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Co-occurrence of Bacillariophyceae-based- and Cryptophyceae-based planktonic food webs in a temperate estuarine ecosystem revealed via eDNA

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ABSTRACT

We examined microbial food webs in Seomjin River and Gwangyang Bay in South Korea, using 18S rRNA amplicon sequencing of environmental DNA (eDNA) with a weighted gene correlation network analysis (WGCNA) – Cytoscape analysis, considering various environmental variables. WGCNA with weight >0.5 revealed two taxon-specific trophic interactions. One was composed of Chlorophyceae and Cryptophyceae-based primary producers and ciliates-based primary consumers. In another one, Bacillariophyceae was a main primary producer while rotifer and ciliates were primary consumers. The Bray-Curtis dissimilarity and correlation matrix of gene abundance with gene significance related to environmental variables showed that the Chlorophyceae and Cryptophyceae-based microbial food web was a function of dissolved inorganic nitrogen in the Seomjin River, whereas the Bacillariophyceae-based microbial food web was a function of dissolved inorganic phosphate in the Gwangyang Bay. Our study provides new insight into microbial food webs while considering the nutrient status of estuarine ecosystems.

"Who eats whom" has long been an interesting topic for researchers when investigating ecological functions of organisms in various ecosystems (Cohen et al., 1994; Roslin et al., 2016). Marine food webs depict the fate of carbon by showing carbon transfer through multiple trophic levels (Pauly et al., 1998) and carbon or nutrient remineralization via microbial food webs (Rivkin and Legendre, 2001; Tian et al., 2000). Planktonic food webs comprising viruses, bacteria, phytoplankton, and protists are responsible for biogeochemical cycles in oceanic regimes (Calbet and Landry, 2004; Calbet and Saiz, 2005; Caron et al., 2017; Church, 2000; Shelford et al., 2012). The importance of trophic interactions between phytoplankton and ciliates in planktonic food webs has been increasingly recognized owing to the pivotal role of ciliates as trophic links from bacteria, picoplankton, and nanoplankton to higher trophic consumers, such as zooplankton and fish (Calbet and Saiz, 2005; Pierce and Turner, 1992).

To assess the ecological role of food webs in marine ecosystems, food web elements were identified and carbon transfer to upper trophic levels was quantified by utilizing various techniques, such as stable isotope analysis (Chen et al., 2018a; Layman et al., 2012), morphological analysis using microscopy (Connell et al., 2017; Sherr and Sherr, 1994), small phytoplankton detection using flow cytometry (Tadonléké et al., 2005; Trombetta et al., 2020), or trawling and diving (Tittensor et al., 2010). Exploring biodiversity of organisms across the life domain is pivotal to identifying aquatic food webs; however, it is challenging to classify organisms simultaneously in contemporary ecosystems. Current food web studies require researcher's expertise to identify organisms in each trophic level, and limited sample sizes limit the reliable identification of food webs (Roslin et al., 2016). Thus, fragmentary studies through field surveys and laboratory experiments have been conducted to illustrate prey and predator interactions in microbial communities (Caron et al., 2017; Connell et al., 2017; Kang et al., 2015).

Following recent advances in DNA metabarcoding, environmental DNA (eDNA) has been applied to marine organism community monitoring across various trophic levels (Chen et al., 2020; Craine et al., 2018; Deutschmann et al., 2019; Djurhuus et al., 2018; Jo et al., 2019; Sawaya et al., 2019; Takahara et al., 2019), exhibiting an excellent capability to classify organisms in contemporary pelagic ecosystems. Recently, marine food webs have been constructed using eDNA (Djurhuus et al., 2020; Zamora-Terol et al., 2020); this technique also provides new insight into trophic interactions in inaccessible environments

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such as the deep sea or hydrothermal vents (Olsen et al., 2014). In addition, applying multiple molecular barcodes, including 16S rRNA, 18S rRNA, cytochrome *c* oxidase subunit I (COI), and 12S rRNA, has facilitated the development of complex trophic networks of marine organisms at a large scale (Djurhuus et al., 2020).

Recent advance in network analysis techniques such as weighted gene correlation network analysis (WGCNA) – Cytoscape analysis highly raised opportunity for scientists to gain new insights in microbial dynamics in conjunction with environmental changes both in open oceans and coastal waters (Gong et al., 2018; Lin et al., 2021; Poff et al., 2021). Although there is a concern that co-occurrence signals may not be able to provide evidence of ecological interactions, particularly when presence-absence data are utilized (Blanchet et al., 2020; Hirano and Takemoto, 2019), WGCNA constructs a weighted gene co-occurrence adjacency matrix as a function of a soft-threshold unlike other co-occurrence networks (Langfelder and Horvath, 2008) and the WGCNA – Cytoscape analysis is applied to identify microbial interactions (Cao et al., 2018; Duran-Pinedo et al., 2011).

In the temperate estuarine ecosystem encompassing Seomjin River and Gwangyang Bay in South Korea, food web studies have largely focused on food sources (Bibi et al., 2020; Kim et al., 2019a) and copepod trophic interactions and feeding selectivity (Chen et al., 2018a) via stable isotope analysis. Comparisons of aquatic monitoring results from microscopy and eDNA metabarcoding suggested that eDNA is efficient for food web assessments (Kim et al., 2019b). Moreover, the spatial distribution of marine organisms in Gwangyang Bay has been successfully examined using eDNA (Jo et al., 2019). A recent effort to identify patterns in multiple communities across trophic stages and reconstruct food web structure elucidated food web dynamics in Gwangyang Bay at a large scale (Kang et al., 2020a). Despite the importance of planktonic food webs in aquatic ecosystems (Legendre and Le Fèvre, 1995; Smetacek, 2002) and continuous efforts to investigate food webs (Bibi et al., 2020; Chen et al., 2018a; Kang et al., 2020a; Kim et al., 2019a), to the best of our knowledge, planktonic food webs including phytoplankton and ciliates have never been examined in Seomjin River and Gwangyang Bay.

