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A Study on the Possibility of Early Warning for *Cochlodinium polykrikoides* Blooms, Using Molecular Methods

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Abstract: The occurrence of HABs is known to be affected by multiple environmental factors such as temperature, salinity, nutrients, light, water chemistry, and biological activity. This study aimed to study the possibility of early warning for *Cochlodinium polykrikoides* blooms in southern Korean waters using qPCR and metabarcoding analysis. We were able to detect *C. polykrikoides* via a sensitive technique, and confirmed the early warning potential of *C. polykrikoides* by quantitative PCR. To identify the microbial organisms related to the bloom caused by *C. polykrikoides*, metabarcoding analysis was conducted using 18 water samples collected from bloom sites in southern Korean waters between 2018 and 2019. Although there was no single group that could explain the successive blooms of *C. polykrikoides*, four taxa, including Gamma proteobacteria (0.588, $p < 0.01$), Verrucomicrobia (0.874, $p < 0.01$), Cyanobacteria (0.975, $p < 0.01$), and Cryptophyta, were related to *C. polykrikoides* blooms. When the HABs that occurred were caused by *C. polykrikoides*, although there were sites where the cells of *C. polykrikoides* were not detected, similar functions of the microbial communities operated in all southern Korean coastal waters. We identified that the phytoplankton community structure was divided into three or four, using metabarcoding analysis by sampling years.

Keywords: *Cochlodinium polykrikoides*; metabarcoding; HABs; qPCR; coastal water



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1. Introduction

Phytoplankton plays an essential role in the marine ecosystem by creating organic compounds from carbon dioxide dissolved in water, which supports biological and geochemical processes [1–3]. However, several phytoplankton taxa are responsible for harmful algal blooms (HABs), with severe impacts on human health and marine ecosystems in many countries and also economic hardship in affected regions. *Cochlodinium polykrikoides* (*C. polykrikoides*), for example, is one of the major dinoflagellates responsible for HABs in Korean coastal waters [4], where massive aquaculture farms are located. The most intense *C. polykrikoides* blooms occur in the southern coastal waters adjacent to the cities of Geoje, Tongyeong, and Yeosu in Korea. *C. polykrikoides* has been introduced to these areas, and large-scale damage to aquaculture has occurred. Since the 2000s, annual losses in the Korean aquaculture industry caused by *C. polykrikoides* blooms have ranged from USD 4 to 18.6 million [3]. It is of great necessity to forecast the outbreaks of blooms, in order to take appropriate measures. To forecast the outbreak of HABs, the National Institute of Fisheries Sciences (NIFS) has conducted regular monitoring of cell counts at designated stations along the coastal waters in the Korean peninsula every March to November since 1981 [5]. However, the traditional counting method has often lacked sensitivity; it has

missed HABs in cases where abundance is low, and at times been inaccurate due to the morphological similarity of HABs to other species, and/or changes in morphology by the addition of fixatives [6,7]. Molecular detection of HABs including the quantitative PCR (qPCR) method, is an alternative to traditional methods and provides rapid, sensitive, and accurate detection and quantification of HABs and toxic microalgae [8,9].

The occurrence of HABs is known to be affected by multiple environmental variables, such as temperature, salinity, nutrient, light, water chemistry, and biological factors [10,11]. However, there is still no clear understanding of the mechanism between those abiotic factors and the onset and explosive multiplication of the blooms [12]. In addition to abiotic factors, bacterial compositions are also known to be involved in the dynamics of HABs caused by particular algal species [13,14]. Bacteria support the growth of HABs by the recycling of nutrients, and phytoplankton produces various algal-derived products, which affect bacterial growth rate and community composition [15,16]. Since there are complicated interactions among microorganisms and algal growth [17–19], toxin production [20], algicidal activity [21–23], and bloom termination [24,25], study of these interactions between them can provide useful information about the dynamics of algal blooms.

Molecular-based methods have been demonstrated capable of identifying the community structures and diversity of microbial organisms. Their development has made possible DNA metabarcoding analysis based on massive sequencing of the amplicon, which is related to the occurrence of HABs [26–28]. Several previous studies have shown that bacterial communities appear to be associated with HABs. The relative abundances of bacteria changed in response to the different phases of *C. polykrikoides* growth in culture [26]. Alphaproteobacteria and Flavobacteria, which exist in high proportions and are present from the early bloom stage to the late bloom stage, correlated positively with *C. polykrikoides*, but gamma proteobacteria were negatively correlated with *C. polykrikoides* [11]. However, Shin et al. [27] reported that Gammaproteobacteria and Alphaproteobacteria were the main components of the bacterial community associated with *C. polykrikoides* in culture. The interaction between bacteria and phytoplankton can be analyzed through metabarcoding analysis with multiple simultaneous markers [29].

In this study, we performed qPCR and metabarcoding to analyze 16S rDNA and p23S rDNA sequenced from 18 samples collected from southern Korean coastal waters. Our objectives included: (i) comparing cell counting (traditional method) and copy number of *C. polykrikoides* rDNA (molecular method) for the detection and quantification of *C. polykrikoides*; (ii) identifying specific and repetitive microbial (particularly bacterial) and phytoplankton communities in *C. polykrikoides* bloomed waters in 2018 and 2019, and (iii) identifying microbial species potentially related with *C. polykrikoides*, which could be utilized for early warning.

2. Materials and Methods

2.1. Sample Collection and Genomic DNA Extraction

Sea water samples were collected from nine sites in southern Korean coastal waters during harmful algal blooms (HABs) that occurred from 31 July to 2 August 2018 and from 27 August to 30 August 2019 (Figure 1). Two surface water samples (100 mL × 2) were collected in each site, each of which was further filtered through a GH membrane with 0.45 µm pore size (Pall Corporation, New York, NY, USA). Filtered membranes were directly stored at −70 °C until used for genomic DNA extraction. Genomic DNA was extracted from the membrane using a DNeasy® plant mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Isolated DNA was quantified using an ND-1000 nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at −70 °C until used for library construction.

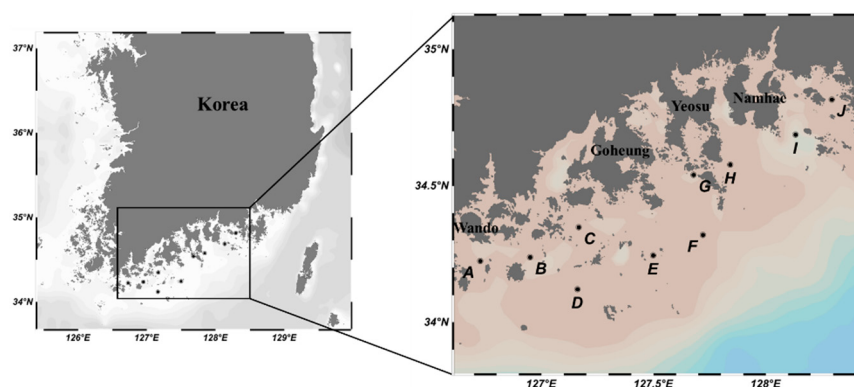


Figure 1. Sampling sites for the metabarcoding analysis of *C. polykrikoides* bloom in the Southern Korean coastal waters.

2.2. Quantitative PCR for Microbial Communities, Phytoplankton Communities, and *C. polykrikoides*

qPCR was adopted for the quantitative analysis of bacterial and phytoplankton abundance. The standard curve for each taxon was constructed using the partial insulin-like growth factor 1 (Igf1) sequences from *Mus musculus*, flanking 16S universal primers or p23S universal primers, respectively [30,31] (Table 1). The amplified products stained with Dyne Loading STAR (Dynebio, Seongnam, Korea) were confirmed by 1% agarose gel electrophoresis. The amplicons with the expected size ranges (444 bp for 16S and 410 bp for p23S) were purified DNA fragments, and were cloned into a pGEM[®]-T easy vector (Promega, Madison, WI, USA) then transformed into *Escherichia coli* DH5 α competent cells. The plasmids were then extracted using an AccuPrep[®] Plasmid Mini Extraction Kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. The concentrations of the plasmids were measured using a Quantus TM Fluorometer (E6150, Promega, Madison, WI, USA), and were sequenced (Macrogen, Daejeon, Korea). The standard curve for the quantitative PCR (qPCR) was constructed using the serially diluted plasmid harboring the target gene (from 10^{-1} ng/uL to 10^{-9} ng/uL). The relative copy numbers of microorganisms and phytoplankton were measured by qPCR, with two different universal primer sets; 16S universal primers for total microorganisms and p23S universal primers for phytoplankton [30,31]. Thermal cycling was performed with a DNA Engine Chromo 4 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) under the following conditions: initial template denaturation (94 °C for 3 min); 35 amplification cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s) and a final extension step at 72 °C for 3 min. Each PCR mixture (20 μ L) contained 10 μ L of 2 X SYBR Green premix Ex Taq II (Takara Bio Inc., Shiga, Japan), 4 μ L of template, 1 μ L of forward and reverse primers (10 pmol), and 4 μ L of PCR-grade water.

Cochlodinium polykrikoides was detected in water samples, using quantitative PCR.

qPCR was performed using the MIC Real-Time PCR (MIC, Bio Molecular Systems, Upper Coomera, Australia). The following reagents were added for a 20 μ L reaction mixture: 10 μ L of Premix Ex Taq for real-time PCR (Takara Bio), forward and reverse primers at a final concentration of 200 nM, TaqMan probe at a final concentration of 150 nM, 2 μ L of template DNA, and PCR-grade water to a final volume of 20 μ L. The thermal cycling conditions consisted of 2 min at 95 °C followed by 40 cycles of 10 s at 95 °C and 45 s at 60 °C. Fluorescence data were collected at the end of each cycle, and the cycle threshold line was determined automatically by the instrument. Ten-fold serial dilutions of each plasmid containing *C. polykrikoides* rDNA were used to construct standard curves. These standard curves showed a strong linear correlation between log (plasmid DNA copy number) and CT values (correlation coefficient, R^2 of 0.998) for *C. polykrikoides*.

