon's diversity, and evenness) were calculated for all samples using QIIME2. To assess whether sequencing depths were sufficient, rarefaction curves were calculated using QIIME2. The Kruskal–Wallis test was performed in R (v4.0.1) programing environment to compare the alpha-diversity metrics of different seasons, followed by Dunn's post hoc test using the R package "FSA".[42,43] Nonmetric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity was performed to investigate the differences in the distribution of each sample and assess the relationship between microeukaryote communities and temporal variables after converting OTU abundances into frequencies within the samples

using the vegan package.[44] NMDS analysis was performed using the metaMDS function with default parameters, except trymax, which was set to 100. Statistical analysis was performed to evaluate whether there was any difference in microeukaryote community structures among seasons using the ANalysis Of SIMilarity (ANOSIM) test with 9,999 random permutations in the vegan package.[44,45]

To identify the relationship between observed microeukaryote community compositions and environmental factors, environmental factors were mapped to the NMDS plot using the envfit (9,999 permutations) function of the vegan package. Indicator OTU analysis was performed to identify OTUs that were associated with the seasons using the package "indicspecies" with 9,999 permutations.[46] The "pheatmap" package was used to generate heatmaps,[47] whereas NMDS plots and barplots were generated using the "ggplot2" package.[48]

Results

Environmental variables

The environmental variables as per the sampling date are provided in Table S1. The highest and lowest WT values were recorded in June 2019 (26.3°C-26.5°C) and December 2020 (3.8°C-7.6°C), respectively. The pH value during the year of the study was 7.0 and 8.5 in September 2019 and April 2020, respectively. A higher DO value was observed in winter and spring compared with that in summer and autumn, and the highest value (14.4 mg/L) was recorded in December 2019, whereas the lowest value (6.7 mg/L) was recorded in June 2019. The highest (224 ppm) and lowest (102 ppm) salinity values were recorded in December 2019 and September 2019, respectively. The TN and TP levels also fluctuated considerably throughout the sampling period; the highest value was measured in April 2020 and March 2020, respectively. The lowest values for TN and TP was noticed in December 2019.

Sequencing and diversity analysis

A total of 1,790,480 raw reads were generated, of which 432,689 high-quality reads were retained after trimming and filtering. These high-quality filtered sequences with 97% sequence similarity were clustered, and the obtained final dataset comprised 893 OTUs. After the clustering phase, eight OTUs with only one read were removed. Among the remaining 885 OTUs, 2 were discarded because they were taxonomically classified as bacteria and 1 OTU that was not taxonomically assigned to any group in the SILVA database was also removed. Finally, 882 unique OTUs were retained for further analysis. The rarefaction curves of all samples reached near-saturation (Figure S1). For each season, 457, 275, 210, and 177 OTUs were detected in spring, summer, autumn, and winter, respectively (Figure S2). Although OTUs representing various diatoms, metazoans, chlorophytes, and fungi were detected, the analysis was performed by focusing on taxa that could mainly be detected using the V1–V2 and V4 region sequence data from the 18S rRNA gene and their data could be accessed from the SILVA database. The majority of OTUs in the dataset were classified as fungi (234 OTUs, 26.5%), followed by diatoms (132 OTUs, 15.0%), metazoans (105 OTUs, 11.9%), and chlorophytes (96 OTUs, 10.9%). Diatoms (Bacillariophyta) were dominated by the order Thalassiosirales, Fragilariales, and Aulacoseirales, whereas chlorophytes (Chlorophyta) were mainly dominated by the order Spaeropleales. By contrast, metazoans were dominated by the order Diplostraca belonging to Arthropoda and the order Ploima belonging to Rotifera. Fungi were mainly classified up to the phylum level, of which Cyptomycota and Chytridiomycota were dominant (Table S2).

Table 1. Analysis of similarity (ANOSIM) results of alpha and beta diversity by seasonal.