In this study, we explored planktonic food webs in a temperate estuarine ecosystem using an 18S rRNA barcode-applied eDNA technique. We aimed to determine the effectiveness of eDNA metabarcoding in food web studies and to distinguish specific microbial food webs that may be linked to higher trophic levels with fundamental roles in this ecosystem using the WGCNA – Cytoscape analysis. Given the importance of environmental characteristics in controlling plankton dynamics, we also investigated the role of environmental variables in constructing planktonic food webs.

1. Materials and methods

1.1. Sample collection

Sampling was conducted at seven sampling sites between Seomjin River and Gwangyang Bay on September 21 and 22, 2020 (Fig. 1). Surface water samples were collected in 10 L carboys using a Niskin water sampler (General Oceanics, Miami, FL, USA) and stored in ice under dark conditions. Temperature and salinity were measured using a portable YSI (Pro Plus, Yellow Springs, OH, USA). Samples were taken to the laboratory within 2 h of collection. For eDNA analysis, 1 L samples were filtered onto 0.2 μm polycarbonate track etched membrane filters (47 mm diameter; GVS North America, ME, USA) and were flash-frozen in liquid nitrogen and then stored at −80 °C until further analysis. For dissolved nutrients, 20 mL samples were filtered onto pre-combusted (2 h at 450 °C) GFF filters (Whatman, 47 mm of diameter; Cyvita, MA, USA) and stored at −20 °C. Nutrients samples were analyzed on a QuAAtro nutrient auto-analyzer (Seal Analytical Ltd., Southampton, UK) at the National Institute of Fisheries Science in Yeosu, Republic of Korea. NO_2, NO_3^-, NH_4^+, PO_4^{3-}, and SiO_2 were analyzed in duplicate by

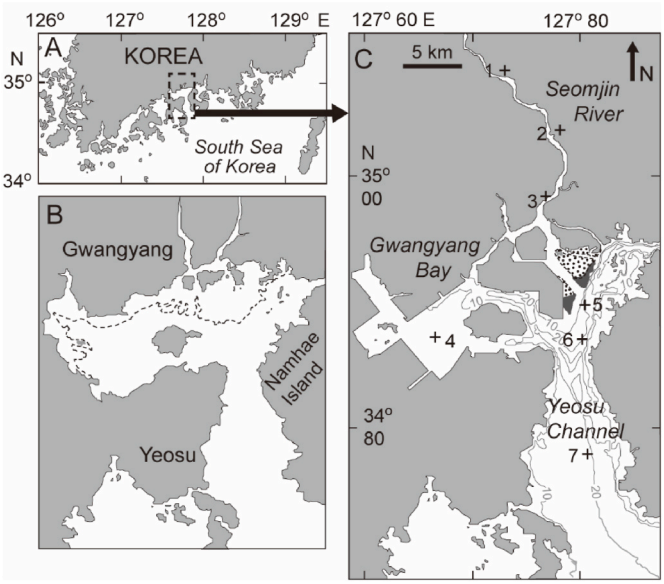

Fig. 1. Map showing sampling stations from Seomjin River to Gwangyang Bay through the Yeosu Channel.

standard spectrophotometric methods (Parsons et al., 1984). Dissolved inorganic nitrogen (DIN) refers to combination of NO_2, NO_3^-, and NH_4^+.

1.2. DNA extraction

DNA extraction and sequencing were performed at Macrogen Inc. (Seoul, Korea; www.macrogen.com) according to their pipeline. DNA was extracted using the DNeasy powerMax soil kit (Qiagen, Carlsbad, CA, USA) following the manufacturer's protocol. Each sequenced sample was prepared according to the Illumina 16S Metagenomic Sequencing Library protocols (San Diego, CA, USA; Amplicon et al., 2013) but different PCR conditions were applied as below. DNA quality and quantity were determined using a PicoGreen dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The 18S rRNA genes were amplified using 18S V9 primers according to Tragin et al. (2018) as 18S Amplicon PCR Forward Primer: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGT TGTACACACCGCCCGTCGC 3′, 18S Amplicon PCR Reverse Primer: 5′ GTCTCGTGGG CTCGGAGATGTGTATAAGAGACAGCCTTCYGCAGGTTCACCTAC 3'. while multiplexing indexes and Illumina sequencing adapters were added. First, PCR was performed on extracted DNA to amplify the target region with one cycle of 3 min at 95 °C, and 25 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, and 5 min at 72 °C. To produce indexing PCR, the second PCR was performed with one cycle of 3 min at 95 °C, 8 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, and 5 min at 72 °C. The final products were normalized and pooled using the PicoGreen assay, and library size was verified using TapeStation DNA Screen Tape D1000 (Agilent Technologies, Santa Clara, CA, USA). The library was sequenced using the MiSeq™ platform (Illumina, San Diego, USA) at a depth of 0.06G per sample.