Table 1. Primer list for qPCR and metabarcoding analysis.

Primer	Sequences (5' to 3')	References
16S universal primer F 16S universal primer R	CCTACGGGNGGCWGCAG GACTACHVGGGTATCTAATCC	[30]
p23S universal primer F p23S universal primer R	GGACARWAAGACCCTATGMAG AGATYAGCCTGTTATCCCT	[31]
Nex 16SF Nex 16SR	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGG NGGCWGCAG GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGT ATCTAATCC	
Nex p23SF Nex p23SR	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGACARWAAGACCC TATGMAG GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGATYAGCCTGTTATCCCT	
CPLSUF CPLSUR CPLSUP (probe)	GCCGAGGATACCTGCAAAG TGTCAGGACCCACGATCA FAM-CTCACATGATCAGCGCCGAGTACTAA-BHQ1	[32]

2.3. Library Preparation and Sequencing

The library for MiSeq sequencing was constructed by the Nextera XT kit (Illumina, San Diego, CA, USA) indexing the amplicons generated by the universal primers: 16S universal primers for the microbial community [30], p23S universal primers for phytoplankton community [31] (Table 1). The expected amplicon size for each universal primer set was 580 bp for microbial communities (16S universal primers), and 540 bp for phytoplankton communities (p23S universal primers). PCR conditions for each universal primer set were the same as in the previous respective studies [30,31]. Amplicons were purified using an AccuPrep[®] gel purification Kit (Bioneer, Daejeon, Republic of Korea), and indexing of each amplicon was conducted according to the manufacturer's instructions. The quality and quantity of the constructed libraries were measured using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and MiSeq sequencing was conducted with the MiSeq 600-cycle Reagent Kit v3 (Illumina, San Diego, CA, USA).

2.4. Bioinformatics Analysis of Metabarcoding Data

The raw reads with low quality (QV < 20) and those shorter than 100 nucleotides were removed using the CLC Genomic Workbench v.8.0 (CLC Bio, Aarhus, CA, USA). The reads longer than 7 bp overlapping sequences without any mismatches were pair-assembled. The merged reads with the expected size ranges (400–500 bp for 16S and 350–450 bp for p23S) were selected and their primer sequences were trimmed using Mothur software v.1.35.0 [33]. The obtained merged reads were clustered into operational taxonomic units (OTUs) at 99% similarities, and chimeras were removed using UCHIME software v.8.1 [34]. OTUs with lower than 10 read numbers and below 0.1% of the total read numbers were eliminated from further analysis. The species name for each OTU was assigned by a similarity search using a blast search with BLAST +2.2.30 on the NCBI non-redundant nucleotide database (<ftp://ftp.ncbi.nlm.nih.gov/blast/db/>; accessed on 21 May 2020) [35]. For each OTU with higher than 98% sequence identity in the database, the top-scored species name was assigned. The OTUs with 90–98% identities in the database were described as “Genus name with highest score” followed by “sp.” OTUs with less than 90% identity were classified as “Unknown”.

2.5. Statistical Analysis

Non-metric multidimensional scaling (nMDS) analysis was performed based on the Bray–Curtis similarity with group average on square-root transformed abundance data, to compare the microbial and phytoplankton communities among different samples, using the PRIMER-E software v. 6 (Primer-E Ltd., Plymouth, UK). Canonical correspondence

analysis (CCA) was used to link variations in microbial communities to environmental factors, using Canoco 5 software (Microcomputer Power, Ithaca, NY, USA). Functional analysis was performed in which bacterial gene functions were predicted from 16S rRNA gene-based microbial compositions, using the PICRUST algorithm to make inferences from KEGG pathways. Normalized values were calculated using Mothur software v.1.35.0 [36], as the measured abundance divided by the number of each copy number predicted for each OTU. The diversity indices for each sample were calculated using Mothur software and PRIMER version 5 (PRIMER-E Ltd., Lutton, United Kingdom), based on the normalized values. Analysis of similarity (ANOSIM), and permutational multivariate analysis of variance (PERMANOVA) were performed to determine differences in microbial and phytoplankton communities among samples and sampling years. These analyses were conducted on transformed data by using the Bray–Curtis similarity. The correlations of *C. polykrikoides* copy numbers to environmental parameters were analyzed using the Pearson correlation coefficient. Correlation analysis was performed based on the copy numbers of the *C. polykrikoides* rDNA with microbial OTUs and phytoplankton OTU, generated by metabarcoding analysis.

3. Results

3.1. Environmental Parameters

The temperature, salinity, nutrient concentration, and chlorophyll-a (Chl-a) content were measured at each sample site (Table 2). In 2018, the surface water temperature ranged from 22.78 to 27.52 °C, with an average of 25.78 ± 1.78 °C, which was 3.26 °C lower than in 2019 (26.04 ± 4.32 °C). A slightly higher average salinity ($32.60 \text{ PSU} \pm 0.41$) was identified in 2018, compared to its value ($31.94 \text{ PSU} \pm 0.50$) in 2019. The average ammonium (NH₄⁺) concentration in 2019 was 0.70 ± 0.46 (μM), which was approximately 4.6 times higher than its value in 2018 (0.15 ± 0.16 μM). The mean nitrate (NO₃[−]) concentrations were 0.49 ± 0.29 μM in 2018 and 0.94 ± 0.66 μM in 2019, respectively. The average DIN in 2019 (14.19 ± 11.47) was 15-fold that in 2018 (0.92 ± 0.54), while the average chlorophyll a concentration was higher in 2018 (10.26 ± 23.17) than in 2019 (5.44 ± 6.11 ug/L).

Table 2. Environmental parameters during *C. polykrikoides* blooms in Korean coastal waters.

Year	Station	Temp. °C	Salinity PSU	NH ₄ -N μM	NO ₂ -N μM	NO ₃ -N μM	DIN μM	PO ₄ -P μM	SiO ₂ -Si μM	Chl-a ug/L
2018	A	22.78	33.04	0.22	0.03	0.11	1.00	0.01	3.59	2.98
	B	23.03	32.97	0.42	0.05	0.36	1.00	0.11	5.41	5.12
	C	26.06	32.73	0.15	0.02	0.56	1.00	0.04	11.65	2.66
	D	27.39	31.66	0.11	0.03	1.00	2.00	0.03	5.32	0.22
	E		32.66	0.37	0.02	0.55	1.00	0.05	4.89	1.24
	H	27.52	32.46	0.00	0.02	0.56	0.58	0.12	7.90	67.47
	I	27.16	32.50	0.00	0.00	0.12	0.12	0.01	5.40	0.28
	J	26.09	32.90	0.00	0.03	0.67	0.70	0.06	9.93	2.08
2019	A	22.16	32.19	1.32	0.24	2.24	18.01	0.29	4.48	2.00
	C	23.80	32.20	0.97	0.05	0.88	19.28	0.06	2.99	3.04
	D	22.83	30.76	0.88	0.02	1.08	17.69	0.08	2.39	1.10
	E	33.46	31.71	0.85	0.01	0.85	34.39	0.00	3.33	16.24
	F	25.18	32.18	1.06	0.02	1.14	18.99	0.05	3.52	2.82
	G	33.46	31.71	0.82	0.01	1.18	17.61	0.02	3.15	1.70
	H	23.72	32.19	0.40	0.27	1.03	1.69	0.43	10.97	15.97
	I	24.79	32.14	0.00	0.01	0.06	0.06	0.16	5.30	2.04
	J	25.00	32.42	0.00	0.00	0.00	0.00	0.24	4.82	4.05

3.2. Quantitative Analysis of Microorganisms, Phytoplankton, and *C. polykrikoides*

The quantitative analysis of the total microorganisms, phytoplankton, and *C. polykrikoides* was conducted by qPCR (Table 3). The average copy number of the total microorganisms in 2019 was $899,913.23 \pm 331,554.94$ copies/mL, which was 1.76-fold higher than in 2018

(512,004.86 $\pm$ 336,315.44 copies/mL). The highest copy number of microorganisms was identified at J in 2018 (1,179,845.14 copies/mL) and J in 2019 (1,556,795.40 copies/mL), while the lowest numbers were detected at D in 2018 (214,395.14 copies/mL) and at G in 2019 (463,332.43 copies/mL), respectively. The average copy number of phytoplankton in 2019 (93,034.61 $\pm$ 30,967.44 copies/mL) was 1.08-fold higher than in 2018 (86,089.62 $\pm$ 50,155.64 copies/mL). Compared to the ratio in 2018 (5.95), the average ratio of total microorganisms to phytoplankton was 9.67 in 2019, which was approximately 1.63-fold higher than in 2018 (Table 3). The average copy numbers of *C. polykrikoides* were 2,102,482.86 $\pm$ 2,590,794.70 copies/mL and 30,342,731.76 $\pm$ 81,746,202.15 copies/mL in 2018 and 2019, respectively—14.43-fold higher in 2018 than in 2019. However, high degrees of regional differences in copy numbers were observed.

Table 3. Copy numbers of *C. polykrikoides* and microorganisms and phytoplankton according to quantitative PCR.

