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Original Research

Influence of Conditioned Media of *Halomonas* sp. DH-e on Phycosphere Bacterial Community Dynamics of *Prorocentrum donghaiense*

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Abstract

Prorocentrum donghaiense has become one of the major algae that cause phytoplankton blooms. A marine bacterium, *Halomonas* sp. DH-e with high algicidal activity to *P. donghaiense* was isolated in previous studies. However, the mechanism underlying the algicidal activity of DH-e was unknown. In the present study, the post-culture medium of DH-e was supplemented into the culture of *P. donghaiense*. Samples of algae cells and phycosphere bacteria were collected at different times. Illumina Miseq sequencing was used to evaluate bacterial community dynamics in the phycosphere of microalgae. Taxonomic analysis identified 3 phyla, including Proteobacteria, Bacteroidetes and Verrucomicrobia, with Proteobacteria dominating and accounting for more than 95% of all bacterial species. *Alteromonadaceae* increased gradually, while *Rhodobacteraceae* decreased during the culture with the post-culture medium. Principal component analysis suggested that bacterial community composition was similar during 0-12 h, but changed significantly at 24 h, 36 h and 48 h. Redundancy analysis revealed a close correlation between phycosphere bacterial community composition and pH, chlorophyll a and algae cell mass. In conclusion, the results suggested that algicidal activity of DH-e was mediated via the secretion of unidentified components in the post-culture medium, which induced variation of phycosphere bacterial community and subsequently contributed to the lysis of algae cells.

Keywords: algicidal bacteria, phycosphere bacteria, Illumina Miseq sequencing, *Prorocentrum donghaiense*

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Introduction

There are many close interactions between bacterioplankton and phytoplankton dynamics in aquatic systems [1], in which the extracellular products of photosynthetic algae stimulate the bacterial growth [2] and in return the marine bacteria have abilities to promote or inhibit phytoplankton growth [3-4]. This intimate microenvironment between algal cells and bacteriawas is defined as ‘Phycosphere’ [5].

Phytoplankton-bacteria interactions are multifarious [8] and often highly sophisticated and can span the spectrum of ecological relationships from cooperative to competitive [6]. At the simplest level, the relationship between these organisms is based on resource provision and can be either reciprocal or exploitative in nature. Aquatic heterotrophic bacteria obtain a large, albeit variable, fraction of their carbon demand directly from phytoplankton [7], with up to 50% of the carbon that is fixed by phytoplankton is ultimately consumed by bacteria [9-10]. Bacterial consumption of phytoplankton-derived organic material primarily involves the assimilation of the large quantities of typically highly labile, dissolved organic carbon (DOC) released by phytoplankton cells into the surrounding water column [11-12], but also includes the consumption of more complex algal products (for example, mucilage and polysaccharides) and senescent or dead phytoplankton biomass [13-15].

To understand the close relationship between *P. donghaiense* and its bacteria [16-17], we used Illumina Miseq sequencing of the V3-V4 regions of the 16S rRNA gene to explore the bacterial communities during the culture of *P. donghaiense* with the post-culture medium of DH-e. The results showed that phycosphere bacterial community composition changed significantly during the culture, and a close correlation could be found with pH, chlorophyll a and algae cell mass.

Materials and Methods

Bacterial and Algae Strains and Conditions of Cultivation

The algicidal bacterium, *Halomonas* sp. DH-e, was isolated from red tide areas in the Zhejiang coast, and identified as having efficient algicidal activity on *P. donghaiense* by means of secreting extracellular substance. 100μL of the strain were inoculated in 50mL LB medium (tryptone 10 g, yeast extract 5 g, fixed capacity to 1 L using seawater, pH 7.2) at 28ºC with shaking at 160 r/min [15]. It has been deposited in the China National Culture Collection Centre (No. CCTCC NO: M2014541). The accession number at GenBank are KP144872. (GenBank: www.ncbi.nlm.nih.gov/Genbank/submit.html)

P. donghaiense were provided by the College of Life Science and Technology, Jinan University. The algae were cultured in f/2 culture medium (without silicate) at 20±1ºC [18] under a 12:12 h light-dark cycle with a light intensity of 4000 Lux.

