

Article

Environmental DNA in a Biofilm Can Be Used to Assess Diatom Ecological Health in Stream Water Ecology

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Abstract: In urban and agricultural streams, assessing aquatic ecosystem health is critical due to widespread pollution. Traditional methods for evaluating attached diatoms crucial for ecosystem monitoring face limitations such as species misidentification and sample damage. This study was conducted in the Miho River within the Geum River system and highlights the effectiveness of environmental DNA (eDNA) techniques for more accurate and efficient genetic-based analysis than conventional microscopic analysis methods. When eDNA-based assessments were compared with traditional microscopic methods, this study found that eDNA analysis often revealed poorer ecosystem health. Notably, eDNA assessments showed a stronger correlation with phosphorus concentrations, underlining their precision and importance in ecological studies. These findings suggest that eDNA has potential as a valuable tool for comprehensive biomonitoring. However, the use of international genetic barcode databases in eDNA analysis could lead to the identification of unrecorded species in Korea. Therefore, this study recommends developing a localized genetic barcode database and constructing eDNA information through meta-barcoding, focusing on native species. This approach is crucial for enhancing the accuracy and applicability of eDNA-based health assessments in national biomonitoring efforts.

Keywords: eDNA; biofilm; diatom; TDI; water ecological assessment



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1. Introduction

The physicochemical method for assessing water quality can only evaluate the quality of a specific sample during the assessment, indicating its limitation in accurately representing the real-time variability of water quality. In other words, analyzing numerous pollutants in the water using chemical methods is highly inefficient. Assessing corresponding increase in harm associated with increased concentrations of pollutants is even more challenging [1,2]. However, biological methods utilizing indicator organisms can provide an estimation of the average water quality during a specific period, which allows for the assessment of past pollutant outflows and takes into account cumulative effects of pollutants. Indicator organisms living in the water reflect a synthetic and cumulative state of physicochemical water quality and habitat status. Thus, biological evaluation using indicator organisms ultimately reflects the health of the aquatic ecosystem [3,4].

The diatoms are the primary producers in the stream ecosystem, accounting for a significant proportion of the ecosystem's metabolism and trophic level [5,6]. In particular, these organisms are useful for determining water quality because its recovery rate for disturbances their very quick compared to other biota. In addition, its changes in biomass

due to environmental changes are clear [7]. In addition, since it lives attached to a substrate, it can be used to identify cumulative changes in the water system [8]. The current practice of assessing water quality using diatoms is based on ‘Trophic Diatom Index (TDI)’ [9,10]. Calculation of TDI relies on the utilization of pollution sensitivity and weighting of the index based on occurrence frequency and relative density of diatoms. Thus, the community structure of diatoms has a significant impact on the calculation of TDI [11]. Therefore, it can be stated that the accuracy of community structure analysis is directly related to the accuracy of water quality assessment utilizing diatoms.

Currently, the method for identifying diatoms and analyzing communities is based on morphological characteristics observed with optical microscopes or electron microscopes. Such morphological characteristics for identifying diatoms are very detailed, including the shape and length of diatom shell valves, the arrangement and spacing of holes in the striae present on the shell, the shape of the apex, the shape of the tip of the raphe, and the length of the stigma, which shows a wide variation, with differences ranging from 3 to 10 μm , or more [12–14]. These morphological differences can primarily be distinguished using optical microscopes with a 1000 \times magnification, while finer variations can be observed with an electron microscope. As a result, identifying certain large genera such as *Cymbella*, *Navicula*, and *Nitzschia* can be easily done using an optical microscope set at 1000 \times magnification, accurately observing features of the striae and central area. However, identifying small species such as *Acnathes* and some *Navicular*, *Fragillaria*, and *Nitzschia* species becomes challenging at the optical microscope level [15,16]. Additionally, the ambiguity surrounding the distinction between the arrangement of striae and the shape of the end of the raphe can result in different identifications depending on the observer, which can lead to varying results when identifying diatom species, depending on the observer’s expertise in identification and the equipment used.

To reduce ambiguity in morphological identification criteria and minimize variance in results among different observers, species identification begins to rely on gene sequencing through a metabarcoding process of environmental DNA (eDNA) [17,18]. Environmental DNA (eDNA) refers to DNA derived from organisms present in the water, soil, and atmosphere. It contains genetic information from various organisms living in the environment. In addition, eDNA metabarcoding is a method that can disassemble genomes into countless pieces and read each piece simultaneously. It is a new sequencing analysis method used in a wide variety of fields such as medical diagnosis, forensic science, and genomics [19–23]. Since one researcher can quickly analyze a vast amount of genetic information, eDNA metabarcoding can minimize variations in analysis by observers and shorten the time required for analysis [24,25]. In addition, microbial communities present in samples collected from a specific environment can be analyzed with great precision and comprehensiveness. Through nucleotide sequence analysis, rare species that might have been overlooked during microscopic analysis can be identified [26,27].

In this study, metabarcoding using eDNA present in stream substrates of various sizes was performed and a community of diatoms was identified based on gene sequences. In addition, health assessment of diatoms was conducted using eDNA metabarcoding and the applicability of eDNA-based diatom health assessment was determined by comparing results with existing microscope-based health assessment results.

2. Materials and Methods

2.1. Sampling Sites and Biofilm Sampling

Biofilms (Figure S1) attached to substrates were collected four times throughout the year—in March, May, July, and September—at 12 different samples sites along the *Mihogang* River, which flows into the *Geumgang* River basin (Figure 1). Biofilms were collected following the guidelines stated in the National Biomonitoring Survey and Assessment Manual [10]. Biofilms collected from rapids of the stream at survey sites can represent changes in the underwater environment through horizontal cross-sections. Collected biofilms consisted of 3 to 6 pebbles or boulders with flat surfaces, ranging from 10 to 20 cm

in diameter, that were submerged in the water. The substrate was collected within a 100 cm² area in a quadrat at the site scraped by a toothbrush. It was then placed in a wide-mouthed bottle using distilled water, stored at a low temperature, and transported to the laboratory.