Year	Station	Microorganisms (Copies/mL)	Phytoplankton (Copies/mL)	<i>C. polykrikoides</i> (Copies/mL)	<i>C. polykrikoides</i> (Cells/mL)	<i>C. polykrikoides</i> (Copies/Cell)
2018	A	373,039.66	75,191.72	226,496.55	0.00	-
	B	410,109.67	131,904.62	143,526.43	0.00	-
	C	693,074.64	129,993.01	374,770.09	7.50	49,969.35
	D	214,395.18	12,760.27	5538.65	0.00	-
	E	362,322.25	69,390.50	1,472,837.75	12.00	122,736.48
	F	668,274.68	98,505.39	2,391,768.23	26.40	90,597.28
	H	28,896.15	4428.02	6,740,167.32	1200.00	5616.81
	I	678,086.36	114,823.30	103,142.69	0.10	1,031,426.90
	J	1,179,845.14	137,809.77	5,367,153.82	250.00	21,468.62
Ave.		512,004.86 $\pm$ 336,315.44	86,089.62 $\pm$ 50,155.64	1,869,489.06 $\pm$ 2,522,253.95	166.22 $\pm$ 396.03	220,302.57 $\pm$ 399,725.81
2019	A	1167,211.57	134,914.67	867,252.99	1.38	628,444.20
	C	1,082,885.87	148,788.00	3,089,347.13	450.00	6865.22
	D	738,466.68	82,077.02	36,526.34	0.00	-
	E	581,862.02	70,706.48	248,181,801.83	1240.00	200,146.61
	F	843,365.30	106,511.24	4,958,103.49	7.50	661,080.47
	G	463,332.43	60,937.70	5,726,129.65	0.00	-
	H	913,077.22	81,334.28	9,081,941.97	24.00	378,414.25
	I	752,222.62	64,549.08	1,143,482.48	0.30	3,811,608.27
	J	1,556,795.40	87,493.06	0.00	0.00	-
Ave		899,913.23 $\pm$ 331,554.94	93,034.61 $\pm$ 30,967.44	30,342,731.76 $\pm$ 81,746,202.15	212.40 $\pm$ 443.89	947,759.83 $\pm$ 1,425,071.09

In 2018, the copy numbers of *C. polykrikoides* ranged from 5538.65 copies/mL at D to 6,740,167.32 copies/mL at H (Table 3). Regional differences in the copy numbers in 2019 were higher than in 2018, ranging from zero at J to 248,181,801.83 copies/mL at E (Table 3). The copy numbers of *C. polykrikoides* obtained by qPCR were further compared with the direct cell counts (Figure 2).

The qPCR results exhibited a much higher sensitivity in quantification as well as qualification. The microscopic observation failed to detect *C. polykrikoides* cells at six sites, while qPCR detected them at all the examined sites except one, at which microscopic observation also found no cells (Table 3). In addition, the cell-counting methods failed to detect any cells even at sites where millions of rDNA copies were identified. The number of copies per cell varied from 5616.81 to 1,031,426.90 copies/cell in 2018 and from 6865.22 to 3,811,608.27 copies/cell in 2019. The average copy numbers per cell were 11,246.93 $\pm$ 6368.82 copies/cell (2018) and 947,759.84 $\pm$ 1,425,071.09 copies/cell (2019). There was a low degree of correlation ($r^2 = 0.4676$) between the direct cell counts and copy numbers according to qPCR (Figure 2C). When those values for 2018 and 2019 were separately analyzed, a much higher degree of correlation was identified in each year

($r^2 = 0.7065$ in 2018 and $r^2 = 0.8765$ in 2019, respectively), suggesting different ribosomal DNA copy numbers among two *C. polykrikoides* strains (Figure 2A,B). The correlations of the copy numbers of *C. polykrikoides* to the environmental parameters were analyzed using the Pearson correlation coefficient (Table 4). The abundance of *C. polykrikoides* copies showed a positive correlation to the temperature (correlation coefficient = 0.605, $p < 0.01$), DIN (correlation coefficient = 0.646, $p < 0.01$), and Chl-a (correlation coefficient = 0.752, $p < 0.01$). Phytoplankton was also positively correlated with the total microorganisms and Chl-a (Table 4).

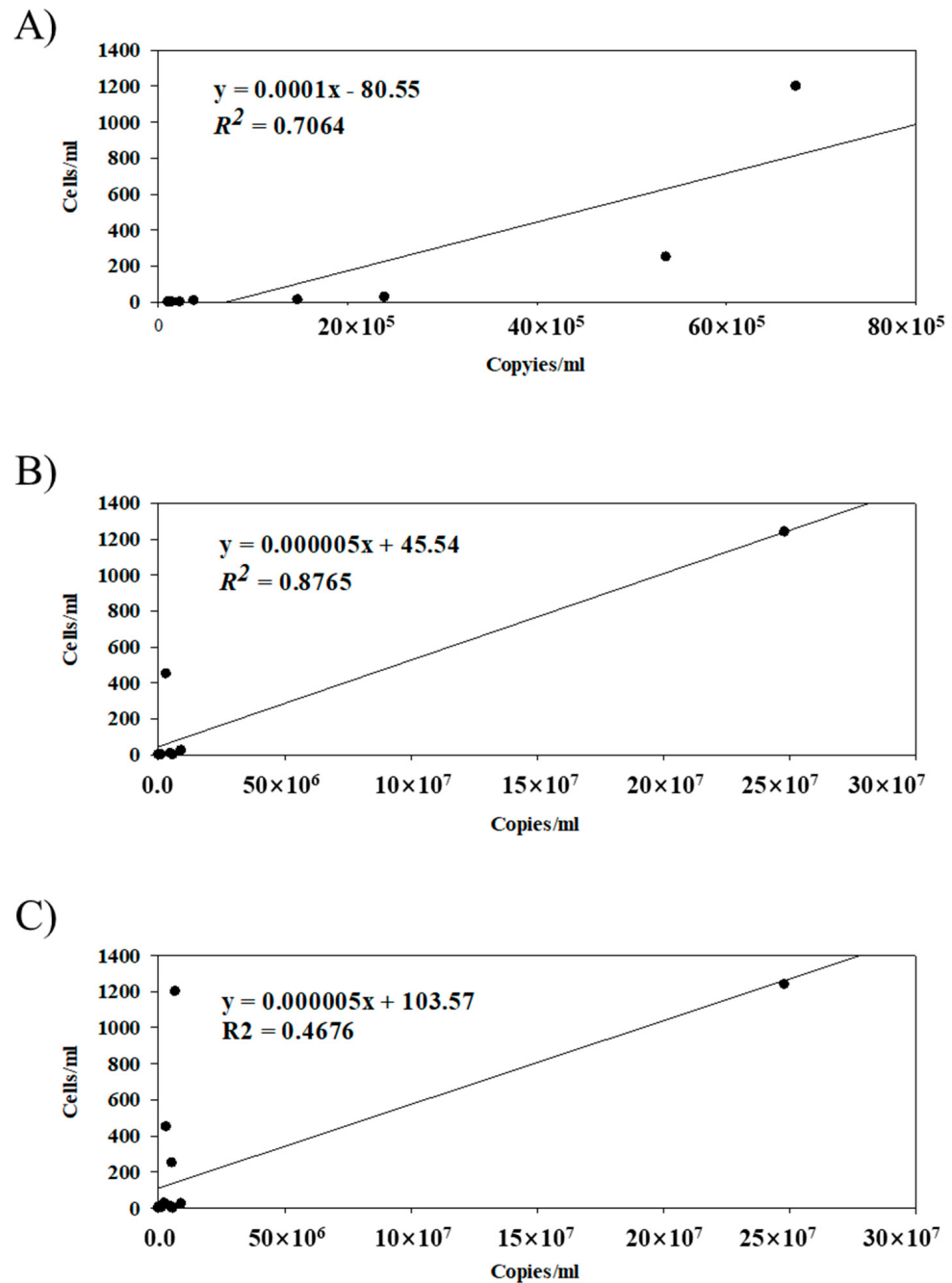


Figure 2. A linear relationship between copy numbers and cell numbers for *C. polykrikoides*. (A) 2018, (B) 2019, (C) 2018 and 2019.

Table 4. Pearson correlation coefficient between environmental variables and *C. polykrikoides* rDNA copies (* $p < 0.05$; ** $p < 0.01$).

	<i>C. polykrikoides</i>	Microorganism	Phytoplankton	Temperature	Salinity	NH ₄ -N	NO ₂ -N	NO ₃ -N	DIN	PO ₄ -P	SiO ₂ -Si
Microorganism	−0.082										
Phytoplankton	−0.124	0.632 **									
Temperature	0.605 **	−0.311	−0.13								
Salinity	−0.247	0.013	0.296	−0.261							
NH ₄ -N	0.241	0.189	0.271	−0.016	−0.461						
NO ₂ -N	−0.112	0.293	0.35	−0.402	0.007	0.361					
NO ₃ -N	0.069	0.131	0.015	0.005	−0.455	0.792 **	0.609 **				
DIN	0.646 **	0.124	0.174	0.386	−0.589 *	0.845 **	0.047	0.591 *			
PO ₄ -P	−0.205	0.453	0.382	−0.439	0.005	0.056	0.808 **	0.253	−0.188		
SiO ₂ -Si	−0.187	0.054	0.176	−0.089	0.443	−0.53	0.309	−0.129	−0.562 *	0.353	
Chl- <i>a</i>	0.752 **	−0.407	0.599 *	0.195	0.065	−0.205	0.024	−0.055	−0.075	0.158	0.266

3.3. Microbial Community Structure during the *Cochlodinium* Bloom

The microbial communities during blooms caused by *C. polykrikoides* were compared (Supplementary Tables S1 and S2). After trimming and clustering the raw reads, a total of 1,206,825 merged reads were obtained using 16S universal primer, with numbers at each site ranging from 25,282 to 129,715 (Supplementary Tables S1 and S2). A total of 367 microbial OTUs were identified from 18 seawater samples, with 99% as the cut-off identity, and were further classified into 15 phyla, including Actinobacteria, Bacillariophyta, Bacteroidetes, Chlamydiae, Chlorophyta, Cryptophyta, Cyanobacteria, Euryarchaeota, Foraminifera, Haptophyta, Marinimicrobia, Ochrophyta, Planctomycetes, Proteobacteria, and Verrucomicrobia. The composition and relative abundance of the microbial community at the different 18 sampling sites were compared at the phylum level (Supplementary Tables S1 and S2 and Figure 3). Proteobacteria, Bacteroidetes, Actinobacteria, and Cyanobacteria were the major dominant phyla, with proportions ranging from 72.23 (18B) to 99.03% (18J) in the southern Korean coastal waters in this study. Proteobacteria exhibited an inverse pattern compared to Cyanobacteria (Figure 3). The Chao1, Pielou's evenness (J'), Shannon index (H'), and Simpson index ($1-\lambda$) in Korean coastal waters during blooms caused by *C. polykrikoides* ranged from 22.71 to 50.20, from 0.47 to 0.70, from 2.85 to 4.19, and from 0.69 to 0.94, respectively (Table 5). These diversity indices were not significantly different at each sample ($F = 0.03$, $p > 0.05$).