ALPHA-DIVERSITY METRIC	ADJUSTED *P*	SIGNIFICANCE
OTUs	0.0862	N.S.
Chao1	0.0862	
Shannon diversity	0.4593	
Evenness	0.0776	
BETA-DIVERSITY METRIC	**ADJUSTED *P***	**SIGNIFICANCE**
Bray-Curtis dissimilarity	0.0005	***

Number represent ANOSIM R-statistic.
Abbreviation: N.S., no significance.
***$P < 0.001$. **$P < 0.01$. *$P < 0.05$.

Alpha-diversity metrics fluctuated during this study, with lowest values in December 2019 (chao1: 23, Shannon: 2.820, and evenness: 0.62), indicating the high dominance of one (or few) taxa within the community (Table S3). The alpha-diversity metrics in different seasons were compared, but no significance difference was observed (Table 1). By contrast, there was a significant temporal difference in microeukaryote community composition among seasons ($P < 0.001$) (Table 1).

Temporal compositions of microeukaryotes

Based on the relative abundance of 18S rRNA gene sequences, classified microeukaryotes were compared at different taxonomic levels. Microeukaryotes were compared at the phylum level up to the lowest taxonomic level, which could be discriminated, to compare the seasonal diversity at the same taxonomic level. Thereafter, the taxonomic compositions of the microeukaryotes were compared at the phylum and order levels. At the phylum level, a high relative abundance of Bacillariophyta (16.9%-95.8%) was observed in all seasons (Figure 2a and Table S4). Compared with other seasons, a high relative abundance of Arthropoda and Chlorophyta was observed in summer compared with that in other seasons (Table S4). A significant variation in the dominant phyla was observed, particularly based on seasons. Rotifera, which exhibited a lower

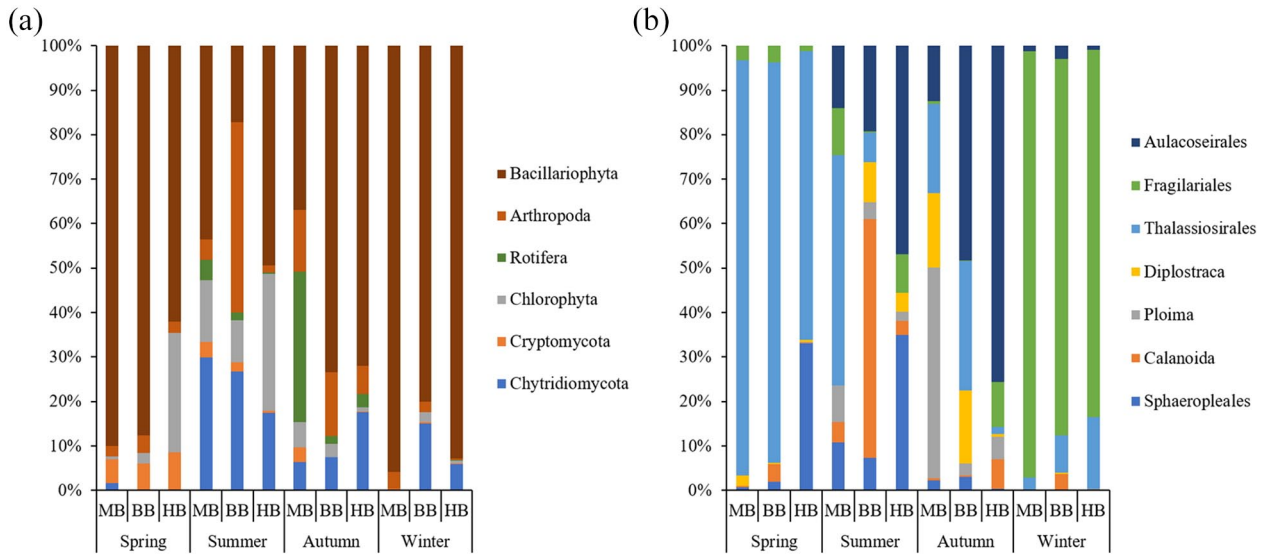

Figure 2. Seasonal taxonomic composition of microeukaryote communities based on the relative abundance of 18S rRNA gene sequences. Bar plots showing a relative abundance in the (a) phylum and (b) order levels.

relative abundance in other seasons, mainly dominated in autumn, whereas Cryptomycota mainly dominated in spring compared with other seasons (Table S4).