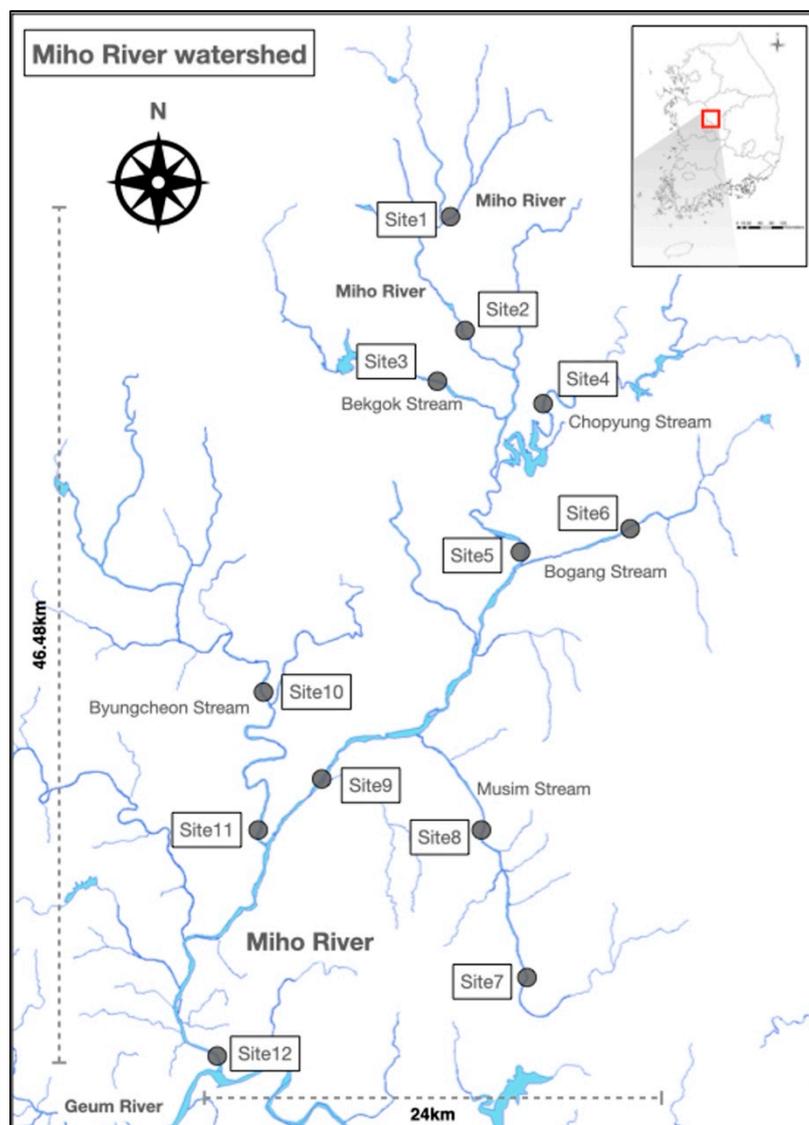


Figure 1. Distribution of survey sites in this study. The red rectangle indicates the watershed of the Mihogang River.

2.2. Extraction of Biofilm eDNA and PCR

Extraction of eDNA from the biofilm of substrate was carried out according to the extraction method presented by the eDNA Society of Japan [28]. Utilizing a disposable brush and tertiary distilled water, biofilm from a 5 cm × 5 cm area was collected and transported to the laboratory. In the laboratory, the biofilm was filtered onto GF/F filter paper (47 mm, Whatman, Maidstone, UK), and environmental DNA (eDNA) was concentrated. The eDNA from the filter paper was then extracted by placing it into a Salivette tube (Sarstedt, Nordrhein-Westfalen, Germany). The extraction of eDNA was performed using the Blood and Tissue kit (Qiagen, Hilden, Germany), following the method outlined by the eDNA Society of Japan [28], involving the mixing of Proteinase K and Buffer AL, which was injected into the filter paper for eDNA extraction. The extracted eDNA was purified using the kit's mini column, and finally, the purified eDNA was eluted in Buffer AE. The extracted DNA sample was stored in a deep freezer (−80 °C) before analysis. Metabarcod-

ing analysis was conducted using an Illumina MiSeq platform to determine the diatom community present in the biofilm of the substrate. Primers developed based on the *rbcL* gene of the diatom [29] (*rbcL*-646F: ATG CGT TGG AGA GAR CGT TTC, *rbcL*-998R: GAT CAC CTT CTA ATT TAC CWA CAA CTG) were used in the primary PCR to amplify the *rbcL* gene of diatom in the eDNA present in the biofilm. For metabarcoding, primers were employed with the attachment of Nextera consensus and sequencing adaptors (Forward: TCG TCG GCA GCG TC-A GAT GTG TAT AAG AGA CAG, Reverse: GTC TCG TGG GCT CCG-AGA TGT GTA TAA GAG ACA G). Primary PCR was performed with the following conditions: heating at 95 °C for 3 min, 35 cycles of 30 s at 95 °C, 30 s at 64 °C, and 30 s at 72 °C, followed by heating at 72 °C for 5 min. Amplified fragments were subjected to 2% agarose gel electrophoreses using an E-gel™ Power Snap Electrophoresis system (Thermo Fisher Scientific, Waltham, MA, USA) and then identified in 352 bp with an E-gel™ Power Snap Camera (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Meta-Barcoding for Diatom Community Analysis

Amplified fragment of the primary PCR had Illumina MiSeq barcode attached using the Nextera XT Index Kit V2. The secondary PCR was performed with the following conditions: heating at 95 °C for 3 min; 8 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C; and followed by heating at 72 °C for 5 min to attach a barcode. Samples with barcodes attached through the secondary PCR were analyzed by an agency specializing in sequencing (Metagenome Bio Life Science Inc., Waterloo, ON, Canada) with an Illumina platform through the paired-end method. Species identified based on the *rbcL* gene sequence, which analyzed approximately 35,000 reads per sample using the IdTaxa classifier (ver. 2.26.0) [30] of the DECIPHER package (ver. 2.26.0) [31]. We used the Genbank database to identify diatom species. And, contigs that were not identified at the species level were selectively chosen for BLAST analysis, and the determination was made at the genus level whenever possible.

2.4. Diatom Community Analysis Using a Microscope

The Diatom community within the biofilm, analyzed using a microscope, underwent a preprocessing procedure following the methods outlined in the 'Biomonitoring Survey and Assessment Manual' [10]. Subsequently, permanent slide mounts were prepared, and attached diatom species were analyzed based on microscopic observations. A brief description of the Manual's methodology is as follows: To facilitate observation of the shell of diatoms, organic matter was removed by acid washing and permanent specimen was created using a mount media solution (Wako, Tokyo, Japan) after a sufficient dilution process using distilled water. The permanent specimen produced was identified with 1000× magnification using an optical microscope (Leica DM 2500, Wetzlar, Germany). To obtain more accurate data, a minimum of 200 or more valves per samples were counted. Diatoms were identified through literature sources [32–36]. Their morphological characteristics were classified based on Simonsen's morphology [37].

2.5. Assessment of Diatom Ecological Health

Trophic diatom index (TDI) for assessing biological health of aquatic ecosystems by utilizing a community of diatoms identified through a microscope was calculated using the following formula [11]:

$$\text{TDI} = [(\text{WMS} \times 25) - 25], \quad (1)$$

$$\text{WMS}(\text{weighted mean sensitivity}) = \frac{\sum A_i S_i V_i}{\sum A_i V_i} \quad (2)$$

where A_i was the population of species i , S_i was the sensitivity value of species i ($1 \leq S \leq 5$), and V_i was the indicator value of species i ($1 \leq V \leq 3$).

TDI was calculated using sensitivity values, indicator values, and populations of species. It was used to determine five grades according to the national Biomonitoring

Survey and Assessment Manual: Very good (grade A), Good (grade B), Fair (grade C), Poor (grade D), and Very Poor (grade E) [10].

In this study, different sensitivity and indicator values were utilized following diatom analysis. Sensitivity and indicator values used in the analysis using a microscope were obtained from the Biomonitoring Survey and Assessment Manual [10]. For health assessment using eDNA, recalculated diatom sensitivity and indicator values of eDNA based on the assessment conducted by Kim [38] were used.