Bray–Curtis cluster analyses of the microbial communities were performed to determine whether there were differences in their structures (Figure 4A). The cluster analysis of the microbial communities showed four main clusters at a similarity level of 60%. Clusters I and II grouped the samples that were collected from the Yoesu coastal waters in 2018 and 2019, respectively. The second cluster (Cluster II) grouped the samples from the two stations located at the leftmost side among the sampling sites (18A, 18B), from 2018. Finally, the fourth cluster (Cluster IV) grouped samples collected in 2019 from the Tongyeong coastal waters. Heatmap plots were constructed to determine significantly different taxa in the microbial communities of the 18 samples. The heatmap analysis of the phyla showed distinct differences among the samples (Figure 5). The samples (except for 18C and 18I) in which millions of copies of *C. polykrikoides* rDNA were detected, including Clusters I and III, showed that the phyla Cyanobacteria were the most significantly different from the other communities (Figure 5). The phyla Verrucomicrobia and Planctomycetes characterized these clusters. The ANOSIM and PERMANOVA analyses of the microbial communities, using the Bray–Curtis dissimilarity measure on the square root-transformed data, indicate that the differences in microbial community structure were dependent on factors associated with the sampling year (Table 6).

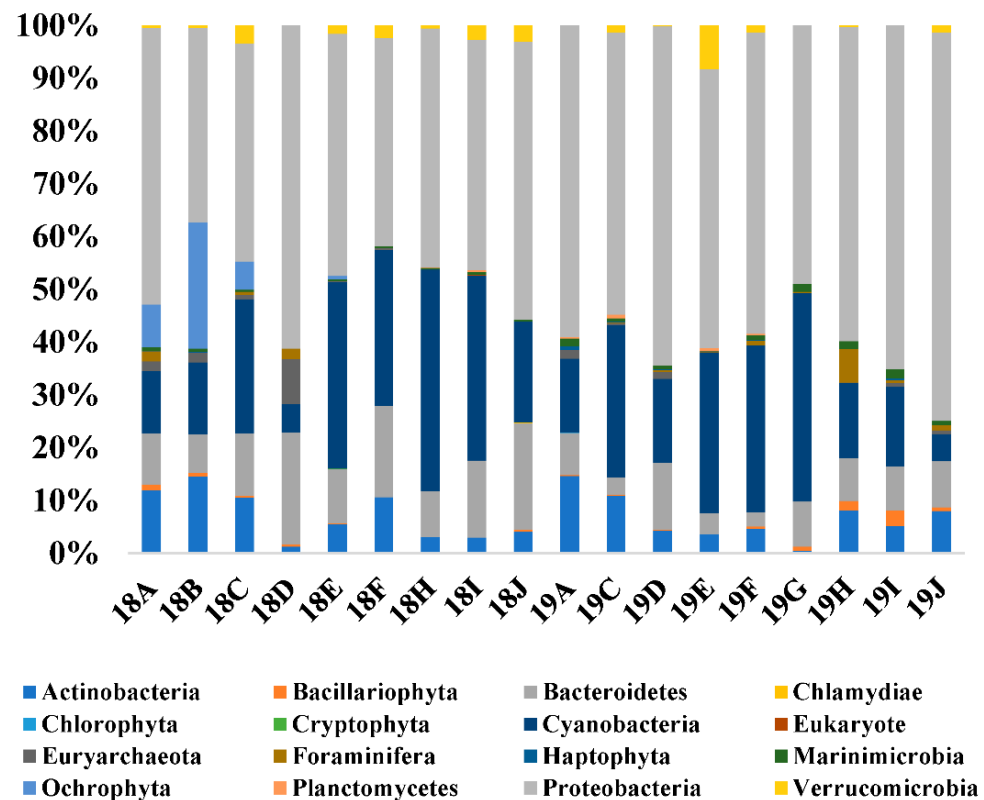


Figure 3. Microbial community structure of samples. Relative abundances of the dominant groups at the phylum level.

Table 5. The diversity indices of microorganisms and phytoplankton.

Year	Station	Microorganisms				Phytoplankton			
		Chao1	Pielou's Evenness (J')	Shannon Index (H')	Simpson Index (1-λ)	Chao1	Pielou's Evenness (J')	Shannon Index (H')	Simpson Index (1-λ)
2018	A	50.2	0.6587	4.154	0.9106	11.35	0.6733	3.256	0.917
	B	39.18	0.6979	4.185	0.9377	8.139	0.6003	2.694	0.8495
	C	36.9	0.6757	4.019	0.9228	10.37	0.611	2.92	0.8904
	D	27.93	0.5303	3.021	0.7385	8.166	0.6651	2.939	0.8485
	E	35.33	0.6375	3.758	0.8977	9.675	0.542	2.538	0.7612
	F	42.78	0.6559	4.044	0.9177	8.69	0.5656	2.575	0.822
	H	38.79	0.5546	3.381	0.8673	10.77	0.5123	2.444	0.733
	I	41.44	0.5689	3.51	0.8759	9.885	0.4516	2.114	0.6819
	J	43.79	0.6404	3.978	0.9086	7.445	0.379	1.675	0.5861
2019	A	39.13	0.5584	3.379	0.8041	10.72	0.732	3.467	0.9392
	C	34.66	0.5391	3.203	0.8265	10.78	0.5934	2.8	0.8068
	D	34.76	0.5233	3.09	0.7703	11.89	0.6461	3.13	0.881
	E	38.43	0.4983	3.02	0.8101	9.294	0.5108	2.331	0.6994
	F	42.12	0.5048	3.113	0.7995	9.043	0.5999	2.738	0.8439
	G	22.71	0.5229	2.889	0.8293	8.107	0.4168	1.852	0.6464
	H	46.58	0.5956	3.73	0.8427	10.27	0.6942	3.257	0.9148
	I	47.23	0.5318	3.332	0.7915	9.808	0.6582	3.038	0.868
	J	41.5	0.4656	2.85	0.688	10.87	0.6673	3.166	0.9135

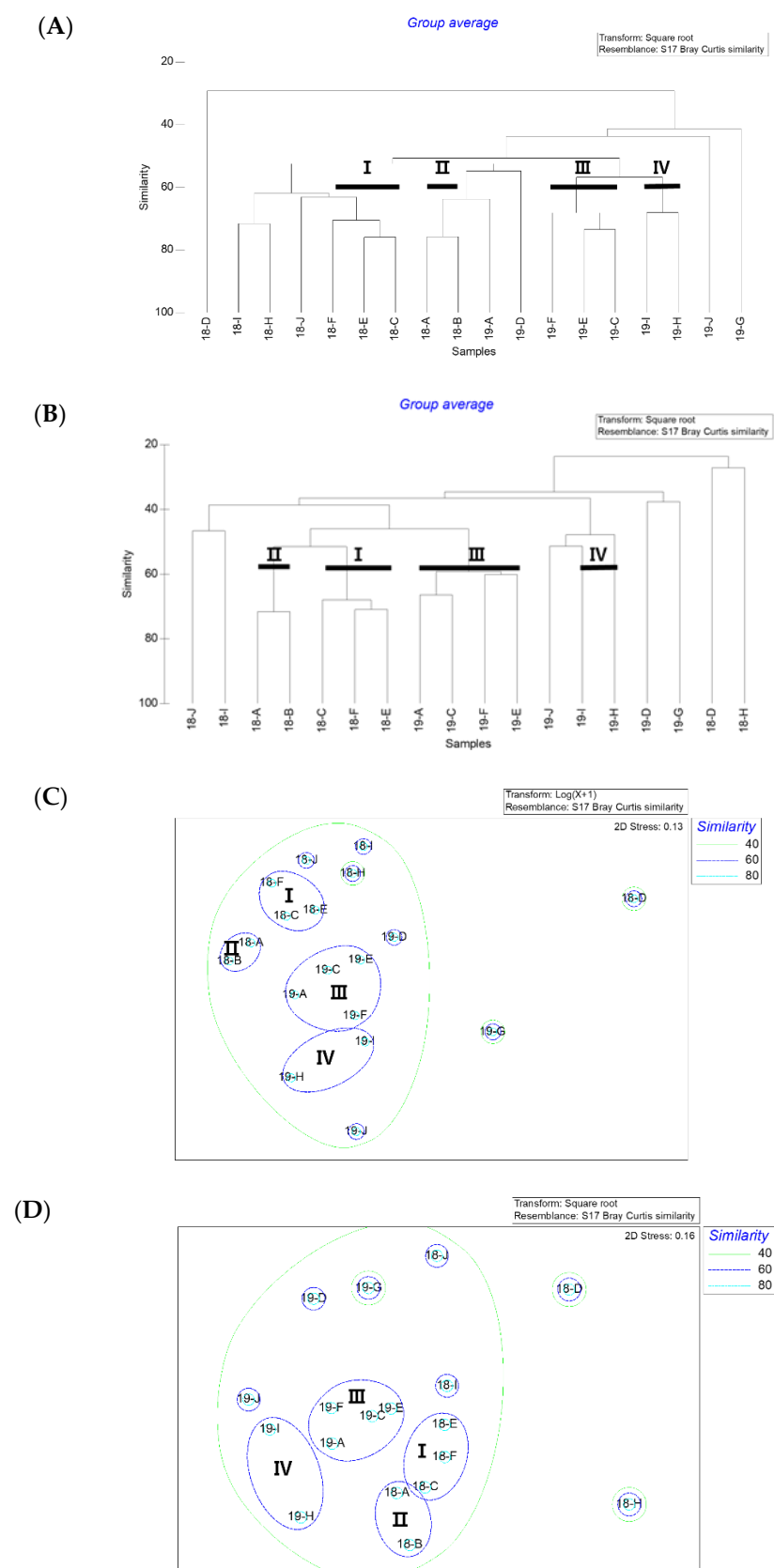


Figure 4. Bray-Curtis similarity-based Cluster at the operational taxonomic Unit (OTU) level illustrating groups ((A): Microbial community, (B): Phytoplankton community) and non-metric multi-dimensional scaling ((C): Microbial community, (D): phytoplankton community) in *C. polykrikoides* bloomed water samples.