Sample Collection

After culturing for 5 days, the bacterial culture was centrifuged at 4000 rpm for 15 min, and supernatant was collected and filtered through 0.22 μm diameter pore-size filters for three times. The above supernatant was used as the post-culture medium and added into the 300 mL logarithmic *P. donghaiense* cultures in 1.0% final concentration. Three parallel samples of algae cells and phycosphere bacteria were collected at 0, 12, 24, 36 and 48 h by filtering the culture through 0.22 μm membrane. The collected samples were designated as S1_1, S1_2, S1_3 for 0 h, S2_1, S2_2, S2_3 for 12 h, S3_1, S3_2, S3_3 for 24 h, S4_1, S4_2, S4_3 for 36 h, S5_1, S5_2, S5_3 for 48 h. The samples were stored in a freezer at –80ºC.

Measurement of Environmental Parameters

In order to obtain the environmental parameters, the concentrations of total nitrogen (TN), total phosphorus (TP), ratio of nitrogen to phosphorus (N/P), chlorophyll a (Chl a) of *P. donghaiense* cultures [19] and the density of algae cells (DAC) were measured according to standard methods [20]. The *P. donghaiense* cells were dyed with Lugol’s iodine and the number of cells were counted under an optical microscope.

DNA Extraction, PCR Amplification and Sequencing

DNA was extracted using a soil DNA kit (OMEGA, USA) according to the manufacturer’s protocol. The DNA concentration and quality were determined by agarose gel electrophoresis (1% wt/vol agarose in Tris-acetate-EDTA buffer) and using a NanoDrop 2000 spectrophotometer (Thermo Scientifc, USA). DNA was stored at -20ºC until used for PCR amplification.

A fragment of the 16S rRNA gene was amplified using the universal primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) [21] and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) [22], covering the V3-V4 hyper-variable region, 15 libraries were constructed and sequenced using the Illumina MiSeq sequencing platform PE300. The sequencing was performed at Majorbio Biomedical Technology Co., Ltd, Shanghai, China.

Data Analysis

Quality control was performed on the raw data. The reads were processed by removing tags and primer, accepted only reads with a mean quality score above 20 and read lengths more than 245 bp. Sequences with more than one ambiguous base call were removed using a Ribosomal Database Project sequencing pipeline (RDP) [23-24]. The data of chloroplast and mitochondria were eliminated, and the resulting sequences were used for final analysis. The sequence with similarity greater than 0.97 was categorized as an operational taxonomic unit (OTU). The number of OTUs contained in each sample was analyzed and the rarefaction curve was drawn. The diversity and richness of the samples were studied by calculating the Simpson, Shannon, Chao and Ace indexes [25-26] with MOTHUR version v.1.30.1 [27].

The community structure was analyzed at the phylum and the family levels. Principal component analysis (PCA) results were displayed using the vegan package, and the figures were draw using ggplot2 packange in R [28-29]. Linear discriminant analysis (LDA) was performed using the online LEfSe Program. Significant differences in abundance between groups were identified.

The correlations between microbial communities and environmental factors were analyzed with ordination methods using CANOCO software for Windows, version 4.5. Redundancy analysis (RDA) was used to illustrate the relationship between microbiota and environment factors because detrended correspondence analysis run on the bacterial OUT (97% similarity) profile matrix indicated that the length of the first axis was <3. The environmental factors were normalized and served as the environmental input.

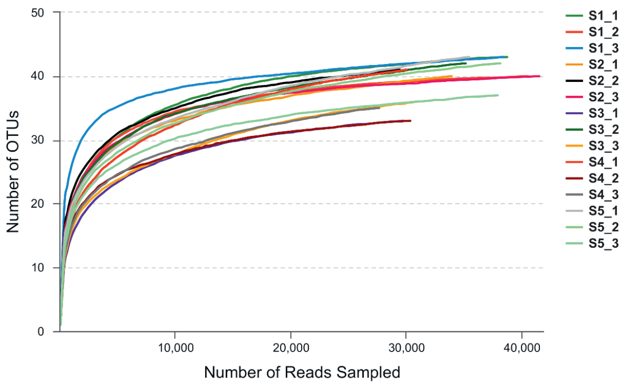

Fig. 1. Rarefaction curves of operational taxonomic units (OTUs) sharing ≥97% similarity for the 15 samples. Samples were treated with the post-culture medium for 0 h (S1_1, S1_2, S1_3), 12 h (S2_1, S2_2, S2_3), 24 h (S3_1, S3_2, S3_3), 36 h (S4_1, S4_2, S4_3), 48 h (S5_1, S5_2, S5_3).