2.6. Statistical Analysis

To investigate the correlation between the Trophic Diatom Index (TDI) derived from both microscopy and environmental DNA (eDNA) methods in this study and water quality, bivariate correlations between each index and concentrations of total phosphorus and phosphate were analyzed. The correlation analysis included the presentation of a 95% confidence interval, and Spearman correlation coefficients (r values) were calculated, validating statistical significance at the 0.001 and 0.05 levels. Statistical analyses were conducted using IBM SPSS Statistics (version 29.0.1.0, IBM, Armonk, NY, USA), and Plot graphs with 95% confidence intervals were displayed using DataGraph (version 5.2, Visual Data Tools, Inc., Chapel Hill, NC, USA).

3. Results

3.1. Difference of Diatom Community between Morphology and Biofilm eDNA Analyses

Dominant and subdominant species as a result of conducting analysis using a microscope four times at 12 different survey points are shown in Table 1. In the fourth survey, dominant and subdominant species among all identified species were shown to be *Nitzschia inconspicua* and *Navicula subminuscula* (relative density: 48.6% and 8.1%, respectively), with the relative density of *N. inconspicua* being nearly six times higher than that of *N. subminuscula*. In March and May surveys conducted before seasonal rainfall, *N. inconspicua* (20.3%), *N. cryptocephala* (14.3%), *Fragilaria elliptica* (13.9%), and *N. inconspicua* (13.8%) were identified as dominant and subdominant species, respectively. At certain survey points, *F. elliptica* mostly found in low PO₄-P concentrations exhibited dominance. However, in the July survey conducted after the rainfall, *N. inconspicua* and *N. subminuscula* were found to dominate and subdominate, accounting for 24.0% and 23.4%, respectively. In the September survey, which shows a decrease in the frequency and intensity of rainfall, *N. inconspicua* and *Nanicula minima* were found to be dominant and subdominant species with relative densities of 35.2% and 18.3%, respectively.

Table 1. Dominance and subdominance of diatom species based on light microscope (LMS).

| Site | | Dominance Based on LMS | | Subdominance Based on LMS | |
|-----------|-----|------------------------------------|--------|-------------------------------|--------|
| Site Name | | Species | RA (%) | Species | RA (%) |
| Site 1 | 1st | <i>Nitzschia inconspicua</i> | 26.1 | <i>Navicula subminuscula</i> | 19.6 |
| | 2nd | <i>Nitzschia inconspicua</i> | 27.9 | <i>Navicula subminuscula</i> | 25.9 |
| | 3rd | <i>Nitzschia amphibia</i> | 36.0 | <i>Nitzschia inconspicua</i> | 33.9 |
| | 4th | <i>Nitzschia inconspicua</i> | 33.7 | <i>Navicula minima</i> | 29.3 |
| Site 2 | 1st | <i>Navicula cryptocephala</i> | 41.3 | <i>Surirella minuta</i> | 8.6 |
| | 2nd | <i>Navicula subminuscula</i> | 48.6 | <i>Nitzschia inconspicua</i> | 15.4 |
| | 3rd | <i>Nitzschia inconspicua</i> | 59.1 | <i>Navicula minima</i> | 20.6 |
| | 4th | <i>Nitzschia inconspicua</i> | 38.0 | <i>Navicula minima</i> | 15.9 |
| Site 3 | 1st | <i>Gomphonema olivaceum</i> | 18.0 | <i>Fragilaria capucina</i> | 17.2 |
| | 2nd | <i>Achnanthes alteragracillima</i> | 20.3 | <i>Fragilaria elliptica</i> | 13.1 |
| | 3rd | <i>Navicula minima</i> | 30.1 | <i>Nitzschia amphibia</i> | 18.6 |
| | 4th | <i>Navicula minima</i> | 24.6 | <i>Achnanthes subhudsonis</i> | 23.2 |

Table 1. Cont.

| Site | | Dominance Based on LMS | | Subdominance Based on LMS | |
|-----------|-----|--|--------|--|--------|
| Site Name | | Species | RA (%) | Species | RA (%) |
| Site 4 | 1st | <i>Achnanthes minutissima</i> | 48.2 | <i>Cymbella silesiaca</i> | 10.6 |
| | 2nd | <i>Navicula minima</i> | 16.4 | <i>Nitzschia sinuata</i> var. <i>tabellaria</i> | 15.4 |
| | 3rd | <i>Navicula minima</i> | 29.7 | <i>Fragilaria elliptica</i> | 14.8 |
| | 4th | <i>Navicula minima</i> | 41.0 | <i>Fragilaria elliptica</i> | 22.5 |
| Site 5 | 1st | <i>Navicula cryptocephala</i> | 43.4 | <i>Nitzschia inconspicua</i> | 18.4 |
| | 2nd | <i>Navicula subminuscula</i> | 34.8 | <i>Nitzschia inconspicua</i> | 16.1 |
| | 3rd | <i>Navicula minima</i> | 50.7 | <i>Nitzschia inconspicua</i> | 15.0 |
| | 4th | <i>Navicula subminuscula</i> | 30.5 | <i>Nitzschia inconspicua</i> | 28.6 |
| Site 6 | 1st | <i>Nitzschia inconspicua</i> | 30.0 | <i>Nitzschia amphibia</i> | 23.1 |
| | 2nd | <i>Fragilaria elliptica</i> | 44.2 | <i>Nitzschia palea</i> | 11.7 |
| | 3rd | <i>Navicula minima</i> | 34.6 | <i>Navicula subminuscula</i> | 27.0 |
| | 4th | <i>Nitzschia inconspicua</i> | 59.8 | <i>Nitzschia frustulum</i> | 13.7 |
| Site 7 | 1st | <i>Nitzschia inconspicua</i> | 26.9 | <i>Navicula minima</i> | 13.0 |
| | 2nd | <i>Navicula minima</i> | 18.0 | <i>Navicula seminulum</i> | 14.2 |
| | 3rd | <i>Navicula subminuscula</i> | 53.4 | <i>Nitzschia inconspicua</i> | 14.5 |
| | 4th | <i>Nitzschia inconspicua</i> | 35.0 | <i>Cymbella turgidula</i> var. <i>nipponica</i> | 25.1 |
| Site 8 | 1st | <i>Nitzschia inconspicua</i> | 27.7 | <i>Navicula minima</i> | 14.2 |
| | 2nd | <i>Gomphonema minutum</i> | 11.4 | <i>Fragilaria elliptica</i> | 10.4 |
| | 3rd | <i>Navicula subminuscula</i> | 42.9 | <i>Nitzschia inconspicua</i> | 15.5 |
| | 4th | <i>Navicula minima</i> | 48.4 | <i>Navicula subminuscula</i> | 24.2 |
| Site 9 | 1st | <i>Nitzschia inconspicua</i> | 50.8 | <i>Navicula cryptocephala</i> | 11.3 |
| | 2nd | <i>Navicula minima</i> | 24.2 | <i>Nitzschia inconspicua</i> | 23.7 |
| | 3rd | <i>Navicula subminuscula</i> | 35.9 | <i>Navicula minima</i> | 25.2 |
| | 4th | <i>Nitzschia inconspicua</i> | 36.5 | <i>Navicula minima</i> | 28.3 |
| Site 10 | 1st | <i>Nitzschia amphibia</i> | 16.1 | <i>Navicula cryptocephala</i> | 15.4 |
| | 2nd | <i>Fragilaria elliptica</i> | 21.7 | <i>Cocconeis pediculus</i> | 18.7 |
| | 3rd | <i>Navicula minima</i> | 33.5 | <i>Nitzschia palea</i> | 13.6 |
| | 4th | <i>Navicula viridula</i> var. <i>rostellata</i> | 22.5 | <i>Nitzschia palea</i> | 15.0 |
| Site 11 | 1st | <i>Nitzschia inconspicua</i> | 21.9 | <i>Navicula cryptocephala</i> | 21.1 |
| | 2nd | <i>Fragilaria elliptica</i> | 26.5 | <i>Nitzschia paleacea</i> | 13.7 |
| | 3rd | <i>Fragilaria elliptica</i> | 51.7 | <i>Fragilaria construens</i> f. <i>binodis</i> | 10.0 |
| | 4th | <i>Navicula minima</i> | 53.2 | <i>Nitzschia inconspicua</i> | 18.5 |
| Site 12 | 1st | <i>Nitzschia inconspicua</i> | 33.4 | <i>Gomphonema parvulum</i> | 25.6 |
| | 2nd | <i>Nitzschia inconspicua</i> | 32.1 | <i>Fragilaria elliptica</i> | 14.4 |
| | 3rd | <i>Nitzschia inconspicua</i> | 60.9 | <i>Navicula subminuscula</i> | 18.9 |
| | 4th | <i>Nitzschia inconspicua</i> | 69.5 | <i>Navicula subminuscula</i> | 14.2 |
| Total | | <i>Nitzschia inconspicua</i> | 48.6 | <i>Navicula subminuscula</i> | 8.1 |