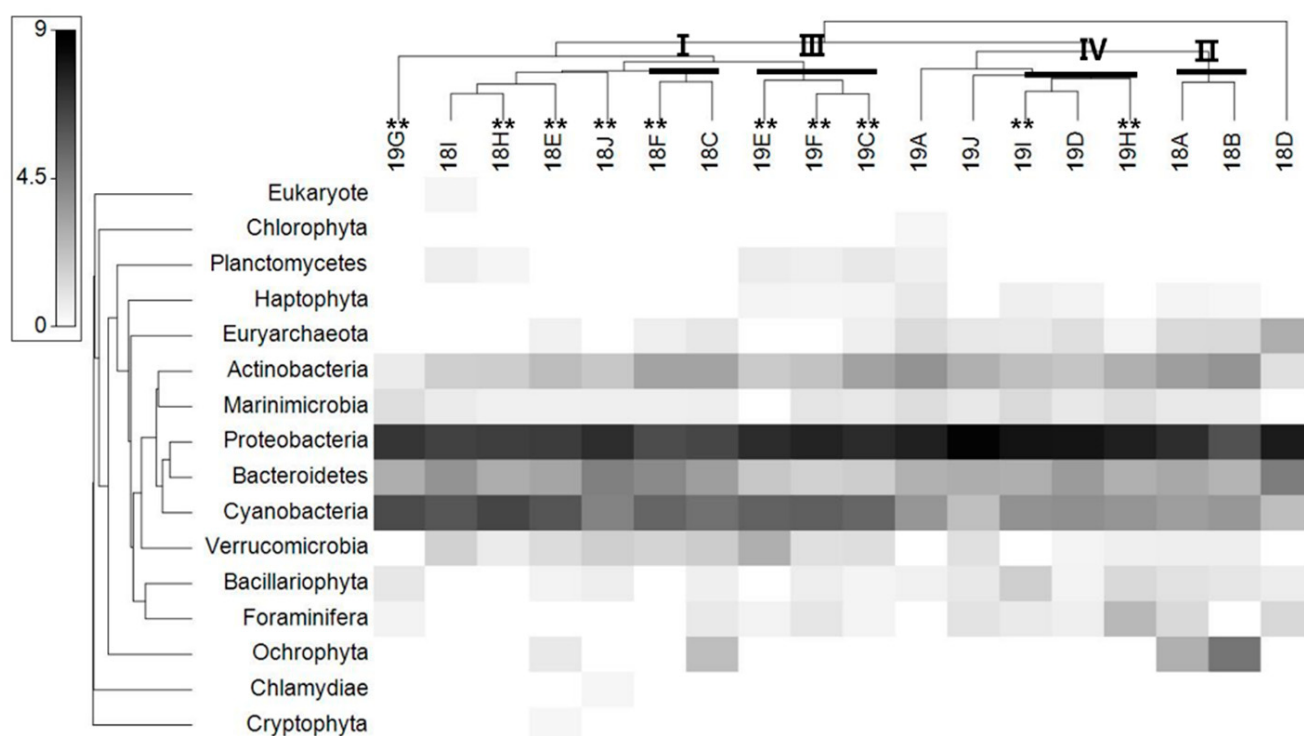


Figure 5. Shade plot illustrating the microbial communities at the phylum level by sites for 18 samples. The plot depicts the relative abundance of phyla within microbial communities. **: >1,000,000 copies/mL (rDNA of *C. polykrikoides*).

Table 6. Analysis of similarities (ANOSIM) and PERMANOVA results for years and stations.

	Microbial Community		Phytoplankton Community	
	ANOSIM (Global R)	PERMANOVA (Pseudo-F)	ANOSIM (Global R)	PERMANOVA (Pseudo-F)
2018 VS. 2019	0.350	3.730	0.392	4.776
R ²	0.001	0.015	0.001	0.005
A, B, C, D, E, F, G, H, I, J	0.109	1.621	0.134	1.649
R ²	0.245	0.011	0.216	0.006

To identify the significant microorganism for *C. polykrikoides* blooms, correlation analysis was performed based on the copy numbers of the *C. polykrikoides* rDNA and microbial OTUs generated by the metabarcoding analysis. In 2018, three microbial OTUs (correlation coefficient ≥ 0.5 and $p < 0.05$), i.e., Cyanobacterium (16SOTU19), Gamma proteobacterium (16SOTU29), and Rhodobacteraceae bacterium (16SOTU92) showed significant correlation with the abundance of *C. polykrikoides*. Additionally, in 2019, five Gamma proteobacteria OTUs (16SOTU29, 70, 211, 265, 274), four Alpha proteobacterium OTUs (16SOTU102, 146, 153, 351), two Flavobacterium OTUs (16SOTU123, 338), Verrucomicrobia bacterium (16SOTU125), Pelagibacteraceae bacterium (16SOTU308), and Planctomycete sp. (16SOTU350) had strong positive correlations with *C. polykrikoides*. However, only four microbial OTUs—Gamma proteobacterium (16SOTU70), Flavobacteriales bacterium (16SOTU123), Verrucomicrobia bacterium (16SOTU125), and Alpha proteobacterium (16SOTU153)—had strong positive correlations with *C. polykrikoides* from among the total microbial OTUs (2018, 2019) (Table 7). Meanwhile, energy metabolism was the main pathway at station H (Figure 6). Neurodegenerative disease-related functions showed higher levels (about 1.5 times higher) at stations where the numbers of *C. polykrikoides* were

comparatively lower. Functions related to the circulatory system and cardiovascular disease showed similar trends. In contrast, the functions of signaling molecules and interactions, the immune system, and the digestive system showed higher levels (about 1.5 times) in samples where *C. polykrikoides* was more abundant (Figure 6).

Table 7. Microbial OTUs strongly correlated with *C. polykrikoides*.

Year	OTU	Correlation Coefficient	<i>p</i>	Description	Accession	Identify (%)	Phylum	Regression Equation	R ²
2018	16SOTU19	0.688	<0.05	<i>Cyanobacterium</i>	KC687149	100	Cyanobacteria	$y = (2 \times 10^{-8})x + 0.0092$	0.4733
	16SOTU29	0.698	<0.01	<i>Gamma proteobacterium</i>	MH077347	100	Proteobacteria	$y = (1 \times 10^{-7})x + 0.338$	0.4869
	16SOTU92	0.713	<0.05	<i>Rhodobacteraceae bacterium</i>	MK603708	100	Proteobacteria	$y = (4 \times 10^{-7})x - 0.003$	0.5078
2019	16SOTU29	0.707	<0.05	<i>Gamma proteobacterium</i>	MH077347	100	Proteobacteria	$y = (9 \times 10^{-10})x + 0.0859$	0.4997
	16SOTU70	0.678	<0.05	<i>Gamma proteobacterium</i>	HM057639	99.84	Proteobacteria	$y = (2 \times 10^{-9})x + 0.1066$	0.4594
	16SOTU102	0.999	<0.01	<i>Alpha proteobacterium</i>	DQ436547	99.83	Proteobacteria	$y = (8 \times 10^{-10})x - 0.0026$	0.9986
	16SOTU123	0.841	<0.01	<i>Flavobacteriales bacterium</i>	LC314467	99.68	Bacteroidetes	$y = (7 \times 10^{-10})x + 0.0117$	0.7074
	16SOTU125	0.984	<0.01	<i>Verrucomicrobia bacterium</i>	HQ675288	99.21	Verrucomicrobia	$y = (3 \times 10^{-8})x + 0.2271$	0.9685
	16SOTU146	0.999	<0.01	<i>Alpha proteobacterium</i>	DQ187753	99.59	Proteobacteria	$y = (5 \times 10^{-10})x - 0.0014$	0.9986
	16SOTU153	0.864	<0.01	<i>Alpha proteobacterium</i>	JX528177	99.59	Proteobacteria	$y = (1 \times 10^{-8})x + 0.1774$	0.7462
	16SOTU211	0.999	<0.01	<i>Gamma proteobacterium</i>	LC018880	99.06	Proteobacteria	$y = (4 \times 10^{-10})x - 0.0014$	0.9986
	16SOTU223	0.999	<0.01	<i>Flavobacterium sp.</i>	GU230428	99.05	Bacteroidetes	$y = (1 \times 10^{-9})x - 0.003$	0.9986
	16SOTU265	0.701	<0.05	<i>Gamma proteobacterium</i>	AY580810	99.61	Proteobacteria	$y = (5 \times 10^{-10})x + 0.0128$	0.4914
	16SOTU274	0.841	<0.01	<i>Gamma sp.</i>	EF414148	94.33	Proteobacteria	$y = (5 \times 10^{-9})x + 0.0816$	0.7079
	16SOTU308	0.999	<0.01	<i>Pelagibacteraceae bacterium</i>	MH077429	99.75	Proteobacteria	$y = (6 \times 10^{-9})x - 0.0195$	0.9986
	16SOTU338	0.973	<0.01	<i>Flavobacteriales bacterium</i>	KF786847	99.61	Bacteroidetes	$y = (2 \times 10^{-9})x + 0.0109$	0.9468
	16SOTU350	0.999	<0.01	<i>Planctomycete sp.</i>	JN233020	97.42	Planctomycetes	$y = (1 \times 10^{-9})x - 0.0033$	0.9986
	16SOTU351	0.999	<0.01	<i>Alpha proteobacterium</i>	EF471483	99.83	Proteobacteria	$y = (9 \times 10^{-10})x - 0.0029$	0.9986
2018	16SOTU70	0.588	<0.01	<i>Gamma proteobacterium</i>	HM057639	99.84	Proteobacteria	$y = (2 \times 10^{-9})x + 0.1234$	0.3459
And	16SOTU123	0.712	<0.01	<i>Flavobacteriales bacterium</i>	LC314467	99.68	Bacteroidetes	$y = (7 \times 10^{-10})x + 0.0126$	0.5068
2019	16SOTU125	0.874	<0.01	<i>Verrucomicrobia bacterium</i>	HQ675288	99.21	Verrucomicrobia	$y = (3 \times 10^{-8})x + 0.7289$	0.7645
	16SOTU153	0.76	<0.01	<i>Alpha proteobacterium</i>	JX528177	99.59	Proteobacteria	$y = (1 \times 10^{-8})x + 0.2024$	0.5769