Results

General Analyses of Illumina Miseq Sequencing Data

After filtering the low-quality reads and removing all chloroplast/mitochondrial sequences and rarefying the datasets, 30,761 to 44,321 effective sequences were collected from each sample, resulting in a total of 564,527 sequences and 47 OTUs with ≥ 97% sequence similarity from the 15 samples. The coverage ranged

Table 1. Number of reads, observed diversity richness (OTUs), estimated OTU richness (Ace and Chao), diversity index (Shannon and Simpson) and estimated sample coverage for 16S rRNA libraries of the 15 samples. Samples were treated with CM for 0 h (S1_1, S1_2, S1_3), 12 h (S2_1, S2_2, S2_3), 24 h (S3_1, S3_2, S3_3), 36 h (S4_1, S4_2, S4_3), 48 h (S5_1, S5_2, S5_3).

Samples	Reads	OTUs	Coverage (%)	Ace	Chao	Shannon	Simpson
S1_1	40602	43	99.99	44.20	43.60	0.55	0.83
S1_2	43967	40	99.99	41.46	41.00	0.62	0.79
S1_3	42395	43	99.99	47.50	44.50	1.13	0.61
S2_1	35729	40	99.98	45.65	45.00	0.72	0.75
S2_2	31226	41	99.98	44.91	43.50	0.76	0.74
S2_3	44321	40	99.99	41.80	43.00	0.72	0.75
S3_1	32010	33	99.99	34.77	33.50	0.77	0.65
S3_2	39070	42	99.98	48.54	49.00	1.17	0.47
S3_3	33531	36	99.98	43.89	41.25	0.93	0.52
S4_1	33545	41	99.97	48.68	50.33	1.04	0.45
S4_2	33943	33	99.99	35.92	34.50	1.02	0.44
S4_3	30761	35	99.97	52.38	38.50	1.02	0.44
S5_1	39982	43	99.98	51.09	47.67	1.18	0.42
S5_2	41313	42	99.98	46.79	46.20	1.07	0.44
S5_3	42132	37	99.99	40.19	40.33	1.02	0.45

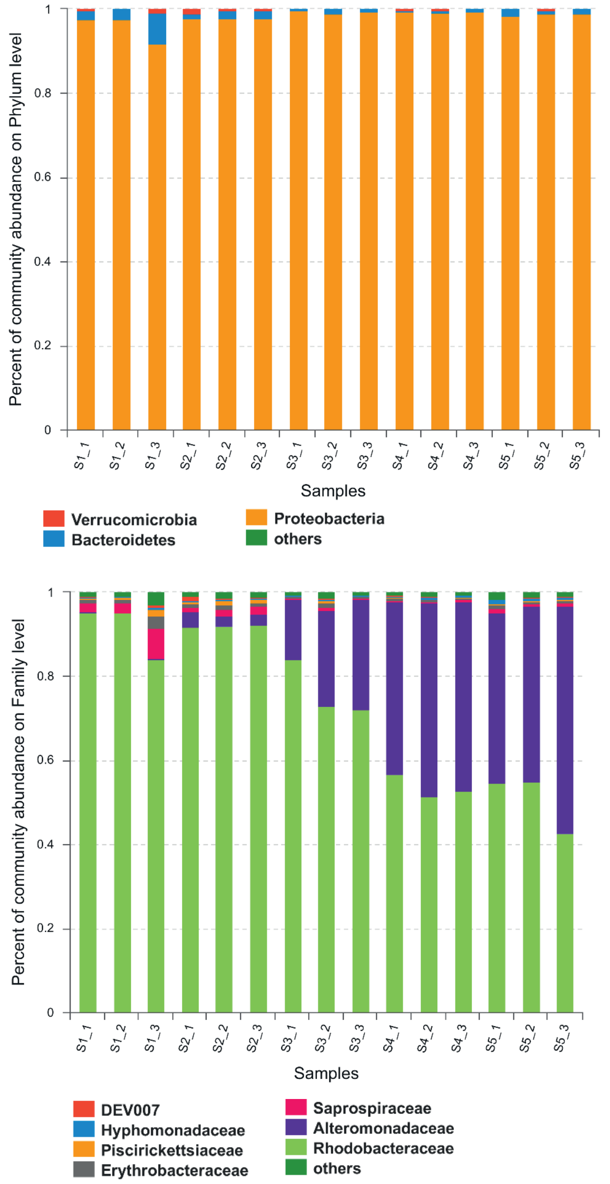

Fig. 2. Taxonomic classification of the sequencing reads at the phylum and family levels. Samples were treated with the post-culture medium for 0 h (S1_1, S1_2, S1_3), 12 h (S2_1, S2_2, S2_3), 24 h (S3_1, S3_2, S3_3), 36 h (S4_1, S4_2, S4_3), 48 h (S5_1, S5_2, S5_3).