1.3. Bioinformatics

After sequencing, FASTQ files were generated using MiSeq raw data. Quality control and preprocessing were performed using fastp software (Chen et al., 2018b), and error-correlation was applied to the overlap region between two reads. After the pair-end data were assembled through FLASH 1.2.11 (Magoč and Salzberg, 2011), the assembled sequences with lengths <100 bp or > 200 bp were eliminated. CD-HIT operational taxonomic unit (OTU) was utilized to cluster obtained sequences using a ≥97% similarity cut-off and the average linkage method

with VSEARCH (Rognes et al., 2016) after sequences characterized by low quality, ambiguous, chimera, and other deformations were removed (Li et al., 2012). The representative OTU sequences were assigned to reference taxa using BLASTN v 2.9.0 with the highest similarity to reference (Zhang et al., 2000). When query coverage of best hit was less than 85%, taxonomy was not defined. Bacteria OTUs that were determined via the Quantitative Insights into Microbial Ecology (QIIME) v1.9 (Caporaso et al., 2010) were deleted to concentrate eukaryotic communities. Supplementary Table 1 exhibits raw data statistics including total read bases (bp), total reads after merging paired-end reads using FLASH, read counts after clustering raw reads using CD-HIT-OTU, GC (%), Q20, and Q30. Supplementary Table 2 illustrates statistics after the sequenced data were assembled using FLASH and clustered using CD-HIT-OUT. Bioinformatics analysis was performed at Macrogen Inc. (Seoul, Korea; www.macrogen.com).

1.4. Data analysis

QIIME 2 was utilized to estimate alpha diversity (Bolyen et al., 2019; Hall and Beiko, 2018), and alpha diversity values were confirmed via rarefraction curve. For abundance indexing of sequencing data, the OTU results were simplified by grouping OTUs by their Class annotation to refer to a taxon as an individual Class, which may include multiple species or genera OTUs. eDNA data sets were processed through multiple steps, including DNA extraction, amplification, and bioinformatics. Such processes can influence the number of OTU reads assigned to a taxon in a given sequencing run (Shelton et al., 2016). Thus, after raw reads were summed by Class, we standardized the dataset by normalizing amplicon read counts as sample sum of squares equal to one across the whole dataset using the decostand function in the "vegan" package (Oksanen et al., 2013) in R (Supplementary Fig. 1; Kelly et al., 2019) with an assumption that amplification efficiency remains constant across samples. This method is identical to the geometric-mean-based normalization in DESeq2 (Love et al., 2014).

Weighted gene correlation network analysis (WGCNA) was performed to identify modules (i.e., clusters of highly interconnected genes) that are highly correlated with environmental variables such as temperature, salinity, DIN, dissolved inorganic phosphate (DIP), and SiO_2. Co-occurring network of taxon abundance (i.e., Class in this study) was expressed mathematically using an adjacency matrix, which exhibiting pairwise similarities. Then, specific modules were generated based on gene significance and module membership, which indicate the significance of gene associations with environmental variables and their correlations with the specific module, respectively using a soft-threshold Pearson's correlation jointly with a topological overlap distance metric and Ward's Hierachical Agglomerative Clustering Method as described below (Langfelder and Horvath, 2008; Zhang and Horvath, 2005). Higher absolute values of gene significance indicate greater biological importance. Module memberships ranging from −1 to 1 show that the gene is highly connected to the module. If the gene has a module