RA: Relative abundance.

When analyzing the diatom community based on eDNA collected from the same biofilm at the same survey point, most of the four surveys showed similar results in microscopic analysis of dominant and subdominant taxa of diatoms (Table 2). In microscopic analysis, *Navicula subminuscula*, *Navicula minima*, *Achnanthes minutissima*, *Nitzschia inconspicua*, and *Nitzschia palea*, which accounted for significant proportions, were dominant and subdominant species in eDNA analysis. However, some survey points showed that *Melosira varians*, *Fragilaria flavovirens* (*Gedaniella flavovirens*), *Gomphonema minutum*, and *Diatoma tenuis* diatoms, which did not account for a significant proportion in microscopic analysis, accounted for a substantial proportion in eDNA analysis (Figure 2). In the March

survey, *Navicula subminuscula* dominated and *Diatoma tenuis* subdominated in all survey points. However, after March, *D. tenuis* did not account for a large proportion in any survey points. In the May survey, *N. palea* and *N. subminuscula* were dominant and subdominant, respectively. In the July survey conducted after the rainfall, *N. palea* exhibited a significant dominance. In the September survey, microscope-based analysis revealed that small *N. inconspicua* or *N. subminuscula* dominated. However, in the eDNA-based analysis, a significant number of contigs of *M. varians* with large cell sizes were found, indicating that *M. varians* dominated. In results of eDNA metabarcoding analysis, no diatoms were identified as species (sp.) in March or May survey. However, from the July survey, *Navicula* sp. contigs that were difficult to identify at the species level accounted for a large proportion. They dominated in July and September surveys.

Table 2. Dominance and subdominance of diatom species based on eDNA metabarcoding (eDNA).

| Site | | Dominance (eDNA) | | Subdominance (eDNA) | |
|-----------|-----|------------------------------------|--------|--------------------------------|--------|
| Site Name | | Species | RA (%) | Species | RA (%) |
| Site 1 | 1st | <i>Navicula subminuscula</i> | 80.6 | <i>Nitzschia palea</i> | 3.7 |
| | 2nd | <i>Navicula subminuscula</i> | 48.3 | <i>Nitzschia amphibia</i> | 19.1 |
| | 3rd | <i>Nitzschia palea</i> | 33.0 | <i>Navicula subminuscula</i> | 22.4 |
| | 4th | <i>Melosira varians</i> | 30.7 | <i>Nitzschia palea</i> | 21.0 |
| Site 2 | 1st | <i>Navicula subminuscula</i> | 30.9 | <i>Nitzschia palea</i> | 15.3 |
| | 2nd | <i>Navicula subminuscula</i> | 64.0 | <i>Gomphonema pumilum</i> | 16.9 |
| | 3rd | <i>Navicula</i> sp. | 63.1 | <i>Nitzschia palea</i> | 54.0 |
| | 4th | <i>Melosira varians</i> | 9.2 | <i>Navicula</i> sp. | 8.5 |
| Site 3 | 1st | <i>Fragilaria construens</i> | 21.2 | <i>Achnanthes microcephala</i> | 14.9 |
| | 2nd | <i>Achnantheidium minutissimum</i> | 29.9 | <i>Fragilaria construens</i> | 20.5 |
| | 3rd | <i>Nitzschia palea</i> | 43.6 | <i>Navicula</i> sp. | 9.1 |
| | 4th | <i>Nitzschia inconspicua</i> | 19.7 | <i>Melosira varians</i> | 13.7 |
| Site 4 | 1st | <i>Achnantheidium minutissimum</i> | 60.5 | <i>Navicula radiosa</i> | 11.4 |
| | 2nd | <i>Achnantheidium minutissimum</i> | 40.1 | <i>Fragilaria construens</i> | 25.3 |
| | 3rd | <i>Nitzschia palea</i> | 32.6 | <i>Fragilaria flavovirens</i> | 26.6 |
| | 4th | <i>Nitzschia palea</i> | 19.3 | <i>Navicula</i> sp. | 15.3 |
| Site 5 | 1st | <i>Navicula subminuscula</i> | 63.4 | <i>Diatoma tenuis</i> | 6.2 |
| | 2nd | <i>Nitzschia palea</i> | 31.5 | <i>Fragilaria flavovirens</i> | 27.5 |
| | 3rd | <i>Navicula subminuscula</i> | 25.7 | <i>Nitzschia palea</i> | 10.3 |
| | 4th | <i>Nitzschia filiformis</i> | 11.7 | <i>Nitzschia amphibia</i> | 9.8 |
| Site 6 | 1st | <i>Navicula subminuscula</i> | 39.2 | <i>Nitzschia soratensis</i> | 25.1 |
| | 2nd | <i>Nitzschia palea</i> | 30.1 | <i>Gomphonema pumilum</i> | 27.8 |
| | 3rd | <i>Nitzschia palea</i> | 31.7 | <i>Navicula subminuscula</i> | 7.2 |
| | 4th | <i>Nitzschia inconspicua</i> | 15.5 | <i>Navicula</i> sp. | 8.4 |
| Site 7 | 1st | <i>Nitzschia soratensis</i> | 30.9 | <i>Navicula subminuscula</i> | 19.2 |
| | 2nd | <i>Gomphonema minutum</i> | 20.0 | <i>Navicula minima</i> | 12.6 |
| | 3rd | <i>Nitzschia palea</i> | 50.9 | <i>Navicula minima</i> | 16.8 |
| | 4th | <i>Melosira varians</i> | 55.0 | <i>Nitzschia palea</i> | 13.8 |
| Site 8 | 1st | <i>Diatoma tenuis</i> | 61.8 | <i>Nitzschia soratensis</i> | 7.7 |
| | 2nd | <i>Gomphonema minutum</i> | 40.8 | <i>Fragilaria construens</i> | 18.3 |
| | 3rd | <i>Nitzschia inconspicua</i> | 20.0 | <i>Navicula subminuscula</i> | 13.7 |
| | 4th | <i>Gomphonema subclavatum</i> | 11.5 | <i>Navicula</i> sp. | 7.7 |