from 99.97% to 99.99%, indicating that almost all bacteria in the samples were identified in this study (Table 1). The rarefaction curves tended to reach the saturation plateau when the number of sequences reached 30000-40000, indicating that the sequencing depth was enough to cover all bacteria and reflect the diversity (Fig. 1).

The microbial complexity was estimated with alpha-diversity indices (Ace, Chao, Shannon and Simpson). The Shannon index increased and Simpson index decreased during treatment with no significant difference of bacterial community composition detected at 0 h and 12 h (Student's t test, $P>0.05$). However, there were significant variations among samples of other time points (Student's t test, $P<0.05$). Ace, Chao indices and the number of OTUs changed indistinctively (Student's t test, $P>0.05$) (Table 1). The results indicated that bacterial diversity of the phycosphere increased when the post-culture medium from DH-e was added.

Composition of Phycosphere Bacterial Community

The phycosphere bacteria community of *P. donghaiense* was mainly composed of 3 phyla, including Proteobacteria, Bacteroidetes and Verrucomicrobia. The dominant bacterial phylum was Proteobacteria, which accounts for more than 95%. Furthermore, the phylogenetic classification of sequences from the samples resulted in 7 different families, including *Rhodobacteraceae*, *Alteromonadaceae*, *Saprospiraceae*, *Erythrobacteraceae*, *Piscirickettsiaceae*, *Hyphomicrobiaceae* and *DEV007* (Fig. 2). Although *Rhodobacteraceae* predominated the bacterial community during the whole cultivation, the abundance decreased from 91.6% to 50.7%. On the other hand, the relative abundance of *Alteromonadaceae* increased from 0.13% to 45.6%.

A metagenomic analysis was performed using the linear discriminant analysis effect size (LEfSe) tool [30] (Fig. 3). We noted a significant enrichment (LDA score, 3.0) of OTU24 (*Saprospiraceae*, *norank_*

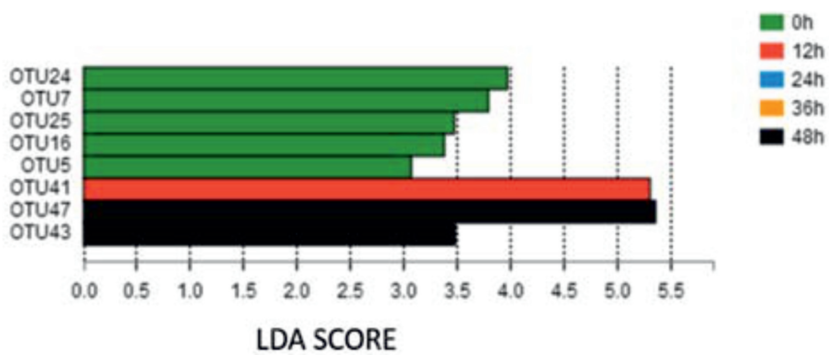

Fig. 3. Linear discriminant analysis (LDA) coupled with effect size measurements identifies the differentially abundant species among different samples. Only taxa exceeding an LDA threshold of 3.0 were considered for analysis. Green, taxa enriched in the 0h library; red, taxa enriched in the 12 h library; and black, taxa enriched in the 48 h library.