When compared at the order level, differences in the predominant taxa of Bacillariophyta were observed in different seasons (Figure 2b). Fragilariales predominated in winter compared with the other seasons (Figure 2b). As the seasons changed, the relative abundance of Fragilariales decreased and that of Thalassiosirales increased in spring (Figure 2b). The high relative abundance of Aulacoseirales (8.2%-12.9%) was observed in summer compared with spring and winter, but it was the most prevalent in autumn (Table S4). Chlorophyta mainly comprised Sphaeropleales, which dominated in spring and summer compared with other seasons (Figure 2b). Overall, Metazoa belonging to Arthropoda was observed more in summer and autumn than in spring and winter. Among metazoans, Calanoida and Poima were mainly dominant in summer and autumn, respectively (Figure 2b).

Environmental effects on microeukaryotes

Based on the NMDS plot, microeukaryote communities were clearly divided in each season (Figure 3). This was also supported by the ANOSIM test, which showed that microeukaryote community compositions significantly differed among seasons (Table 1). The envfit analysis performed to determine the environmental factors that may affect microeukaryote communities revealed the significant influence ($P < 0.05$) of WT ($R^2 = 0.8634$) and DO ($R^2 = 0.6570$), and other factors had no significant influence (Table 2). In particular, the microeukaryote community composition in summer and autumn appeared scattered in a similar pattern and showed a strong positive relationship with WT. By contrast, winter and spring were positively related to DO.

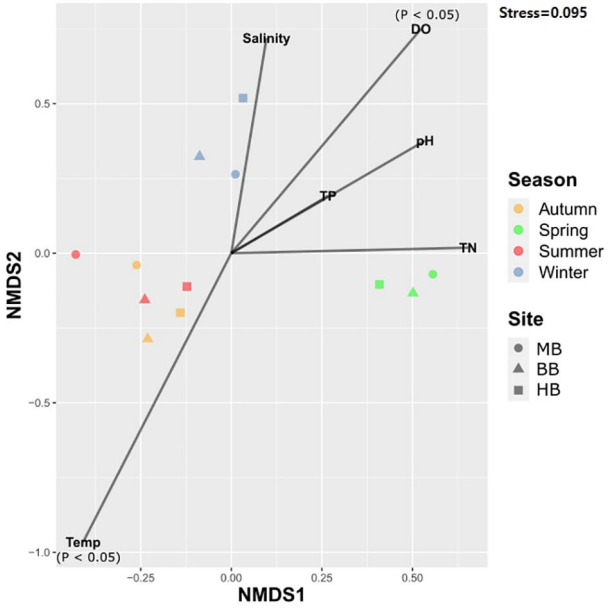

Figure 3. Nonmetric multidimensional scaling (NMDS) plots based on the relative abundance of OTUs using Bray–Curtis dissimilarity. This plot shows the association among temporal, environmental parameters, and microeukaryote communities. The line direction on the NMDS plot indicate correlation with temporal and environmental variables for the microeukaryote communities.

The relationship among individual OTUs in each sample was analyzed using a heatmap, which showed that the variations in the relative abundance of OTUs were >5% of the total number of reads (Figure 4). The taxonomic affiliation of these OTUs is provided in Table S5. Approximately 72% of the OTUs highlighted in Figure 4 represent diatoms and chlorophytes, followed by metazoans (~17%) and fungi (~10%). Diatoms were the predominant taxa in all seasons; however, there were differences in species based on the seasons and

Table 2. Statistical significances in environmental factors were calculated using the envfit function of the vegan package.