Table 2. Cont.

| Site | | Dominance (eDNA) | | Subdominance (eDNA) | |
|-----------|-----|------------------------------------|--------|------------------------------------|--------|
| Site Name | | Species | RA (%) | Species | RA (%) |
| Site 9 | 1st | <i>Nitzschia soratensis</i> | 42.7 | <i>Navicula subminuscula</i> | 13.0 |
| | 2nd | <i>Navicula subminuscula</i> | 35.7 | <i>Navicula minima</i> | 16.8 |
| | 3rd | <i>Navicula subminuscula</i> | 30.3 | <i>Nitzschia palea</i> | 11.2 |
| | 4th | <i>Nitzschia inconspicua</i> | 24.9 | <i>Gomphonema subclavatum</i> | 23.3 |
| Site 10 | 1st | <i>Fragilaria flavovirens</i> | 31.1 | <i>Nitzschia amphibia</i> | 27.7 |
| | 2nd | <i>Cyclotella meneghiniana</i> | 24.3 | <i>Nitzschia palea</i> | 12.3 |
| | 3rd | <i>Achnanthydium minutissimum</i> | 37.4 | <i>Fragilaria construens</i> | 30.3 |
| | 4th | <i>Nitzschia palea</i> | 8.2 | <i>Navicula sp.</i> | 7.5 |
| Site 11 | 1st | <i>Diatoma tenuis</i> | 75.0 | <i>Nitzschia soratensis</i> | 4.7 |
| | 2nd | <i>Cyclotella pseudostelligera</i> | 32.2 | <i>Nitzschia palea</i> | 31.2 |
| | 3rd | <i>Nitzschia palea</i> | 59.1 | <i>Navicula sp.</i> | 12.6 |
| | 4th | <i>Navicula minima</i> | 10.0 | <i>Fragilaria construens</i> | 8.9 |
| Site 12 | 1st | <i>Nitzschia soratensis</i> | 17.1 | <i>Navicula saprophila</i> | 11.3 |
| | 2nd | <i>Nitzschia palea</i> | 62.8 | <i>Cyclotella pseudostelligera</i> | 6.4 |
| | 3rd | <i>Nitzschia inconspicua</i> | 21.3 | <i>Gomphonema subclavatum</i> | 16.8 |
| | 4th | <i>Navicula sp.</i> | 32.1 | <i>Navicula minima</i> | 12.1 |
| Total | | <i>Nitzschia palea</i> | 16.4 | <i>Navicula subminuscula</i> | 11.0 |

RA: Relative abundance.

3.2. Differences of Ecological Health Assessment between Microscope and Metabarcoding

In the four assessments of 12 survey points, the average trophic diatom index (TDI) analyzed based on microscopic assessment was 59.9 points, which was evaluated as a 'C grade' (Figure 3A). There was no significant change after rainfall. However, from the March survey to the September survey, the health of the diatoms tended to gradually improve (Figure 3B). In addition, surveys conducted in March and May prior to the rainfall indicated that the health of diatoms in the mainstream of the Mihogang River was lower than that found in the inflow stream. However, after the rainfall, those found in the mainstream were found to be healthier than those in the inflow stream. The average trophic diatom index (TDI) for the March survey was 49.8 points, resulting in a D grade. However, the average TDI for the May survey slightly increased to 57.5 points, indicating a C grade. July and September surveys indicated improvement, with health scores of 59.3 points (grade C) and 72.9 points (grade B), respectively. In the March survey, there was a significant difference (9.5 to 90.3) in TDI among survey points. In the May survey and the July survey, the deviation between survey points decreased when compared to the March survey. In the September survey, the deviation between survey points was the lowest. All points, ranging from C to B, failed to show significant differences in TDI.

The health assessment value of diatom evaluated using eDNA was lower than that of the microscope-based assessment of diatom at most survey points (Figure 4A). In the eDNA-based assessment, all survey points were evaluated to be below the average C grade in the four assessments, with more than 50% of survey points assessed as a grade of D or E (Figure 4B). However, in the microscope-based assessment, all but some survey points (site 1, site 6) were evaluated as a C grade on average. In particular, site 1 and site 2, both located upstream, had the lowest health with an E grade at both points in the eDNA-based assessment. However, they were evaluated to be better as C and D grades in the microscope-based assessment. In the pre-rainfall surveys (March and May), there was a significant disparity in health based on the analysis technique at several points. However, in the second survey conducted immediately after the rainfall, most of the survey points displayed a significant disparity between eDNA and microscopic analysis. In a survey conducted in July immediately after the rainfall, site 1 and site 5, both located upstream, of the mainstream received a rating of B indicating good health. However, in the eDNA-based

health evaluation, the majority of mainstream points were rated as E. In the September survey, which experienced stabilization after rainfall, the majority of mainstream points were assessed as D grade according to eDNA, with site 12 located downstream receiving an evaluation of E grade.