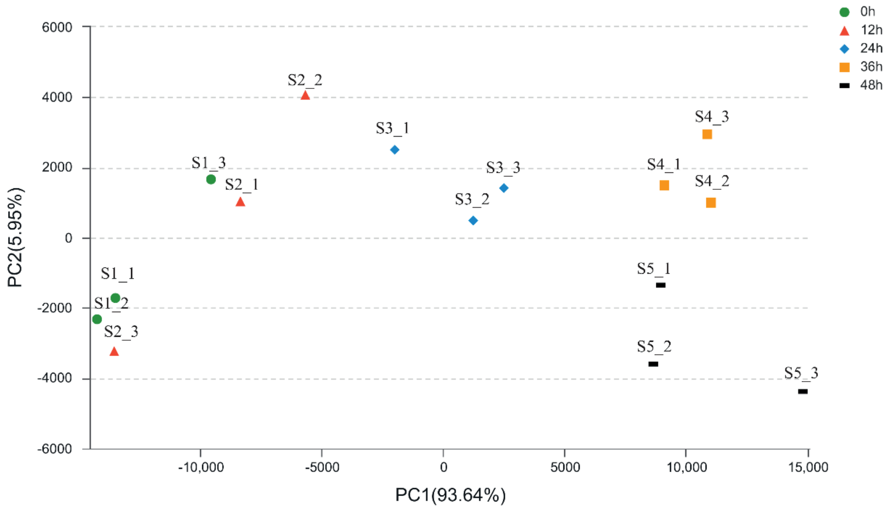

Fig. 4. Principle component analysis of bacterial communities during the algicidal process effected by the post-culture medium. Samples were treated with the post-culture medium for 0 h (S1_1, S1_2, S1_3), 12 h (S2_1, S2_2, S2_3), 24 h (S3_1, S3_2, S3_3), 36 h (S4_1, S4_2, S4_3), 4 8h (S5_1, S5_2, S5_3).

Saprospiraceae), OTU7 (*Saprospiraceae*, *Phaeodactylibacter*), OTU25 (*Piscirickettsiaceae*, *Methylophaga*), OTU16 (*Rhodobacteraceae*, *unclassified_Rhodobacteraceae*), OTU5 (*Rhodobacteraceae*, *unclassified_Rhodobacteraceae*) in the 0 h library, OTU41 (*Rhodobacteraceae*, *Marivita*) in the 12 h library and OTU47 (*Alteromonadaceae*, *Alteromonas*) and OTU43 (*Hyphomonadaceae*, *Maricaulis*) in the 48 h library.

Principal Component Analysis and Correlation Analysis of the Bacterial Community Shift

The PCA results emphasized the differences of bacterial populations among the groups treated with the post-culture medium for different periods of time (Fig. 4). The first and second PCs respectively accounted for 93.64% and 5.95% respectively of the total variation. Scatter plots based on PC1 and PC2 values showed that all samples were clustered into four groups: I, including S1_1, S1_2, S1_3, S2_1, S2_2 and S2_3; II, including S3_1, S3_2 and S3_3; III, including S4_1, S4_2 and S4_3; and IV including S5_1, S5_2 and S5_3. The bacterial communities of the groups treated with the post-culture medium for 0 h showed great similarity to that for 12 h. The samples treated with the post-culture medium for 24, 36 and 48 h were clustered into three groups. The results suggested that the post-culture medium had significant influence on the bacterial community during the culture.

Environmental Characterization

Table 2 lists the environmental parameters. There was slight variation in pH, TN, TP and N/P ratio after incubation with the post-culture medium. The initial pH and TP of *P. donghaiense* were 8.18 and 1.12 µg/mL, which decreased to 7.2 and 0.96 µ g/mL respectively after treatment with the post-culture medium for 48 h. Conversely, TN and N/P increased from 10.9 µg/mL and 9.9 to 11.1 µg/mL and 11.7 respectively after 48 h. The density of algae cells and concentration of Chl a reduced significantly owing to the algicidal effect of the post-culture medium. The density of algae cells decreased from 2.53×10^6 cells/mL to 0 cells/mL after 36 h.

Relationship Between Microbial Community and Environmental Parameters

Microbial community structure is not only regulated by biological factors, but also is closely related to environmental factors. In order to determine what environmental characteristics affected the bacterial community at the family level, the relationship between bacterial community composition and environmental characteristic was analyzed by RDA (Fig. 5). The results indicated that pH, TP, Chl a and DAC significantly contributed to the variation of bacterial communities ($P<0.01$), while TN and N/P had almost no correlation ($P>0.05$). The family *Rhodobacteraceae* was positively correlated to pH, Chl a and DAC, whereas *Alteromonadaceae* had negative correlations.

The group treated for 0 h (S1_1, S1_2, S1_3) and the group treated for 12 h (S2_1, S2_2, S2_3) were located promiscuously in the ordination diagram. These two groups were positively correlated with pH, TN, TP, Chl a, and DAC, and negatively correlated with N/P. Samples treated for 24 h (S3_1, S3_2, S3_3) were positively correlated with TN and TP, and negatively correlated with N/P. Samples treated for 36 h (S4_1, S4_2, S4_3) and samples of 48 h (S5_1, S5_2, S5_3) were located closely in the ordination diagram.