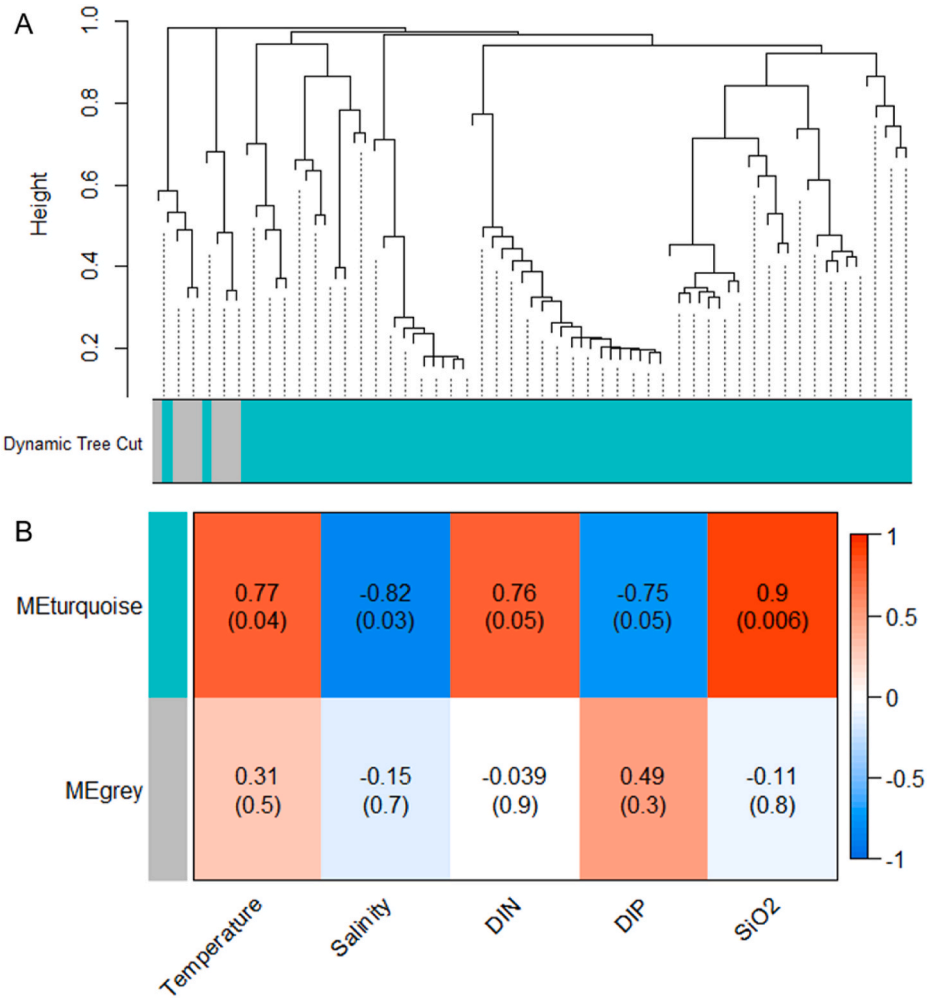

Fig. 2. Weighted gene correlation network analysis (WGCNA) of eDNA associated with environmental variables. A. Dendrogram with clusters of eDNA normalized abundance showing different subnetworks (modules). B. Correlation matrix of the grey and turquoise modules related to the environmental variables. Numbers in parentheses are p values responding to each environmental variable.

membership of 0, then the gene is not part of the module (Langfelder and Horvath, 2008). All taxa were categorized into grey and turquoise modules, and the correlation matrix was created to determine important environmental variables significantly associated with grey and turquoise modules, respectively. Taxa belonging to the grey module were ignored because they exhibited non-significant correlations with environmental variables (Fig. 2; $p > 0.05$; correlation matrix). Weight between two nodes showing the strength of connection was calculated by topological overlap matrix. Results from the turquoise module pertaining to sources, targets, and weights were exported as text file to further investigate the network. Phytoplankton or green-algae (hereafter, phytoplankton) sources in the turquoise module were selected to generate food web structures (Supplementary Table 3). Data analysis was performed using the "WGCNA" package (Langfelder and Horvath, 2008) in R. Network analysis was executed in Cytoscape 3.8.2 (Cline et al., 2007) to visualize the chosen module (i.e., turquoise modules); then, the subnetwork was selected when weight exceeded 0.5. Organisms in the selected subnetwork were categorized as primary producers (mainly phytoplankton) and primary consumers (mainly microzooplankton). Bray-Curtis dissimilarity was determined for the indexed data to cluster the dataset by region using the "vegan" package (Oksanen et al., 2013) in R. Figures were generated using "ggplot2" (Wickham, 2016) and "ggdendro" (De Vries and Ripley, 2016) packages in R. R analyses were performed with R version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria).

2. Results

2.1. Biodiversity and weighted gene correlation networks

A total of 5104 OTUs were identified with a mean of 729 OTUs via eDNA metabarcoding (Table 1; Supplementary Table 4). Richness (Chao1) ranged from 697 at ES3 to 897 at ES6 with a mean of 791, while evenness (Shannon) ranged from 3.61 at ES3 to 8.85 at ES1 with a mean of 5.13 (Table 1). WGCNA clustered the whole data set into two modules according to the dynamic tree cut: grey and turquoise modules (Fig. 2A). The grey module was excluded due to non-significance with environmental variables ($p > 0.05$; correlation matrix), whereas the turquoise module, which contained 70 taxa (Classes), exhibited significant correlation with environmental variables (Fig. 2B). Taxa in the turquoise module were negatively correlated with salinity (correlation coefficient $= -0.82$, $p = 0.03$; correlation matrix) and DIP (correlation coefficient $= -0.75$; $p = 0.05$) but positively correlated with temperature (correlation coefficient $= 0.77$; $p = 0.04$), DIN (correlation coefficient $= 0.76$; $p = 0.05$), and SiO_2 (correlation coefficient $= 0.9$; $p = 0.006$; Fig. 2B).

2.2. Subnetwork characteristics

Subnetworks whose weights exceed 0.5 were extracted from the turquoise module and utilized to construct two distinct planktonic food webs. Chlorophyceae-Chrysophyceae-Cryptophyceae-based (small phytoplankton–based) trophic interaction demonstrated that Cryptophyceae were the main primary source for the planktonic food web with weights of 0.7482, 0.7482, and 0.7533 with the ciliates Heterotrichea, Karyorelictea, and Nassophorea, respectively (Fig. 3A). The Bacillariophyceae–Chlorodendrophyceae (large phytoplankton–based) trophic interaction showed that Bacillariophyceae were the main primary producers, with weights of 0.5767, 0.6615, and 0.6991 with the microzooplankton Litostomatea, Bdelloidea, and Oligohymenophorea, respectively (Fig. 3B).

The relative abundance of Bacillariophyceae sharply increased to 78% from ES4 to ES7, and Chlorophyceae and Cryptophyceae were the main primary producers with 40% and 30% of relative abundance, respectively (Fig. 4A). Litostomatea dominated across all stations, and various ciliates were observed at the Seomjin River stations (ES1 – ES3) with 20% of relative abundance (Fig. 4B). Ciliates with high weight values with Cryptophyceae exhibited at ES1 where the relative abundance of Cryptophyceae was the highest (Fig. 4). Chlorophyceae had the largest number of OTU ranging from 0 at ES4 to 78 at ES1 and the number of OTU in Bacillariophyceae and Cryptophyceae was relatively stable across all stations with a mean of 10 and 12 OTU, respectively (Fig. 4C). The number of Litostomatea (mean of 5 OTU) were highest throughout all stations, whereas the number of other microzooplankton was higher at the Seomjin River stations (ES1 – ES3) than at the Gwangyang Bay stations (ES4 – ES7; Fig. 4D). The Bray-Curtis dissimilarity based on the normalized OTU abundance included two clusters: ES1 – ES3 and ES4 – ES 7 (Fig. 4E).