ENVIRONMENTAL FACTOR	R^2	ADJUSTED *P*	SIGNIFICANCE
Water temperature (WT)	0.8634	0.0003	***
pH	0.3359	0.1591	N.S.
Dissolved oxygen (DO)	0.6570	0.0075	**
Salinity	0.4137	0.0967	N.S.
Total nitrogen (TN)	0.3359	0.1662	N.S.
Total phosphorus (TP)	0.0849	0.6587	N.S.

Number represent ANOSIM R-statistic.
Abbreviation: N.S., no significance
***$P < 0.001$. **$P < 0.01$. *$P < 0.05$.

environmental variables. OTU_490 showed a high relative abundance in summer and autumn, and these seasons were mainly positively related to WT. By contrast, OTUs 344, 447, 461, 574, 766, and 929 were mainly observed in winter and negatively related to WT and positively related to DO. Likewise, OTUs 372, 454, 864, and 1056, which had low relative abundance in winter but high relative abundance in spring, were positively related to DO. OTU_675 was observed most frequently at BB in summer when DO was the lowest among all samples.

Detection of potential indicator OTUs

Multilevel pattern analysis was performed using the package "indicspecies" to identify potential indicator OTUs that reflect the temporal and environmental characteristics of the Han River. Significant potential indicator OTUs were observed as per season ($P < 0.05$). Although some OTUs were not classified up to the species level, 31 OTUs were detected as potential indicator OTUs, of which 84% belonged to diatoms and chlorophytes (Figure S3 and Table S6). Among the 31 OTUs, 13 were identified in spring, whereas 8, 6, and 4 OTUs were detected in winter, autumn, and summer, respectively. The majority of these OTUs (10/31) belonged to Coscinodiscophyceae, followed by Chlorophyceae (4/31). Various Coscinodiscophyceae were observed as potential indicator OTUs depending on the seasons. Although various Coscinodiscophyceae belonging to Thalassiosirales, Melosirales, and Aulacoseirales were detected as potential indicator OTUs, only one OTU belonging to Aulacoseirales (OTU_490) was found in autumn. Fragilariales was detected as a potential indicator taxonomic group during spring and winter, of which the genus *Fragilaria* (OTU_997) was found only in spring and the genera *Synedra* (OTUs 447 and 461) and *Asterionella* (OTU_344) were found only in winter. Moreover, two species (OTUs 696 and 739) belonging to Chlamydomonadales (Chlorophyta) were detected as potential indicator OTUs only in summer.

Discussion

In this study, DNA metabarcoding was employed to depict the temporal dynamics of microeukaryotes in the Han River over four seasons at three different locations. The seasonal diversity was investigated by focusing on relatively abundant taxa, including diatoms, metazoans, chlorophytes, and fungi. There was no significant difference of alpha-diversity metrics. It is expected that this result was caused by the low number of sample examined as in each season, a single sample was collected for site. In future studied, we will select more sampling sites and increase the number of sample to investigate variations of the microeukaryotic community according to spatial and temporal changes for long-term monitoring. There was a significant difference in the seasonal diversity of microeukaryote communities in the Han River in different seasons. In addition, environmental factors such as WT and DO influenced microeukaryote communities by season during the study period. WT and DO appeared to be correlated with the abundance of diatoms, chlorophytes, and arthropods.