Table 2. Environmental variables from 15 P. donghaiense cultures treated with the post-culture medium for 0 h (S1_1, S1_2, S1_3), 12 h (S2_1, S2_2, S2_3), 24 h (S3_1, S3_2, S3_3), 36 h (S4_1, S4_2, S4_3), 48 h (S5_1, S5_2, S5_3).

Samples	pH	TN (μg/mL)	TP (μg/mL)	N/P	Chl a (μg/mL)	DAC ($\times 10^5$ cells/mL)
S1_1	8.33	10.93	1.12	9.72	0.021	25
S1_2	8.06	11.07	1.21	9.11	0.016	27
S1_3	8.16	11.00	1.01	10.86	0.018	24
S2_1	8.18	11.11	1.03	10.74	0.031	21
S2_2	8.13	11.28	1.10	10.23	0.030	19
S2_3	8.22	11.23	1.03	10.92	0.035	17
S3_1	7.62	11.09	1.12	9.87	0.014	9
S3_2	7.55	10.90	1.02	10.67	0.008	10
S3_3	7.66	11.08	1.10	10.07	0.009	8
S4_1	7.29	11.05	1.04	10.68	0.007	0
S4_2	7.24	10.98	1.09	10.03	0.010	0
S4_3	7.19	11.05	1.05	10.52	0.004	0
S5_1	7.23	11.00	1.04	10.59	0.005	0
S5_2	7.18	11.08	0.80	13.87	0.002	0
S5_3	7.2	11.02	1.04	10.65	0.003	0

Abbreviations: TN, total nitrogen; TP, total phosphorus; N/P, ratio of nitrogen to phosphorus; Chl a, chlorophyll a; DAC, density of algae cells

These two groups were positively correlated with N/P and negatively correlated with pH, TN, TP, Chl a and DAC.

Discussion

Variation of Phycosphere Bacterial Community of *P. donghaiense*

Ace, Chao, Shannon and Simpson indices were used to estimate microbial community diversity [31]. Our results showed that Shannon and Simpson indices had significant difference, indicating that the bacterial diversity increased during the culture. It was already known that algae cells can release dissolved organic carbon (DOC) to phycosphere, and the concentration of DOC remains high even when algae cells were lysed [32-33]. Some bacteria in the phycosphere, like Proteobacteria and Bacteroidetes, can use these substrates as carbon sources [34-36]. It was not difficult to explain why Proteobacteria dominated (>95%). As reported by many researchers, Proteobacteria, Bacteroidetes, Verrucomicrobia and Actinobacteria were found mostly in coastal areas or the phycosphere [11, 37-38]. However, only a minor fraction of sequences (<5%) identified in our study were Bacteroidetes and Verrucomicrobia, which have been shown to be associated with phytoplankton [39]. At the family level, the most abundant bacteria were *Rhodobacteraceae* and *Alteromonadaceae*. Interestingly, the abundance of *Alteromonadaceae* increased and *Rhodobacteraceae* decreased during the lysis of algal cells. It has been reported that *Rhodobacteraceae* could benefit from phytoplankton bloom and its relative abundance had positive correlation with Chl a concentration [40]. Our results drew a similar conclusion, which showed that the density of algae cells and Chl a concentration reduces gradually during the algicidal process due to algae cell lysis (Table 1), and *Rhodobacteraceae* was positively correlated with Chl a and DAC (Fig. 5). Increasingly low Chl a concentration was correlated with the decrease of *Rhodobacteraceae* (Table 1). A large amount of DOC was released during algae cell lysis [41]. *Alteromonadaceae* could rapidly respond to the disturbance and profited from allochthonous carbon input [40], explaining the increased abundance in the anaphase of algicidal process (Fig. 3). It was worth mentioning that the most common algicidal bacteria reported in the literature were Proteobacteria, including *Alteromonas* or the *Bacteroides* [11, 42]. If the concentration of *Alteromonadaceae* has not reach an effective killing density, the compound contained in conditioned media may stimulate the growth of *Alteromonadaceae*. We speculated that the increase of *Alteromonadaceae* and the lysis of algae cells form a positive feedback loop. Our speculation was confirmed by PCA results, which demonstrated that samples were clustered into four groups with variation tendency of bacterial flora. Behrenfeld et al. [43] and Chen et al. [44]