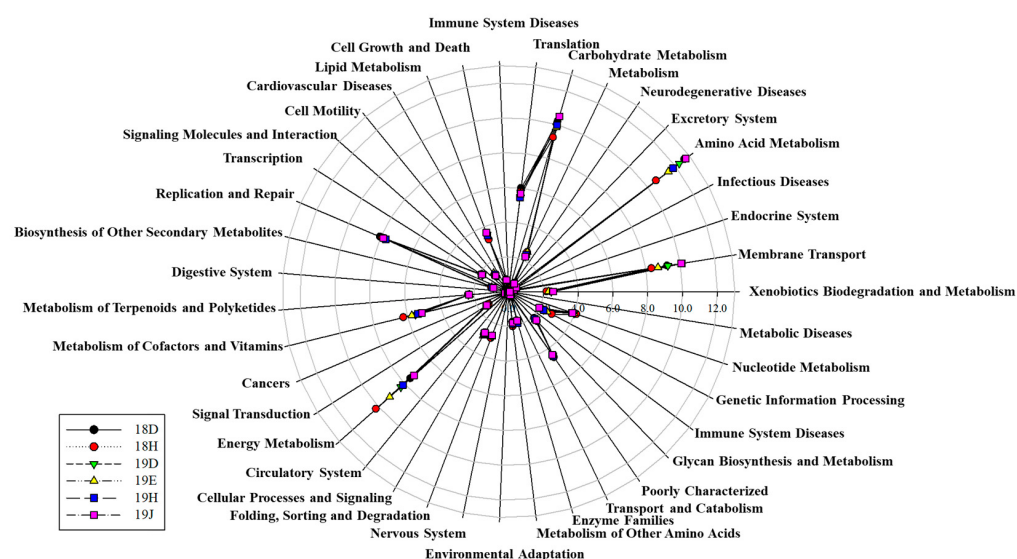


Figure 6. Microbial OTUs associated with *C. polykrikoides* are related to gene functional pathways. Gene functions were predicted from 16S rRNA gene-based microbial compositions using the PICRUSt algorithm to make inferences from KEGG pathways.

3.4. Phytoplankton Community Structure during the *Cochlodinium* Bloom

Amplified sequences from p23S universal primer sets were subjected to a series of bioinformatics and quality processes, resulting in 21,417 to 84,125 merged contigs (Supplementary Tables S3 and S4). Clustered with a 99% similarity cut-off, 261 phytoplankton OTUs were identified from 18 water samples taken from southern Korean coastal waters in 2018 and 2019. The numbers of OTUs in each sample ranged from 31 (18F) to 52 (18D, 19C, and 19D). A total of 261 OTUs were classified into the unknown group with less than 90% sequence identity, and including nine phyla, i.e., Cyanobacteria, Bacillariophyta, Cercozoa, Chlorophyta, Cryptophyta, Euglenophyta, Haptophyta, Miozoa, and Ochrophyta (Supplementary Tables S3 and S4, Figure 7). The diversity indices of the phytoplankton communities of the 18 water samples are shown in Table 5. Values for Chao1, Pielou's evenness (J'), Shannon index (H'), and Simpson index ($1-\lambda$) in Korean coastal waters during blooms caused by *C. polykrikoides* ranged from 7.45 to 11.89, 0.38 to 0.73, 1.68 to 3.47, and 0.59 to 0.94, respectively (Table 5). The minimum values of the four diversity indices occurred at 18J and 19G, but these diversity indices were not significantly different in each sample ($F = 0.04$, $p > 0.05$).

There was a significant positive correlation between the copy number of *C. polykrikoides* rDNA and the Cyanobacteria taxa (0.468, $p < 0.05$) which ranged from 4.59% (18B) to 85.12% (18J). However, Haptophyta, which is composed mainly of *Chrysochromulina parva* (MG520331) and *Phaeocystis globosa* (KC900889), was the dominant group at station D. In 2018, a relatively high proportion of the Ochrophyta group accounted for up to 73.49% (B) at relatively neritic sites, and was identified as the *Heterosigma akashiwo* species. Overall, the proportion of Haptophyta was higher in the 2019 samples than in those from 2018, excluding station D, and the proportion of Ochrophyta was higher in 2018 than in 2019. Similar to the microbial communities, cluster analysis based on the phytoplankton communities showed four main clusters (Clusters I to IV) at a similarity level of 60% (Figure 4B,D). Cyanobacteria were the most significant phyla in the phytoplankton communities with *C. polykrikoides*. The ANOSIM and PERMANOVA analyses based on the phytoplankton community using the Bray–Curtis dissimilarity measure on the square root-transformed data indicated that the differences in the microbial community structure were dependent on factors associated with the sampling year (Table 6).

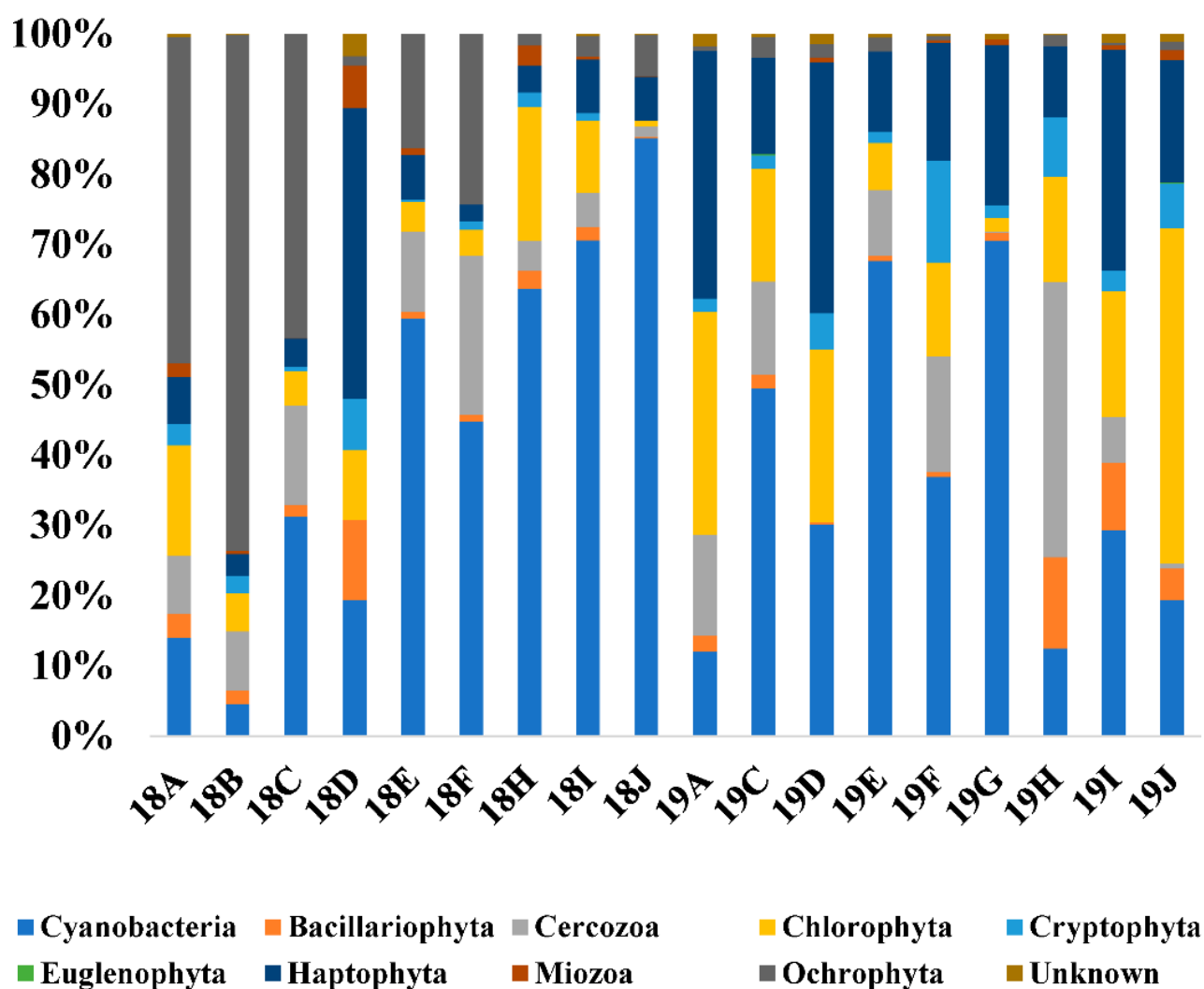


Figure 7. Photosynthetic phytoplankton community structures of samples. Relative abundances of the dominant groups at the phylum level.