2.3. Correlation with environmental variables

To determine which taxon is the most correlated with environmental variables in a given module, unique properties such as gene significance and module membership were estimated via WGCNA (Supplementary Fig. 2), which elucidated correlations between gene significances and module memberships (Supplementary Fig. 3). Cryptophyceae, Chrysophyceae, and Chlorophyceae exhibited the highest module membership with 0.877, 0.837, and 0.742, respectively (Table 2). The Bacillariophyceae-based trophic interaction had positive gene significances with salinity, whereas the small phytoplankton–based trophic interaction had negative gene significances with salinity (Table 2). Bacillariophyceae had the highest positive gene significances with DIP while Cryptophyceae had the highest positive gene significances with DIN and SiO_2 (Table 2).

Comparison between the Seomjin River and Gwangyang Bay showed distinct ecosystems exhibiting different planktonic food web and environmental elements. Normalized OTU abundance of major primary producers and consumers were presented, indicating a significant difference between the Seomjin River and Gwangyang Bay (Fig. 5). While the normalized OTU abundance of Bacillariophyceae were fairly similar between two regions ($p = 0.8597$; Mann Whitney U test; Fig. 5A), the abundance of other primary producers, including Chlorophyceae, Chrysophyceae, and Cryptophyceae, were significantly different between the regions ($p < 0.05$; Fig. 5C – E). The abundance of most microzooplankton excluding Litostomatea were significantly different ($p = 0.3768$; Fig. 5F – J). Along with the normalized OTU abundance of the major taxon, DIN and SiO2 were significantly higher ($p < 0.05$; Mann Whitney U test; Fig. 6C and E) and salinity was lower in the Seomjin River ($p < 0.05$; Fig. 6B). Conversely, DIP and salinity were significantly higher and somewhat higher in the Gwangyang Bay, respectively ($p < 0.05$ for DIP, $p = 0.116$ for salinity; Fig. 6B and D).

Table 1
Biodiversity indices in the Seomjin River and Gwangyang Bay including operational taxonomic units (OTUs), richness (Chao1), and evenness (Shannon).

Station	OTUs	Chaol	Shannon
ES1	662	700	8.85
ES2	778	858	4.80
ES3	611	697	3.61
ES4	700	756	5.40
ES5	721	754	4.58
ES6	822	897	4.41
ES7	810	873	4.28

3. Discussion

In this study, 18S rRNA gene marker-applied eDNA was utilized to investigate planktonic food webs in Seomjin River and Gwangyang Bay, South Korea. Two potential planktonic food webs were identified: One was based on small phytoplankton, including Chlorophyceae, Chrysophyceae, and Cryptophyceae, with Cryptophyceae exhibiting high significance in the subnetwork. The other food web was based on Bacillariophyceae and Chlorodendrophyceae with Bacillariophyceae

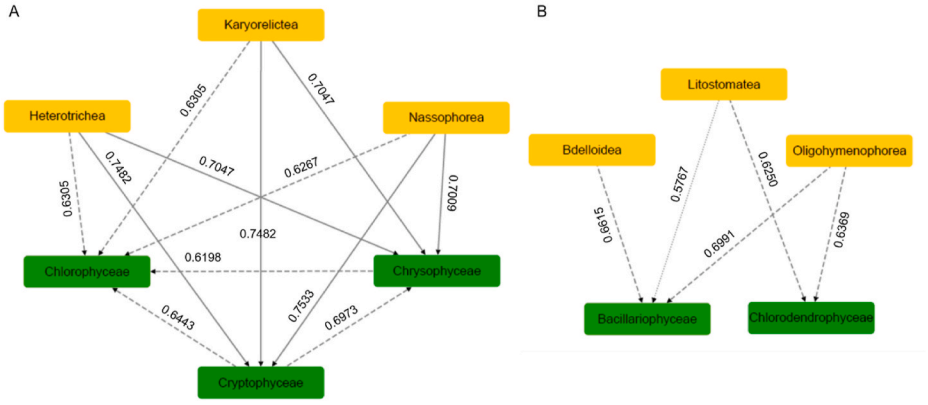

Fig. 3. Subnetwork visualization of planktonic food webs in a turquoise module showing top-down interactions of two distinguished microbial communities. A. Network demonstrating the interaction of small phytoplankton (Chlorophyceae, Chrysophyceae, Cryptophyceae) with ciliates. B. Network demonstrating the interaction of diatoms (Bacillariophyceae) and Chlorodendrophyceae with rotifer (Bdelloidea) and ciliates. Data presented in subnetworks have weight >0.5.