Changes in WT in freshwater ecosystem appeared to have a great influence on diatom community composition, which leads to changes in its dominant species.[49] In this study, among diatoms, Aulacoseirales generally dominated in summer and autumn when WT increases, whereas Fragilariales dominated in winter when WT decreases (Figure 2). Aulacoseira granulata (OTUs 490 and 714), which showed the highest abundance among species belonging to Aulacoseirales, exhibited a high relative abundance in summer and autumn (Figure 4 and Table S5). *A. granulata*, which is known to grow well in high temperature conditions, showed a high abundance in eutrophic waters, primarily in summer and autumn in the Han River.[50] By contrast, Asterionella formosa (OTU_344), belonging to Fragilariales, exhibited a high relative abundance in winter (Figure 4 and Table S5); it grows well at lower WT compared with other diatoms.[51] These results show that WT may be a major environmental factor affecting the growth of diatoms, and changes in the dominant diatom species have been observed according to various temperature conditions corresponding to optimal growth.[52] Likewise, Stephanodiscus hantzschii (OTU_766), which belong to Thalassiosirales, showed a high relative abundance in winter and spring (Figure 4 and Table S5). A previous study reported that *S. hantzschii* is

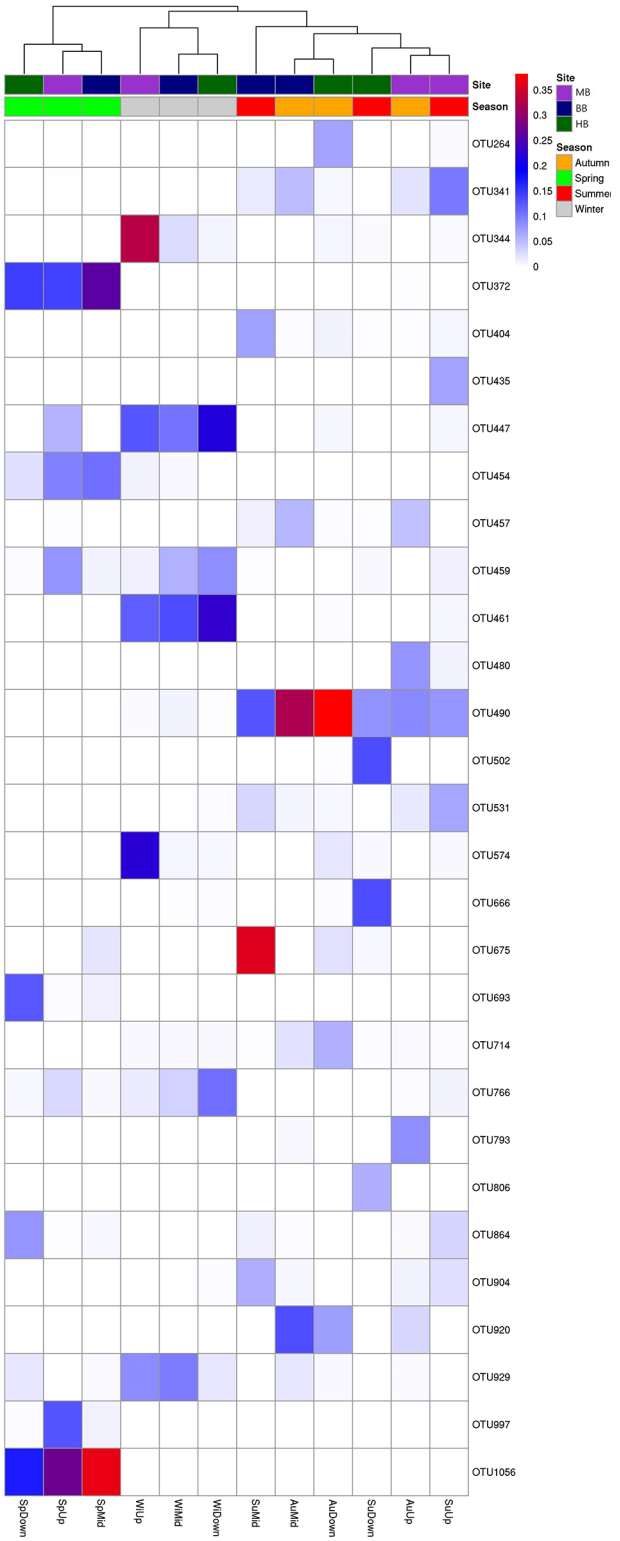

Figure 4. Heatmap shows the relative abundance of OTUs representing various taxonomic groups. The relationship between the dominant OTUs individually, with the relative number of reads being >5% of the total number of reads, and environmental variables was investigated via heatmap. Colors from red to white indicate the high and low relative abundances.