In order to identify the significant phytoplankton for *C. polykrikoides* blooms, a correlation analysis was performed to assess the relation between the abundance of *C. polykrikoides* and phytoplankton OTUs generated by metabarcoding analysis. *Teleaulax minuta* (23SOTU11), which is a phytoplankton OTU, had a strong negative correlation, and eleven phytoplankton OTUs—*Bathycoccus* sp. (23SOTU16), *Micromonas pusilla* (23SOTU26), *Attheya* sp. (23SOTU58), *Actinocyclus subtilis* (23SOTU59), *Kryptoperidinium foliaceum* (23SOTU72), *Emiliana huxleyi* (23SOTU107), *Mallomonas* sp. (23SOTU116), *Thalassiosira oceanica* (23SOTU160), and *Synechococcus* sp. (23SOTU193, 211, 212) had strong positive correlations with *C. polykrikoides* in 2018. However, in 2019 *Teleaulax minuta* (23SOTU11) showed a strong negative correlation, and *Mallomonas* sp. (23SOTU116, 150), *Synechococcus* sp. (23SOTU125, 215), and *Ochromonas* sp. (23SOTU171) each had a strong positive correlation. Only four OTUs—Prymnesiophyte sp. (23SOTU25), *Mallomonas* sp. (23SOTU150), *Ochromonas* sp. (23SOTU171), *Synechococcus* sp. (23SOTU215)—had a strong positive correlation with *C. polykrikoides* in the phytoplankton communities (Table 8). Findings showed that the pattern of species correlated with *C. polykrikoides* differed from year to year, and the Cyanobacteria group was closely related to the blooms of *C. polykrikoides* (Table 8).

Table 8. Strongly correlated phytoplankton OTUs with *C. polykrikoides*.

Year	OTU	Correlation Coefficient	<i>p</i>	Description	Accession	Identify (%)	Phylum	Regression Equation	<i>R</i> ²
2018	23SOTU11	−0.747	<0.05	<i>Teleaulax minuta</i>	KP142642	99.73	Cryptophyta	$y = (-6 \times 10^{-8})x + 0.3042$	0.5574
	23SOTU16	0.895	<0.01	<i>Bathycoccus</i> sp.	FO082259	96.95	Chlorophyta	$y = (5 \times 10^{-8})x - 0.0296$	0.8004
	23SOTU26	0.724	<0.05	<i>Micromonas pusilla</i>	L42847	98.56	Chlorophyta	$y = (3 \times 10^{-8})x - 0.0193$	0.5244
	23SOTU58	0.724	<0.05	<i>Attheya</i> sp.	KJ958479	97.49	Bacillariophyta	$y = (3 \times 10^{-8})x - 0.0181$	0.5244
	23SOTU59	0.724	<0.05	<i>Actinocyclus subtilis</i>	KP826904	98.11	Bacillariophyta	$y = (3 \times 10^{-8})x - 0.0215$	0.5244
	23SOTU72	0.713	<0.05	<i>Kryptoperidinium foliaceum</i>	GU591328	98.74	Miozoa	$y = (2 \times 10^{-7})x - 0.1419$	0.5088
	23SOTU107	−0.67	<0.05	<i>Emiliana huxleyi</i>	JN022705	98.83	Haptophyta	$y = (-5 \times 10^{-8})x + 0.3755$	0.4485
	23SOTU116	0.728	<0.05	<i>Mallomonas</i> sp.	KM817948	92.94	Ochrophyta	$y = (8 \times 10^{-8})x + 0.0021$	0.5297
	23SOTU160	0.724	<0.05	<i>Thalassiosira oceanica</i>	GU323224	99.01	Bacillariophyta	$y = (2 \times 10^{-8})x - 0.011$	0.5244
	23SOTU193	0.719	<0.05	<i>Synechococcus</i> sp.	CP006882	99.01	Cyanobacteria	$y = 0.0859x - 0.2161$	0.3983
	23SOTU211	0.724	<0.05	<i>Synechococcus</i> sp.	CP006882	99.01	Cyanobacteria	$y = (2 \times 10^{-8})x - 0.0134$	0.5244
	23SOTU212	0.724	<0.05	<i>Synechococcus</i> sp.	CP006882	99.28	Cyanobacteria	$y = (2 \times 10^{-8})x - 0.0153$	0.5244
2019	23SOTU11	0.73	<0.05	<i>Teleaulax minuta</i>	KP142642	99.73	Cryptophyta	$y = (1 \times 10^{-9})x + 0.0535$	0.5322
	23SOTU116	0.668	<0.05	<i>Mallomonas</i> sp.	KM817948	92.94	Ochrophyta	$y = (6 \times 10^{-10})x + 0.0177$	0.446
	23SOTU125	0.745	<0.05	<i>Synechococcus</i> sp.	LC455658	99.28	Cyanobacteria	$y = (9 \times 10^{-10})x + 0.0215$	0.5553
	23SOTU150	0.999	<0.01	<i>Mallomonas</i> sp.	KM817983	92.73	Ochrophyta	$y = (6 \times 10^{-10})x - 0.0018$	0.9986
	23SOTU171	0.999	<0.01	<i>Ochromonas</i> sp.	KJ877675	92.27	Ochrophyta	$y = (5 \times 10^{-10})x - 0.0015$	0.9986
	23SOTU215	0.975	<0.01	<i>Synechococcus</i> sp.	CP006882	99.01	Cyanobacteria	$y = (5 \times 10^{-9})x + 0.0192$	0.95
2018	23SOTU25	0.616	<0.01	<i>Prymnesiophyte</i> sp.	HM565909	97.84	Haptophyta	$y = (8 \times 10^{-10})x + 0.0123$	0.3795
And	23SOTU150	0.999	<0.01	<i>Mallomonas</i> sp.	KM817983	92.73	Ochrophyta	$y = (6 \times 10^{-10})x - 0.0014$	0.9977
2019	23SOTU171	0.999	<0.01	<i>Ochromonas</i> sp.	KJ877675	92.27	Ochrophyta	$y = (5 \times 10^{-10})x - 0.0012$	0.9977
	23SOTU215	0.975	<0.01	<i>Synechococcus</i> sp.	CP006882	99.01	Cyanobacteria	$y = (5 \times 10^{-9})x + 0.0037$	0.9513

3.5. Correlation of *C. polykrikoides* with Environmental Factors

To analyze the responses of *C. polykrikoides* in Korean coastal waters to environmental factors, CCA analysis was conducted on the relationship between *C. polykrikoides* abundance and environmental parameters (i.e., temperature, salinity, nutrients, chlorophyll a). The first two ordination axes of CCA explain 100% of the variance in rDNA copies of *C. polykrikoides*, relative to the environmental factors. The eigenvalues for axis 1 (horizontal) and axes 2 (vertical) were 0.249 and 0.005, respectively, and they explain the *C. polykrikoides* rDNA copies' variance of 82.7%. Samples H in 2018 and E in 2019 were significantly affected by NH₄N, DIN, and chlorophyll a, and were positively correlated (Figure 8).

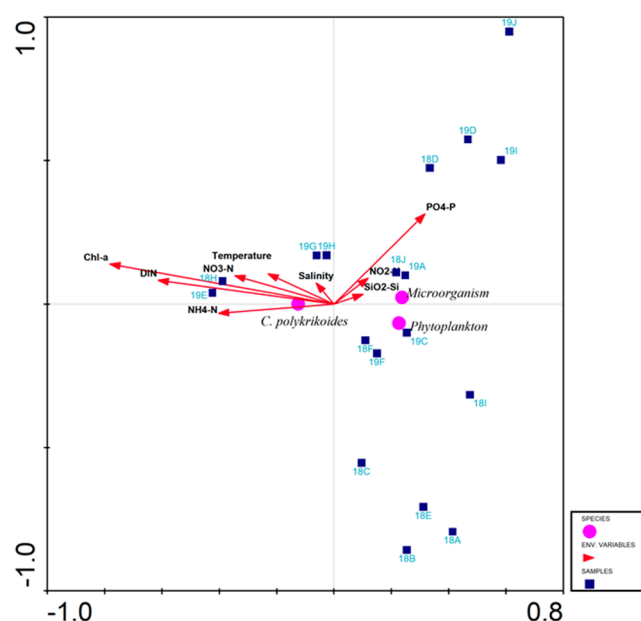


Figure 8. Canonical correspondence analysis (CCA) of microbial communities (OTUs), phytoplankton communities, and environmental parameters sampled during *C. polykrikoides* bloom.

4. Discussion

Recently, molecular methods such as quantitative PCR have been applied for the detection of various species of phytoplankton. Quantitative PCR methods that can reliably and accurately quantify the number of cells, even if only one cell of the target species is included in the sample, have been developed for a variety of HAB species, including *Alexandrium*, *Aureococcus* [37], *Cochlodinium* [32], *Chattenella*, *Heterosigma* [36], *Gambierdiscus*, *Karenia* [38,39], *Lingulodinium* [40], *Ostreopsis* [41], and *Pfiesteria*. This method has been shown to be powerful for the quantitative detection of HAB species in environmental samples [32,42,43].

In this study, cell-counting methods failed to detect any single cells even in sites where millions of rDNA copies were identified (at least 5538.65 at D in 2018, to up to 5,726,129.65 copies/mL at G in 2019) (Table 3). *C. polykrikoides* exist in various forms in environmental samples, from single cells up to 16 cells that form chains. Since DNA dissolves in water as environmental DNA, many more copies than the number of cells can be detected. As a simple and sensitive technique, several previous qPCR studies have reported a strong correlation between the dinoflagellate cell number and the copy number of the rDNA [38,44,45]. However, we found that there was a low degree of correlation ($r^2 = 0.4676$) between the direct cell counts and the copy numbers obtained by qPCR (Figure 2C). When those values were separately analyzed for 2018 and 2019, a much higher degree of correlation was identified within each year ($r^2 = 0.7065$ in 2018 and $r^2 = 0.8765$ in 2019, respectively), suggesting different ribosomal DNA copy numbers between the two *C. polykrikoides* strains (Figure 2A,B). These results may suggest that several things should be considered by researchers before applying qPCR to estimate cell numbers of *Cochlodinium* species. First, rDNA copy numbers may vary even in the same species depending on the growth stage or strain [42,46]. Few data are available in the literature concerning rDNA gene copies in dinoflagellates: approximate values of 65, 350, 600, 1000, and more than 12,000 have been reported, respectively, for *Prorocentrum nux*, *Amphidinium carterae*, *Prorocentrum minimum*, *Alexandrium minutum*, and *Akashiwo sanguinea* [47,48]. However, in the case of *C. polykrikoides*, exact rDNA copies per cell are not yet known. Second, when the density of *C. polykrikoides* cells was low, qPCR values showed large fluctuations [43]. Based on forecast-breaking news from the National Institute of Fisheries Science (NIFS), red tides caused by *C. polykrikoides* began on 23 July 2018, and remained for 18 days, and the maximum number of *C. polykrikoides* cells was 4500 cells/mL (NIFS,

2018). In 2019, *C. polykrikoides* blooms started on August 20 and ended on September 28, with a reported maximum concentration of 15,120 cells/mL (NIFS, 2019). During the survey period, we detected zero to 1250 *C. polykrikoides* cells/mL, and the rDNA copies of *C. polykrikoides* showed more fluctuation. Nevertheless, we were able to detect *C. polykrikoides* with a sensitive technique. Because of its sensitivity, there have been attempts to use qPCR for early warning of HABs [43,49,50].