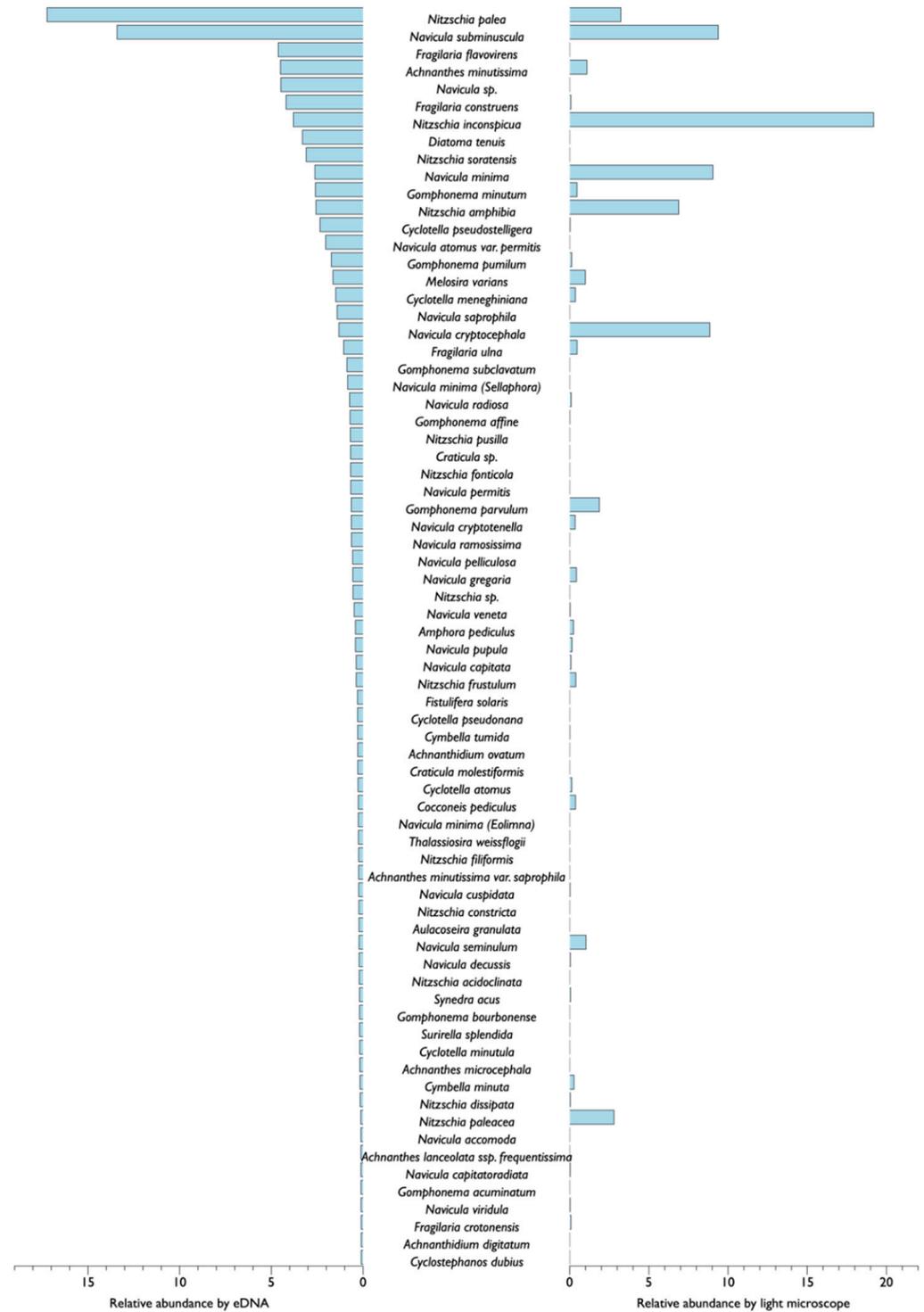


Figure 2. Taxonomic differences in benthic diatom species occurrence based on diatom species identification methods: eDNA metabarcoding (left bar) and light microscope (right bar). Diatom species targeted over 0.1% relative abundance in eDNA metabarcoding.

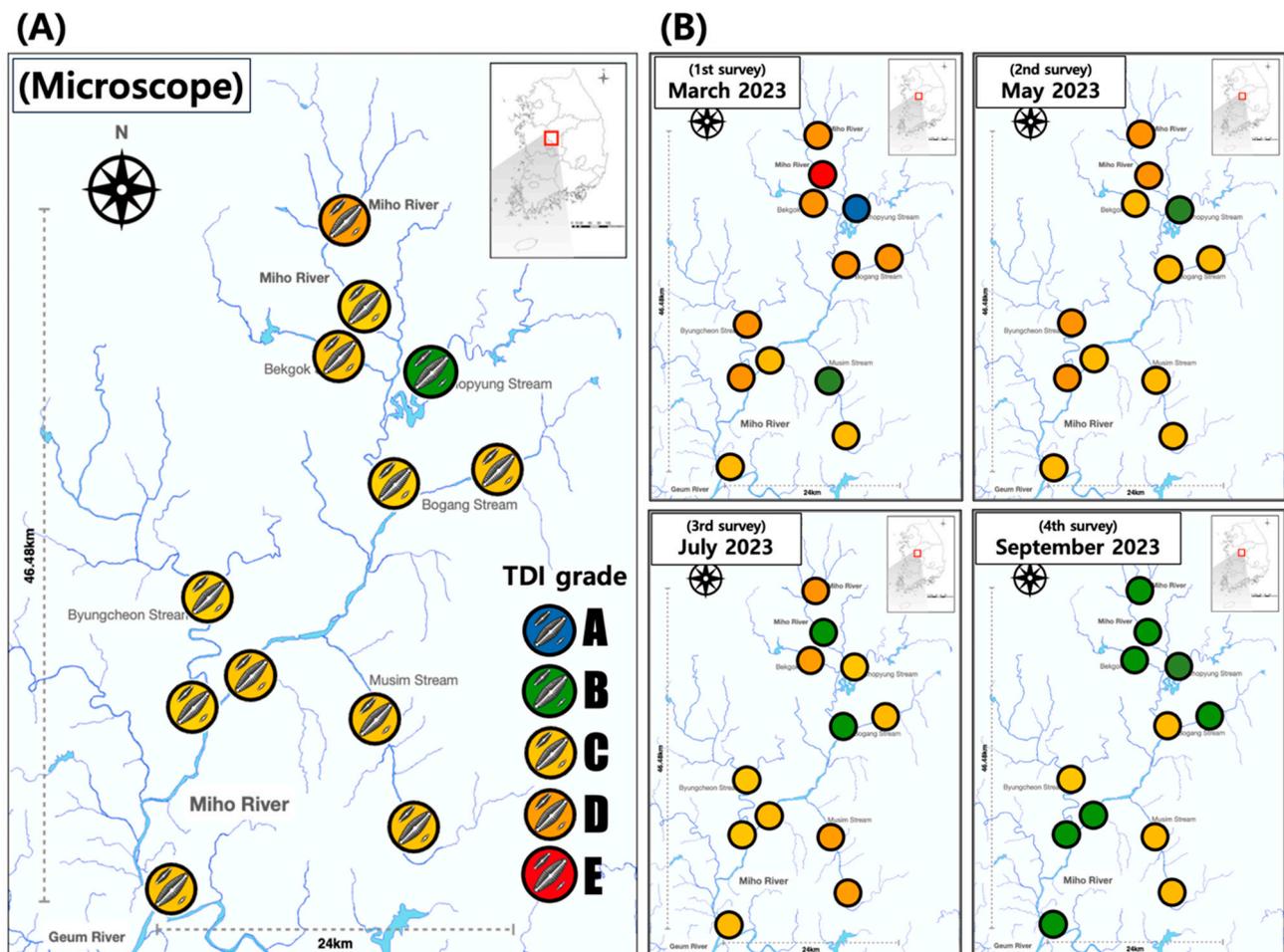


Figure 3. Assessment of ecological health for benthic diatom based on light microscope. (A) Averaged during research period. (B) Assessment on each survey. In the TDI assessment results, the colors inside the circles represent the following: Blue: Very good, Green: Good, Yellow: Moderate, Orange: Poor, Red: Very bad.

In four assessments at 12 survey points, results of eDNA-based health assessment of diatom and existing microscope-based health assessment of diatom varied depending on the survey point. There were no differences in grades among the five survey points with a score difference of less than 10 points. However, there was a difference of 20 points or more in TDI evaluation values at seven survey points. There were occasions with a difference of 1 grade or more. In particular, the eDNA-based health assessment of diatom at site 1 in the upper stream was assessed as E grade with an average of 28.9 points. However, in the microscope-based health assessment of diatom, it was evaluated as a C grade with 52.3 points, indicating a difference of two grades. Site 9, a downstream site, exhibited the largest difference, with a gap of 31.2 points between eDNA-based diatom health assessment value (average of 34.3 points) and microscope-based assessment value (average of 65.5 points), although the difference in grade was not significant due to the score range.