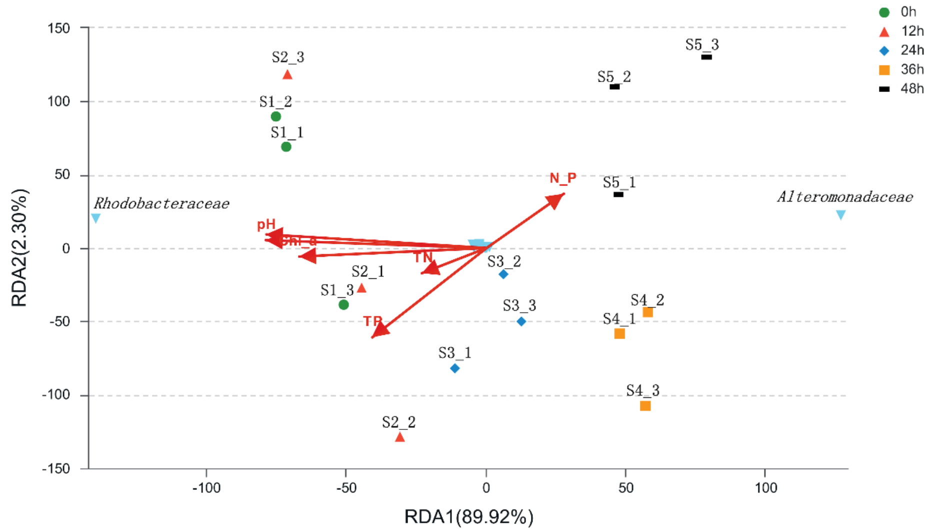

Fig. 5. Redundancy analysis (RDA) ordination diagram showing the relationship among samples, bacteria and environmental factors. TN, total nitrogen; TP, total phosphorus; N/P, ratio of nitrogen to phosphorus; Chl a, chlorophyll a; DAC, the density of algae cells.

proposed that bacterial community compositions were resilient to phytoplankton blooms to a certain extent, whereas our results showed that bacterial community compositions changed irreversibly after treatment with conditioned media. Collectively, our results supported bacterial community interactions that were crucial factors and that regulated the growth and death of algae cells, and we speculated that the variation of bacterial community composition was one of the factors that induced the lysis of algae cells.

Relationship Between Bacterial Community Composition and Environmental Factors

The density of algae cells and Chl a concentration was reduced gradually during the algicidal process because of the algae cell lysis (Table 1). Moreover, pH reduced gradually and the water samples changed from alkaline to neutral. The pH of samples increased in the initial state because algae cells could maintain photosynthesis in low CO_2 condition when the number of algae cells were high; however, pH turned increasingly lower as the algae cell number became less and less under the influence of conditioned media [44]. Consistently, the samples treated with conditioned media for 0 h and 12 h were positively correlated with pH, Chl a and DAC. However, the samples treated with conditioned media for 36 h and 48 h were negatively correlated with these indices. TN and TP of samples showed no significant variation (P>0.05) on account of the consumption of nitrogen and phosphorus by algae cells, and the release by lytic algae cells simultaneously [11]. Consistently, the bacterial community compositions had no correlation with TN and TP (P>0.05).

What's more, *Rhodobacteraceae* and *Alteromonadaceae* had a strong oppositional correlation because *Rhodobacteraceae* was positively correlated with pH, Chl a and DAC, while *Alteromonadaceae* was negatively correlated with them. The result was also consistent with the report that *Rhodobacteraceae* could benefit from phytoplankton bloom and its relative abundance had a positive correlation with Chl a [40].

In conclusion, we isolated *Halomonas* sp. DH-e, which had an algicidal effect on *P. donghaiense* from China's Zhejiang coast in previous studies. In the present study, the results showed that bacterial community composition in phycosphere of *P. donghaiense* was influenced by conditioned media of *Halomonas* sp. DH-e. We demonstrated that phycosphere bacterial community structure shifted dynamically and the diversity varied with the treatment time extended. Proteobacteria was a core microbial community due to its higher relative abundances than others. Interestingly, the abundances of *Alteromonadaceae* increased and *Rhodobacteraceae* decreased significantly as treatment time was prolonged. We speculated that the increasing concentration of *Alteromonadaceae* and the lysis of algae cells formed a positive feedback loop and that the bacterial community composition played a significant role in the lysis of algae cells.

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Conflict of Interest

The author declares no conflict of interest.

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