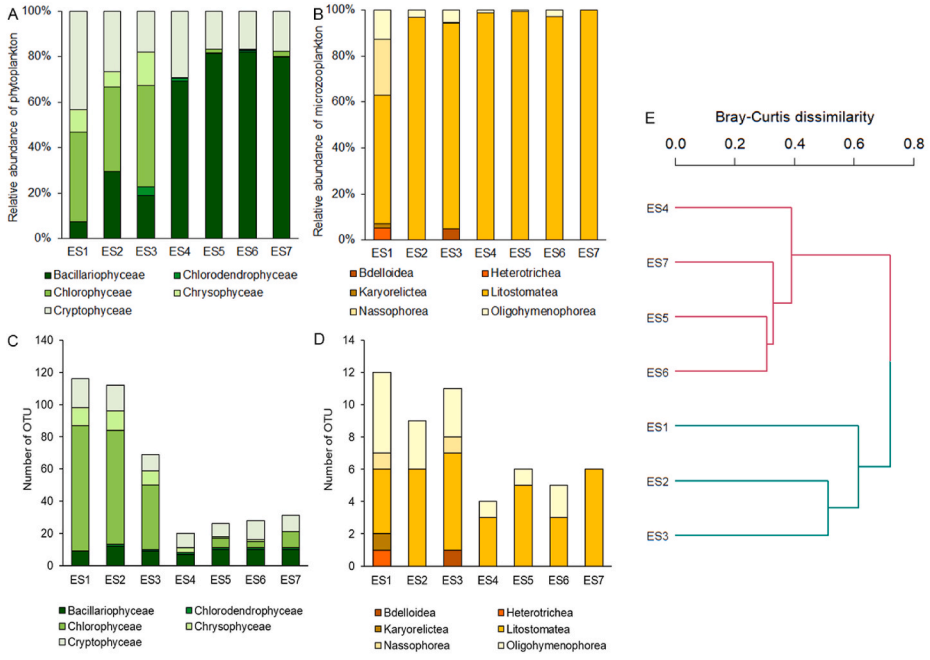

Fig. 4. Primary producers and consumers in a turquoise module. A. Relative abundance of primary producers (phytoplankton), B. Relative abundance of primary consumers (microzooplankton), C. Number of OTU in primary producers, D. Number of OTU in primary consumers, E. Bray-Curtis dissimilarity of eDNA normalized abundance of OTUs.

exhibiting high significance, indicating a large phytoplankton–based planktonic food web. WGCNA combined with network visualization showing edges (connections) with high weights (weight >0.5) revealed taxon-specific trophic interactions between primary producers and primary consumers. In addition, gene significances obtained from the WGCNA elucidated the significant correlation of Bacillariophyceae with DIP and that of Cryptophyceae with DIN and SiO_2, indicating that the locale of taxon in the ecosystem is important because nutrient compounds originate from different sources; thus, the biogeochemical interactions of primary producers are linked to taxon-specific top-down interactions with microzooplankton. Our study provides novel information on planktonic food webs in Seomjin River and Gwangyang Bay. Our findings may provide guidance for the development of a food web model utilizing biogeochemical and hydrodynamic modeling in the future.

3.1. Construction of whole food web network via WGCNA and network visualization

A food web network of the turquoise module, which was identified using WGCNA, was constructed using Cytoscape. As the 18S rRNA-applied eDNA identified various eukaryotic components of an aquatic food web structure across multiple trophic levels, WGCNA revealed a significantly connected food web structure (Supplementary Fig. 4). The whole network of the turquoise module contained three unique subnetworks, including a Cryptophyceae-based food web, Bacillariophyceae-based food web, and top-down trophic interactions from Sagittoidea (Supplementary Fig. 4). Interestingly, eDNA from the surface water indicated benthic-pelagic interactions that are directly connected primary producers (mainly phytoplankton) and benthic worms (Sagittoidea) or benthic filter feeders (Bivalves), although this subnetwork was excluded due to low weight values (weight <0.5). This indicates that tidal flows and anthropogenic activity such as reclamation

Table 2
Module memberships and gene significances associated with environmental variables. Absolute values of module memberships indicate greater biological importance. Module memberships ranging from −1 to 1 show that the gene is highly connected to the module (Langfelder and Horvath, 2008). Gene significances indicate the significance of gene associations with environmental variables (Langfelder and Horvath, 2008).

Class	Modulemembership	Gene significance				
		Temperature	Salinity	DIN	DIP	SiO_2
Cryptophyceae	0.877	0.738	−0.798	0.720	−0.661	0.833
Chryophyceae	0.837	0.706	−0.757	0.707	−0.633	0.792
Chlorophyceae	0.742	0.690	−0.717	0.575	−0.498	0.686
Nassophorea	0.637	0.582	−0.621	0.536	−0.450	0.593
Heterotrichea	0.619	0.581	−0.620	0.507	−0.422	0.576
Karyorelictea	0.619	0.581	−0.620	0.507	−0.422	0.576
Oligohymenophorea	0.330	0.074	−0.0578	0.476	−0.450	0.323
Bdelloidea	0.188	−0.065	0.081	0.358	−0.353	0.185
Litostomatea	0.157	−0.137	0.133	0.302	−0.382	0.152
Chlorodendrophyceae	−0.049	−0.114	0.284	0.073	−0.0183	−0.099
Bacillariophyceae	−0.319	−0.061	0.169	−0.544	0.446	−0.373

Abbreviations are as following; DIN = dissolved inorganic nitrogen and DIP = dissolved inorganic phosphate.