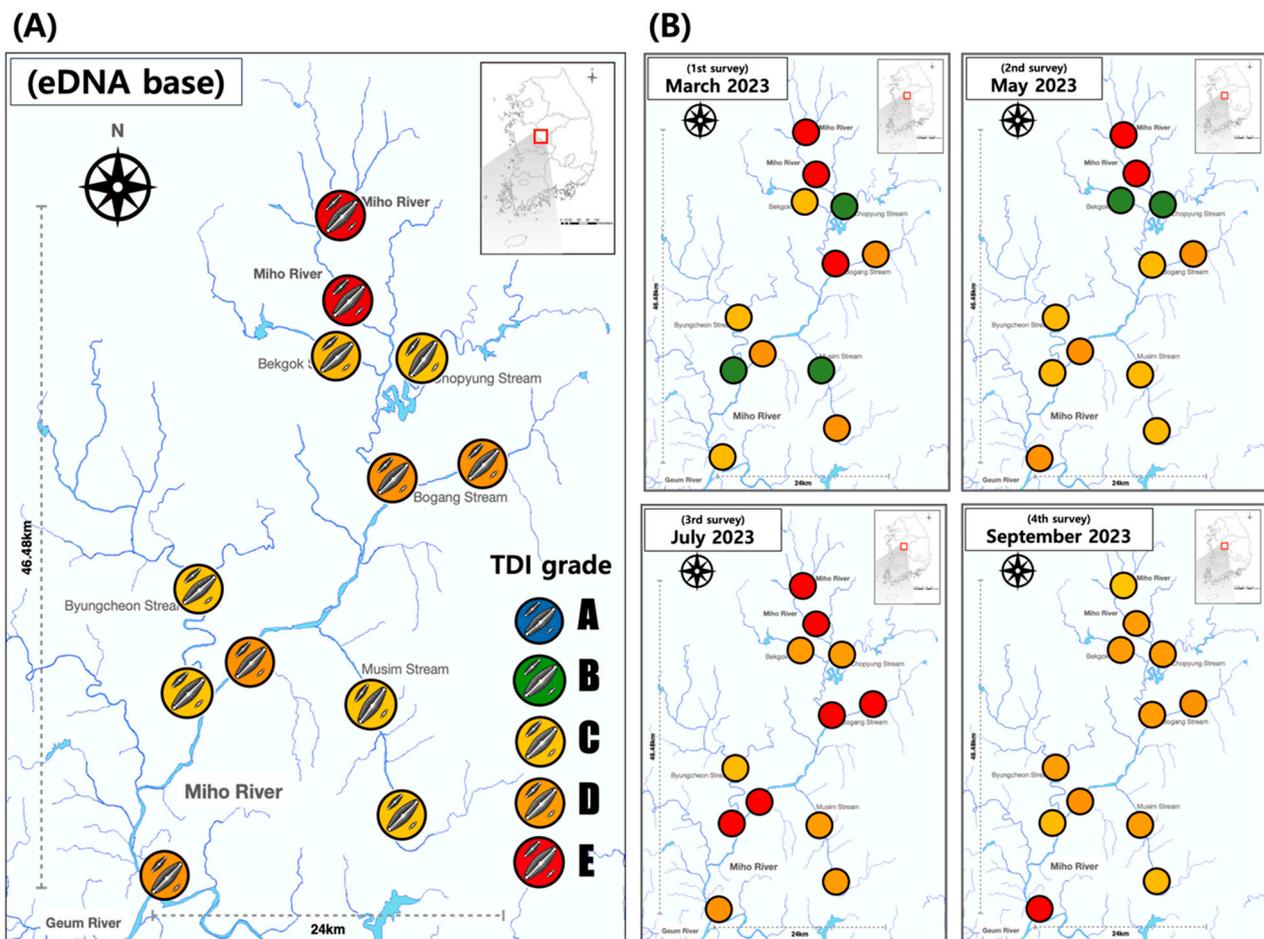


Figure 4. Assessment of ecological health for benthic diatom based on eDNA metabarcoding. (A) Averaged during research period. (B) Assessment on each survey. In the TDI assessment results, the colors inside the circles represent the following: Blue: Very good, Green: Good, Yellow: Moderate, Orange: Poor, Red: Very bad. In the TDI assessment using eDNA, no A-grade was detected.

3.3. Correlation with Water Quality (Total Phosphorus, TP; Phosphate, $PO_4\text{-P}$)

To confirm the accuracy of the health assessment value of diatom using eDNA, the concentration and correlation (Spearman's rho) of phosphate ($PO_4\text{-P}$) and total phosphate (TP) were determined and compared with the existing health assessment value of diatom using a microscope (Figure 5). Only eDNA-based health assessment values of diatoms in both $PO_4\text{-P}$ and TP showed statistically significant correlations, with a correlation coefficient being nearly twice as high as that obtained from microscopic-based health assessment. Results of the eDNA-based health assessment for diatoms showed correlations with $PO_4\text{-P}$ and TP (coefficients of -0.603 and -0.609 , respectively), all of which showed a statistical significance ($p < 0.001$). However, microscope-based health assessment values for attached stoma showed relatively lower correlation coefficients with $PO_4\text{-P}$ and TP (-0.267 and -0.286 , respectively), although these corrections were not statistically significance ($p > 0.50$).