primarily affected by WT changes in the downstream region of the Han River and Yeong-San River.[53,54] Although *S. hantzschii* has an optimal growth temperature of 13°C, it has been reported to dominate both during spring and winter as it can grow sufficiently when WT decreases to 3°C if appropriate environmental factors are met.[55]

Arthropoda primarily dominated in summer and autumn when WT increases, and the abundance of Arthropoda has been shown to be positively correlated with WT. In this study, Bosmina longirostris (OTU_457), which belong to Diplostraca, exhibited a high relative abundance in autumn at approximately 22°C (Figure 4 and Table S5), and similar results have been reported from Lake Dong in China, where the genus *Bosmina* was found in high density when WT was >20°C.[56] Previous studies, which investigated zooplankton density changes in major domestic river ecosystems in Korea, indicated a high relative density of the genus *Bosmina* in autumn.[57] In addition, when WT decreases, Diplostraca exhibited a low relative abundance because they had a dormant period in the sedimentary layer at that time. Chlorophyta exhibited a high relative abundance mainly in spring and summer. In previous studies, the growth of Chlorophyta showed a positive correlation with WT and nutrients in freshwater ecosystems.[58] Among chlorophytes, the genus *Mychonates* (OTUs 693 and 864) primarily exhibited a high relative abundance in spring and summer. A previous study reported that the genus *Mychonates* showed a high relative abundance in summer in the Geum river, Korea.[59] Along with WT, certain biological factors, such as predation, can affect the abundance of certain microeukaryote communities.[9] When WT is low, the abundance of Chlorophyta, a food source for arthropods, decreases. Hence, the relative abundance of certain arthropods, such as Calanoida and Ploima, decreases. Of note, rotifers exhibited a high relative abundance in autumn under similar WT conditions of approximately 20°C, but low relative abundance was found in summer. Rotifers are the most preferred prey of copepods; therefore, they showed a low relative abundance in summer when copepods exhibited a high relative abundance.[8]

The correlation with other seasonal environmental factors is also important. The DO value was relatively higher in winter and spring than in summer and autumn because DO was more consumed by larger and various biotic communities during summer and autumn. Moreover, the respiration rate of aquatic animals and plants as well as photosynthesis affect DO in water. DO is an important factor that determines the variability of diatoms in freshwater, and the strong positive correlation between DO and diatoms is well known.[60] By contrast, Diplostraca exhibited a low relative abundance at high DO value, similar to a previous study conducted in the NakDong river basin.[61] The results of this small-scale, preliminary study of three locations per season could be used as basic data for the long-term monitoring of various regions and seasons in the Han River in the future. Moreover, additional research is needed to investigate various environmental factors and microeukaryotes in the Han River as dominant species show different reactivities to environmental factors.