Water temperature is one of the most important factors for *C. polykrikoides* blooms [51,52]. Kim et al.'s [52] study showed that *C. polykrikoides* exhibited its maximum specific growth rate of 0.41 day^{-1} at a combination of 25°C and a salinity of 34 psu, and in the laboratory experiment optimum growth rates of $>0.3 \text{ day}^{-1}$ were observed at temperatures ranging from 21 to 26°C at salinities from 30 to 36 psu. Among the environmental parameters in this study, we found that the water temperature (correlation coefficient, 0.605; $p < 0.01$) was strongly correlated with *C. polykrikoides* (Table 4). The average water temperatures were 25.78 ± 1.78 and $26.04 \pm 4.32^\circ\text{C}$ in each sampling year, respectively, within the optimal temperature range ($21\sim 27.5^\circ\text{C}$) for the growth of *C. polykrikoides*, and the salinity also appeared to be in the optimum range (Table 2). Ecological research has suggested the presence of specific algal–microbial interactions based on their co-occurrence in marine ecosystems, which have the potential to affect biogeochemical cycles and their community structures [2,53]. Bacteria support the growth of HABs from the recycling of nutrients, and phytoplankton produce various algal-derived products that affect bacterial growth rates and community composition [15,16]. The interactions between bacteria and phytoplankton can be analyzed through metabarcoding analysis with multiple markers simultaneously [29]. Several previous studies using molecular methods have shown that bacterial communities appear to be associated with HABs. Previous studies have shown that universal primer sets (16S and p23S) provide useful information about algal blooms by metabarcoding analysis [31,54]. In order to identify specific microbial and phytoplankton communities in *C. polykrikoides* blooms, we compared each community within the blooms of *C. polykrikoides* in two years. Microbial OTUs that showed significant correlations differed according to the sampling year. Among the physical factors, N is a key chemical element that influences microbial communities [55]. Specifically, we found that inorganic nutrients, such as DIN, NO_3 , and NH_4 were higher in 2019 than in 2018, and these factors had effects on the microbial community structure. We confirmed that there was a difference in the microbial community structure by year (Figures 3 and 4A,B).

Studies have shown that the Gamma proteobacteria are the most abundant group in HABs [2,11,27]. Park et al. [11] reported that the *C. polykrikoides* bloom that occurred in Korean coastal waters in 2012 had a negative relationship with Gamma proteobacteria [11]. Two genera within the Gamma proteobacteria group, i.e., *Alteromonas* and *Pseudomonas*, are considered algicidal bacteria and seem to be related to the termination of blooms [56,57]. However, a strong positive correlation between the abundance of *C. polykrikoides* and Gamma proteobacteria ($p < 0.05$) was found in this study (Table 7). Shin et al. [27] reported that Gamma proteobacteria was one of the major bacteria communities associated with *C. polykrikoides* in culture. As in the case of *Alexandrium* blooms [2], Gamma proteobacteria act as a buffer, and are thought to enhance the stability of the microbial community structure in *C. polykrikoides* blooms. Thus, types of Gamma proteobacteria may be different depending on the development of the *C. polykrikoides* bloom. Verrucomicrobia are known to grow preferentially on phytoplankton-derived high molecular organic compounds [58]. For this reason, Verrucomicrobia dominated areas where red tide species bloomed in water samples [31,59]. This study also showed that *C. polykrikoides* blooms had a strong positive correlation with Verrucomicrobia. We found in a previous study that the Verrucomicrobia group had a strong positive correlation with red tide species during a *Heterosigma akashiwo* bloom. We hypothesized that the Verrucomicrobia group is closely related to the blooms of red tide species that are found in southern Korean coastal waters. However, we did not find specific or repeated microbial communities that may be associated with *C. polykrikoides* blooms in these waters. We found a pattern of decreasing proteobacteria as Cyanobacteria

in *C. polykrikoides* blooms increased. *C. polykrikoides* can feed on foods such as bacteria, cryptophyta, and diatoms, which are common in Korean coastal waters. In particular, they are able to consume up to 928 *Synechococcus* individuals and up to 9.4 *Teleaulax* sp. individuals daily, and with feeding, the growth rate can double [60,61]. When the nutritional conditions of *C. polykrikoides* blooms were limited, they were able to obtain nutrients by consuming food source species such as *Synechococcus* sp., *Teleaulax* sp., and *Heterosigma akashiwo*. Thus, we suggest that *Synechococcus* sp. and some haptophyta species, such as *Teleaulax* sp., may affect the nutrition of *C. polykrikoides* and potentially the C and N cycles of the microbial community in a red tide. The results of the heatmap analysis also support the above suggestion (Figures 5 and 9).

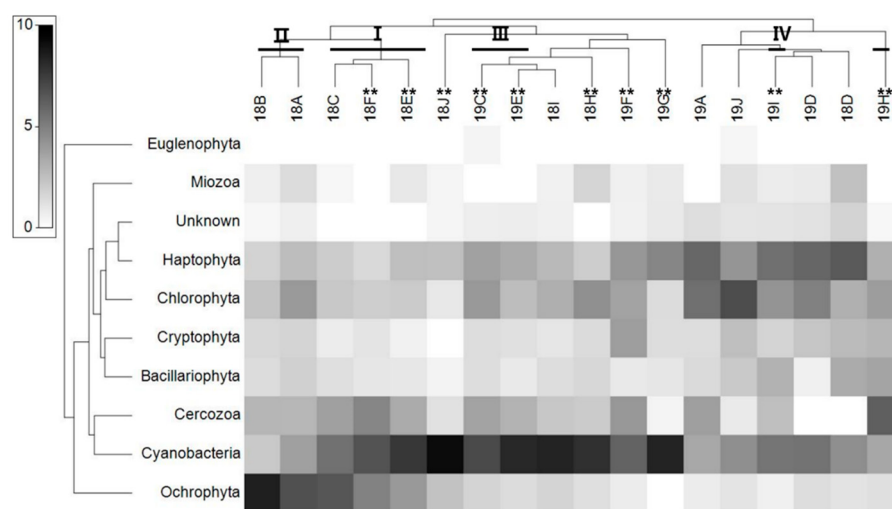


Figure 9. Shade plot illustrating the phytoplankton communities at the phylum level by site, for 18 samples. The plot depicts the relative abundance of phyla within microbial communities. **: >1,000,000 copies/mL (rDNA of *C. polykrikoides*).

Interestingly, although the microbial community at D in 2018 was distinct from other samples, the functional study (PICRUSt) results show only a slight difference in the ratio, and the genetic functions expressed were similar in all samples. Even if HAB species are not found in the coastal waters where HABs have already occurred, it is thought that differences are only in the structure of the microbial communities while they play similar functional roles. Predictive tools (PICRUSt algorithm) [62] to determine the functional potential of microbial communities have revealed different abundances of *C. polykrikoides*. From the functional pathway analysis, we found that genes related to carbohydrate metabolism, amino acid metabolism, and energy metabolism were very abundant during *C. polykrikoides* blooms (Figure 6). Energy metabolism gene expression was maintained at relatively high levels with *Cochlodinium* abundance. Similar results were observed for *Akashiwo sanguinea* and *Alexandrium* sp., through the promotion of nitrogen (ammonium) accumulation, organic acid utilization, and carbon assimilation [2,63], which indicates that S and P availability are important for blooms. These results show that bacterial functions affect algal blooms' metabolic activity. We determined the structure of the microbial community and performed a functional study. To understand functional information gained from the study of HABs, additional analyses are required, including applying "Omics" methods to microbial structure and function, and a time-series investigations from before the outbreak of HABs until they disappear.

5. Conclusions

This work analyzed the microbial and phytoplankton communities from environmental samples during *C. polykrikoides* bloom events, using metabarcoding analysis. We were able to detect *C. polykrikoides* via a sensitive technique, and confirmed potential for early

warning of *C. polykrikoides* using quantitative PCR. No single group could explain successive blooms of *C. polykrikoides*; four taxa, including Gamma proteobacteria (0.588, $p < 0.01$), Verrucomicrobia (0.874, $p < 0.01$), Cyanobacteria (0.975, $p < 0.01$), and Cryptophyta were related to *C. polykrikoides* blooms. When the *C. polykrikoides* bloom events occurred, though there were differences in the relative abundance of *C. polykrikoides*, the value levels differed only in the potential function of the microbial community, and the overall function was similar. The phytoplankton community structure was divided into three or four by sampling years, using metabarcoding analysis. The phytoplankton community structure provided information on the water bodies, using metabarcoding analysis by amplifying the plastid 23S region.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w14193115/s1>, Table S1: Summary of microbial OTUs produced by 16S universal primer set in *C. polykrikoides* bloomed water samples in 2018, Table S2: Summary of microbial OTUs produced by 16S universal primer set in *C. polykrikoides* bloomed water samples in 2019, Table S3: Summary of phytoplankton OTUs produced by p23S universal primer set in *C. polykrikoides* bloomed water samples in 2018, Table S4: Summary of phytoplankton OTUs produced by p23S universal primer set in *C. polykrikoides* bloomed water samples in 2019.

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