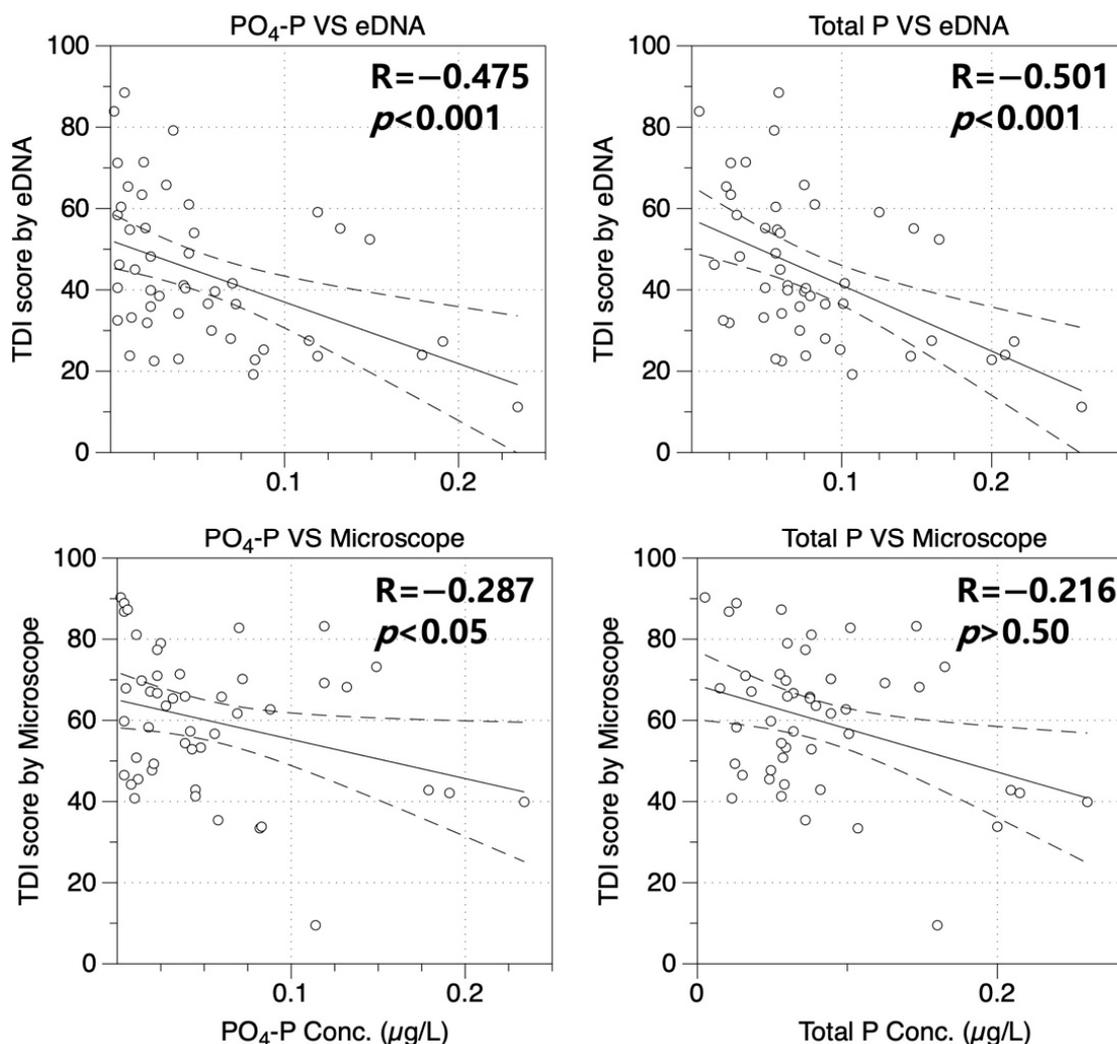


Figure 5. Correlations with water quality indicators $\text{PO}_4\text{-P}$ and TP (Total Phosphorus) according to ecological health assessment method. The solid line represents the slope of the correlation coefficient, while the dashed line indicates the 95% confidence interval. The white circles denote TDI scores corresponding to the respective P concentrations.

4. Discussion and Conclusions

When comparing results of analyses of diatom using eDNA and conventional microscopes, the largest difference was observed in species that appeared. In particular, dominant and subdominant species, as indicated by microscopic analysis and eDNA metabarcoding analysis, were found to be different. In addition, several unrecorded diatoms not previously reported in Korea were discovered through eDNA analysis. This discrepancy was primarily caused by differences in the criteria utilized for species listing and an insufficient sequence database [39,40]. The list of diatom species based on microscopic observations is currently derived from the diatom species record of the “National List of Species of Korea” [10,41]. However, the gene sequence database based on eDNA relies on species names entered by researchers worldwide. These entries are updated to reflect the latest modification of the species name at the time of input [42]. As a result, the most recent species names are mixed up with the gene sequence database from the past. For example, *Eolina subminuscula*, which emerged as a prominent dominant species in the eDNA analysis results, was previously known as *Navicula subminuscula*. It is listed in the National List of Species [10,42]. Rather than assuming that the species had actually appeared, the unrecorded species found in eDNA analysis could be judged as an error caused by misidentification resulting from an insufficient gene sequence database or BLAST of non-specific amplified products

(PCR products) [43–45]. Currently, not all diatoms have information in the gene sequence database existing in the widely used GenBank of the National Centre of Biological Information (NCBI). Most notably, the *Achananthes conversion*, which is most dominant in Korea, is a prominent species in both Korea and Asia. However, it is currently absent from GenBank's gene sequence database [46]. In this study, since the gene sequence information of *Cymbella silesiaca*, which was identified as a subdominant species in microscopic analysis, did not exist in GenBank, it could be concluded that it belongs to another diatom with similar gene sequences.

In addition, in the case of large-sized diatoms (e.g., *Melosira varians*, *Eunotia pectinalis* var. *undulata*, *Cymbella cistula*, *Cymbella aspera*, etc.), the cell envelope might be physically damaged during the process of collecting diatoms, which might result in omission of species identification and counting based on shape from microscopic analysis [47]. However, even if the shell of diatoms is physically destroyed, the DNA of all diatoms still exists in the sample collected from the field. Thus, it can be analyzed using eDNA, which is believed to enable a more accurate community analysis, including taxa that might have been omitted from the current process of field collection.

The use of this analysis technique can lead to differences in the diatom community, which in turn affects health assessment results of diatoms. Existing microscope-based health assessment is performed by treating biofilms collected from the substrate of the site with acid to remove organic matter, followed by identifying and counting species based on the shell of diatoms [48–50]. This acid treatment aims to enhance the accuracy of the identification process by enabling a clearer observation of the shell of the diatom. However, during the acid treatment process and creation of permanent specimen, shells of diatoms may be physically destroyed [48,51]. In addition, it is not possible to determine whether diatoms collected at the site are living or dead cells. However, since eDNA-based health assessment of diatoms relies on DNA present in cells, species identification and contig calculation are typically performed under the assumption of the presence of living cells. Therefore, the difference between microscope-based and eDNA-based structures of diatom communities cannot be disregarded, potentially resulting in variations in the assessment of the health of diatoms.

Additionally, there exists a significant difference in the analytical scale between eDNA analysis and microscopic analysis. Currently, the 'Biomonitoring Survey and Assessment Manual' [10] recommends counting a minimum of 200 valves when analyzing diatom communities under a microscope. However, in this paper, the eDNA analysis involved the examination of 30,000 reads, which can be considered equivalent to counting over 30,000 valves based on microscopic standards. The robust analytical power of eDNA analysis allows for the detection of many rare species that are challenging to identify through microscopic analysis. Consequently, eDNA analysis consistently reveals a higher number of diatom species compared to microscopic analysis. Furthermore, the diatom species list and atlas [10,32–36] used in the National biomonitoring network do not reflect the latest diatom classification system. However, eDNA-based analysis results incorporate the most recent diatom classification system, potentially leading to the discovery of a relatively more diverse assemblage of diatoms.

In the health assessment of diatoms, eDNA demonstrated a stronger correlation with water quality than the existing microscope-based assessment method. Thus, it has a higher utility value as an assessment standard to provide important information that can help us gain a clearer understanding of changes occurring in the aquatic ecosystem. However, in cases where the difference from the microscope is clear, it cannot be said that the health assessment of diatom using eDNA has a high utility value. eDNA metabarcoding offers the advantage of analyzing numerous locations across broad regions and allows for the identification of challenging species without significant variation among analysts. However, in small-scale monitoring scenarios (e.g., fewer than five sites), microscopic analysis can yield faster results, and the taxonomic identification at the genus level may exhibit minimal variation among analysts, even with lower expertise in benthic diatom morphology analysis.

Furthermore, the absence of DNA sequences for certain species does not preclude their identification at the genus level through phylogenetic analysis in eDNA-based approaches. It is anticipated that, during regional development, water quality assessment using diatoms may be applicable to narrow areas beyond the national biomonitoring network, with fewer than three sampling sites. For these reasons, microscopic analysis methods can continue to be employed. Therefore, by generating genus-level diatom indicators from eDNA and subsequently applying them to microscopic analysis, the trends observed in microscopic analysis results are expected to align with those derived from eDNA analysis. In other words, obtaining indicators for the entire diatom community at the species level using precise eDNA analysis and then presenting indicators at the genus level could contribute to the utilization of diatoms in biological water quality assessment across diverse research fields, such as ecological modeling.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d16010008/s1>, Figure S1: The biofilm attached to the gravel substrate found in the river during field surveys.

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