Based on the relative OTU abundance, we could detect potential indicator OTUs that were statistically significant over the seasons. Microalgae such as diatoms are the primary producers in aquatic ecosystems and are used as potential indicators for underwater environments owing to the responsiveness of its communities to changes in water quality as well as in physical and chemical factors. Diatoms are important major producers in freshwater ecosystems, and they are sensitive to some environmental variables, including WT, water flow, salinity, pH, DO, inorganic nutrients, organic carbon, and organic nitrogen.[62] Consequently, they are regarded as the strong indicators of various environmental changes. Most indicator OTUs are diatoms and chlorophytes, possibly owing to their higher sensitivity and turnover ratio of dominant species according to environmental factors.[63] When WT decreases, the relative abundance of some diatoms increases, of which *A. formosa* is more resistant to lower WT than other diatoms (Table S6). Studies have reported that *A. formosa* showed a high relative abundance mainly in the WT of 2.6°C to 19°C, and it dominated in winter and spring when WT decreases in Unmun Dam, Korea.[64] By contrast, when WT increases, *A. granulata* exhibited a high relative abundance in autumn (Table S6). *A. granulata* mainly serves as a potential indicator species in eutrophic waters,[65] and its relative abundance increases when WT increases. Moreover, it is primarily observed in rivers and lakes such as the Han River and NakDong River, Korea, during autumn.[66] *S. hantzschii*, a cold water microdiatom, showed the highest relative abundance in spring, and it is also used as a pollutant indicator species in freshwater ecosystems.[67] It rapidly multiplies when WT and light transmittance increase and when frozen water body thaws in winter.[68] By contrast, the relative abundance of Diplostraca increased in autumn when WT was >20°C. Among them, *B. longirostris* (OTU_457) was detected as a potential indicator species only in autumn (Table S6), and a previous ecological survey in the Han River showed similar results.[28,29] However, it must be noted that the indicator species analysis was performed on a very short time scale of 1 year only. Therefore, it is essential to further investigate the microeukaryotic communities from multiple locations and seasons over a long period of time to confirm the potential indicator species for a given season.

The present study results revealed the relative abundance of the 18S rRNA gene sequences of each identified taxon that may offer an effective measure of the relative biomass of various taxa.[69] In practice, previous studies have reported that the relative abundances of sequences may offer a reasonable measure of the relative biomass of many taxa. Nevertheless, variations in the 18S rRNA gene copy number, number of organismal cells, and biovolume during various developmental stages can induce some bias that might affect the relative density estimation.[70] Moreover, the efficiency of DNA metabarcoding in various conditions, including multi-locations, seasons, and environmental factors, should be validated. Using multiple genetic markers also can enhance the ability of detection and taxonomic resolution of microeukaryote diversity. Therefore, increasing the frequency of sampling and investigation of various spatial and temporal factors would provide an ability to assess the diversity of microeukaryotes more comprehensively in future studies.

Conclusions

In this study, DNA metabarcoding was used to establish an efficient survey and research approach for microeukaryote community analysis in the downstream regions of the Han River, Korea. To investigate the seasonal diversity of microeukaryotes, the relationship among the microeukaryote community, temporal variable, and environmental variables was investigated using the metabarcoding approach, which may offer essential clues to prepare a baseline for future studies. The community composition of microeukaryotes showed a significant difference with temporal variations and environmental factors such as WT and DO. Using the sensitive detection capability of metabarcoding, the discovery of potential indicator species could represent the temporal and environmental characteristics of the Han River. Through further studies, potential indicator OTU found from this study may become an effective tool for evaluating the water quality of the Han River. It is expected that further investigation on these potential indicator species from multiple seasons over a long period will enable us to effectively detect environmental changes in this freshwater ecosystem. Moreover, the efficiency of DNA metabarcoding in various conditions (e.g., various seasons and environmental factors) should be validated, and the use of multiple genetic markers is required to improve the detection and taxonomic resolution of the diversity of microeukaryote taxa. In addition, more studies on spatial and temporal changes are required to further understand the long-term ecosystem stability and response to environmental changes. Establishing a monitoring system using this approach will help identify the mid- to long-term patterns of changes in microeukaryote communities and changes in bioindicator taxa according to environmental changes. This study is a preliminary investigation that may provide a theoretical basis for further studies on long-term monitoring to investigate the relationship between more diverse microeukaryote taxa and biotic and abiotic factors in freshwater ecosystems.

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Availability of Data

The data presented in this study has been submitted to NCBI Sequence Read Archive (SRA) database and will be publicly available upon publication (BioProject metadata is available at https://dataview.ncbi.nlm.nih.gov/object/PRJNA694225?reviewer=995hehh1sn379p6lb5cujc15ff in read-only format).

Supplemental Material

Supplemental material for this article is available online.

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