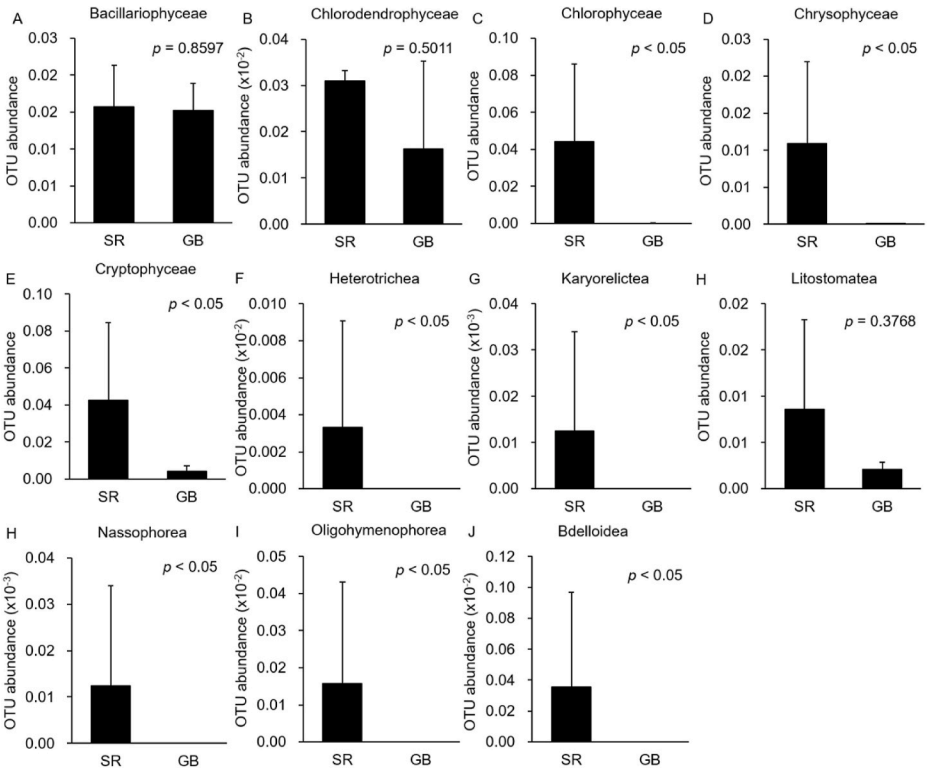

Fig. 5. Normalized OTU abundance in subnetworks of a turquoise module in the Seomjin River (SR) and Gwangyang Bay (GB). A–E. Primary producers including Bacillariophyceae, Chlorodendrophyceae, Chrysophyceae, Cryptophyceae. F–J. Primary consumers including Heterotrichea, Karyorelictea, Litostomatea, Nassophorea, Oligohymenophorea, Bdelloidea.

and dredging in Seomjin River and Gwangyang Bay contributed to the distribution of marine organisms, as benthic organisms were resuspended (Kang et al., 2020b; Lee et al., 2018). This study also revealed the concomitant presence of phytoplankton and fish (Supplementary Table 3) as well as the networking from phytoplankton to fish (Supplementary Table 3), but the connections were rejected due to low significant correlations. Despite the ability of 18S rRNA to detect eukaryotes and monitor eukaryotic plankton (Amaral-Zettler et al., 2009; Djurhuus et al., 2018; Tragin et al., 2018), other gene markers such as 12S rRNA or COI have been prioritized for fishery monitoring (Andruszkiewicz et al., 2017; Fraija-Fernández et al., 2020); thus, multiple gene barcodes can be utilized to increase organism detection rates across the life domain. It is important to note that trophic interactions of concomitantly presenting taxon distinguishing from different trophic levels should be performed carefully because two taxa belonging to different trophic levels from the same location does not necessarily indicate a prey–predator relationship (Blanchet et al., 2020) altough the WGCNA – Cytoscape analysis applied to this study alleviated the limitation as this approach accepts a soft-threshold Pearson's correlation (Langfelder and Horvath, 2008).

3.2. Potential trophic interactions

Complex planktonic food webs were initially constructed and include phytoplankton, zooplankton, crustaceans, and benthic organisms. Using a weight of 0.5, two potential planktonic food webs were identified. Cryptophyceae were the most significant in one subnetwork, and Bacillariophyceae were the most significant in another subnetwork.

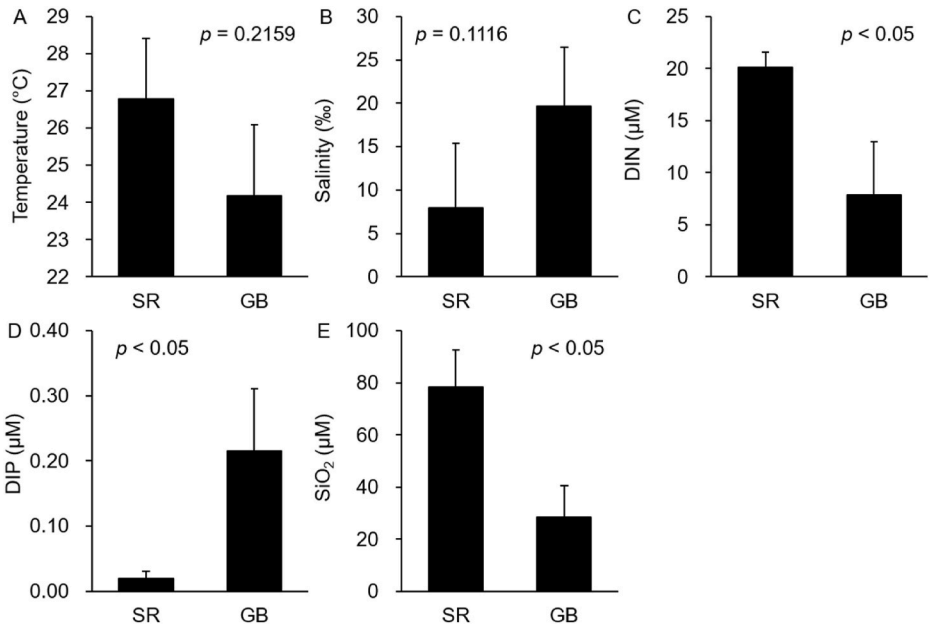

Fig. 6. Environmental variables in the Seomjin River (SR) and Gwangyang Bay (GB). A. Temperature, B. Salinity, C. Dissolved inorganic nitrogen (DIN), D. Dissolved inorganic phosphate (DIP), and E. Silicate (SiO_2).

A close relationship among Cryptophyceae and ciliates has been known for decades (Oakley and Taylor, 1978; Pierce and Turner, 1992), showing that ciliates are a major predator of Cryptophyceae (Posch et al., 2015). Ciliates such as *Mesodinium rubrum* are selective grazers on Cryptophyceae (Johnson et al., 2018), and *Mesodinium* efficiently utilizes algal organelles such as chloroplast–mitochondrial complexes that are obtained from engulfed Cryptophyceae (Kim et al., 2016). A primary consumer, Litostomatea, including *Mesodinium* spp. (Supplementary Table 4), was abundant across sampling stations. Litostomatea was directly connected to Cryptophyceae (Supplementary Fig. 3), although this taxon was closely connected with the large phytoplankton–based planktonic food web (Fig. 3). This indicates that Litostomatea may play a key role as main grazers in the planktonic food webs in the study region.

In our study, gene significances of Cryptophyceae associated with DIN and SiO_2 were highest among the major taxa in the turquoise module. Moreover, strong relationships among Cryptophyceae and ciliates such as Heterotrichea, Karyorelictea, and Nassophorea were detected via WGCNA; the dominance of these ciliates co-occurred with that of Cryptophyceae in Seomjin River, which is the main source of DIN and SiO_2 in the study region. This may suggest that the nutritional status of Seomjin River, such as the abundance of DIN, supported the dominance of Cryptophyceae, thereby promoting ciliate populations. To the best of our knowledge, studies on Heterotrichea, Kryorelictea, and Nassophorea have been conducted with a focus on morphologic and phylogenic descriptions (Taher et al., 2020; Yan et al., 2016; Zhang et al., 2014), and their physiological characteristics and ecological roles have rarely been investigated. Our study distinctly elucidated potential relationships among ciliates and Cryptophyceae. Because 16S rRNA gene markers were not metabarcoded, prokaryotes were not considered in this study. Thus, associations between ciliates and prokaryotes through Cryptophyceae were not explicitly shown (Ferrantini et al., 2009). Given that Cryptophyceae are bacterivorous and comprise a main carbon transfer pathway from bacteria to higher trophic levels (Grujcic et al., 2018; Yoo et al., 2017), and ciliates often exhibit symbiotic relationships with bacteria (Ferrantini et al., 2009), applying the 16S rRNA gene marker will improve the accuracy and detail of planktonic food webs in the future.

WGCNA with network visualization also detected the Bacillariophyceae-based planktonic food web structure with Bdelloidea (rotifers), Litostomatea, and Oligohymenophorea as primary consumers. The coexistence of diatoms and bacteria may be associated with the presence of symbiont ciliates such as Oligohymenophorea (Amin et al., 2012; Fokin et al., 2019); a variety of trophic types in Litostomatea functioning as carnivorous or endosymbiotic organisms suggests a potential association with other trophic organisms as well (Gao et al., 2008; Vďačný et al., 2011). Bacillariophyceae were highly correlated with DIP, which is abundant in Gwangyang Bay compared to that in Seomjin River, indicating that the Gwangyang Bay-originated DIP yielded the dominance of Bacillariophyceae and further affected the trophic interaction with organisms in other trophic levels. Owing to the coexistence of Bacillariophyceae and Litostomatea as well as their dominance, the potential trophic interaction between the two may play a key role in supporting food webs in the Gwangyang Bay ecosystem. Moreover, the physiological responses of Litostomatea to the presence of Bacillariophyceae have not been investigated. eDNA metabarcoding with 16s rRNA gene markers will promote the construction the microbial food web structure in the study region for both Cryptophyceae-based and Bacillariophyceae-based planktonic food webs.

4. Future directions

eDNA has been applied to various aquatic research approaches, including predicting anthropogenic pollution in rivers (Li et al., 2018), determining responses of ecological communities to global changes (Gallego et al., 2020), and controlling and mitigating harmful algal blooms (Liu et al., 2020); in this study, an 18S rRNA gene barcode-applied eDNA technique effectively revealed a food web structure, illustrating the roles of primary producers (phytoplankton, green-algae) and primary consumers (micro- and macro-zooplankton, benthic organisms, crustaceans, etc.). Targeting V9 region of the 18S rRNA gene may be more suitable when focusing on eukaryotic plankton communities (Tragin et al., 2018). Utilizing multiple gene markers will strengthen a comprehensive understanding of aquatic food webs across the life domain (Djurhuus et al., 2020). Particularly, the role of bacteria in microbial food webs requires the use of the 16S rRNA gene barcode to develop accurate food webs (Pomeroy et al., 2007). One limitation of

food web research using eDNA metabarcoding is that carbon fluxes through the trophic levels are not explicitly recognizable. However, merging approaches, such as stable isotope analysis and flow cytometry analysis, can elucidate the amount of carbon transport through food webs. In addition, seasonal shifts in planktonic food webs in response to environmental variations and the function of cryptic species in planktonic food webs that are scarcely identified through conventional approaches can be assessed using eDNA metabarcoding (Djurhuus et al., 2020; Uchii et al., 2016). For decades, planktonic food web models at global and regional scales (Forest et al., 2011; Stock et al., 2014) and sequence read abundance–based models (Kumar et al., 2019) have been simulated; however, the link of eDNA to planktonic food web models has been rarely explored. Our study will provide fundamental information to develop eDNA-based planktonic food web models in the future.

CRediT authorship contribution statement

Y. Kang: Conceptualization, Data Analysis, Writing. I.S. Kwak and C. K Kang: Funding Acquisition.

Data availability statement

Data related to this study are available in the supporting information including OTU read counts and indexes, and supplementary figures.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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