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Comparative metatranscriptomic profiling and microRNA sequencing to reveal active metabolic pathways associated with a dinoflagellate bloom

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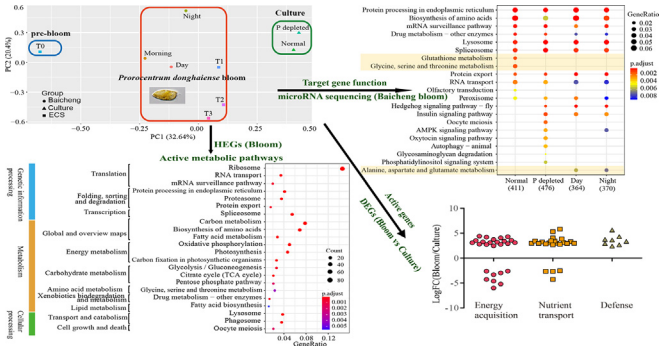
HIGHLIGHTS

- Nutrient-addition bioassays showed that none of phosphate, nitrate and trace metal nutrients was limited for the growth of phytoplankton.
- *P. donghaiense* highly expressed genes related to nutrient uptake, phagotrophy, energy metabolism and carbohydrate metabolism during the bloom.
- Many genes in *P. donghaiense* were up-regulated at night, including phagotrophy and environmental communication genes, and showed active expression in mitosis.
- Eight microbial defense genes were up-regulated in the bloom.
- Seventy-six *P. donghaiense* microRNA were identified, and their target genes strongly regulated amino acid metabolism in the bloom.

GRAPHICAL ABSTRACT



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ABSTRACT

Harmful algal blooms (HABs) have increased as a result of global climate and environmental changes, exerting increasing impacts on the aquatic ecosystem, coastal economy, and human health. Despite great research efforts, our understanding on the drivers of HABs is still limited in part because HAB species' physiology is difficult to probe *in situ*. Here, we used molecular ecological analyses to characterize a dinoflagellate bloom at Xiamen Harbor, China. *Prorocentrum donghaiense* was identified as the culprit, which nutrient bioassays showed were not nutrient-limited. Metatranscriptome profiling revealed that *P. donghaiense* highly expressed genes related to N- and P-nutrient uptake, phagotrophy, energy metabolism (photosynthesis, oxidative phophorylation, and rhodopsin) and carbohydrate metabolism (glycolysis/gluconeogenesis, TCA cycle and pentose phosphate) during the bloom. Many genes in *P. donghaiense* were up-regulated at night, including phagotrophy and environmental communication genes, and showed active expression in mitosis. Eight microbial defense genes were up-regulated in the bloom compared with previously analyzed laboratory cultures. Furthermore, 76 *P. donghaiense*

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Nutrient addition bioassay
Active metabolic pathways

microRNA were identified from the bloom, and their target genes exhibited marked differences in amino acid metabolism between the bloom and cultures and the potential of up-regulated antibiotic and cell communication capabilities. These findings, consistent with and complementary to recent reports, reveal major metabolic processes in *P. donghaiense* potentially important for bloom formation and provide a gene repertoire for developing bloom markers in future research.

1. Introduction

Harmful algal blooms (HABs) result from rapid growth of an algal species spared from grazing or other cell loss processes (Pinckney et al., 1997). The occurrence of HABs has increased in frequency, causative species, geographic extent, and severity of devastating impacts apparently due to anthropogenic activities, global climate and environmental changes (Anderson et al., 2012; Gobler et al., 2017; Hallegraeff, 2015; O'Neil et al., 2012; Wells et al., 2015). Many HAB species produce toxins that directly poison animals or indirectly threaten human health through consumption of seafood (Grattan et al., 2016; Morabito et al., 2018). HABs are caused predominantly by dinoflagellates though also by other groups of algae (Mohamed, 2018).

Our understanding on the causes of HABs has advanced dramatically in the last two decades thanks to the advent of new monitoring and analytical technologies (Elisa et al., 2017; Hess et al., 2017; Sellner et al., 2003). However, although research results converge generally on the associations of HABs with climate change and increasing nutrient discharges by humans, the lack of an identified "smoking gun" for all HABs points to the likelihood that each HAB event is unique to a specific ecosystem or species, and specific set of environmental conditions. This raises a need to investigate the cause of a HAB in a case-by-case manner. It is particularly important to examine the metabolic activities and physiologies of the HAB species during the bloom, which is hardly possible with physiological and ecological methodologies. Metatranscriptomics provides an approach to addressing this need by attributing expressed genes underlying specific metabolic pathways to source organisms and by estimating relative expression levels of these genes to assess the relative importance of these metabolic pathways in the bloom.

Metatranscriptomic studies on phytoplankton in general have provided new insights into microalgal processes in natural marine environments, including dinoflagellate genes not documented previously such as rhodopsins and histones (Lin et al., 2010), molecular responses to varied iron supply in diatoms and dinoflagellates (Marchetti et al., 2012), community structure and function distribution under different marine conditions (Alexander et al., 2015a; Bengtsson et al., 2018; Carradec et al., 2018), diatom nutrient–responsive gene expression patterns (Alexander et al., 2015b). For blooms or HABs, the rare but increasing metatranscriptomic efforts have provided insights into molecular regulation of dinoflagellate blooms (Cooper et al., 2014; Gong et al., 2017; Zhang et al., 2019; Zhuang et al., 2015) and raphidophyte blooms (Ji et al., 2018), particularly shedding light on how the bloom species outcompeted coexisting species from molecular perspectives. For example, Cooper et al. (2014) found that during bloom, the causative dinoflagellate exhibited higher expression of toxin biosynthesis relate genes, whereas Gong et al. (2017) reported enhanced expression of cell growth related metabolic pathways and biotin and thiamine synthesis genes to meet elevated nutrient demands in the bloom. The study on *Alexandrium catenella* (formerly *A. fundyense*) revealed active expression and up-regulation of genes related to nitrogen (N)-scavenging (including cyanate and urea), CO2-concentrating and saxitoxin production during its devastating bloom (Zhuang et al., 2015). And, a time-sequential metatranscriptomic profiling revealed that differential abilities in nutrient acquisition, energy harnessing and microbial defense drive a diatom dominant phytoplankton community to a dinoflagellate bloom (Zhang et al., 2019). These demonstrate the power of metatranscriptomic technique for dissecting bloom outbreak mechanisms and the need for broader exploration of the technique in HAB studies.

In this study, we seized an opportunity of a dinoflagellate bloom that occurred at Baicheng beach of Xiamen Harbor, China, and performed molecular and ecological analyses for samples collected at three time points (night, the early morning or Morning, midday) of the day. We used 18S rDNA metabarcoding and microscopic analyses to identify the bloom species and co-existing phytoplankton species. Nutrient-addition bioassay was carried out to examine if the phytoplankton assemblage was generally experiencing limitation of nitrogen, phosphorus, and trace metal nutrients. Metatranscriptomic analysis was carried out to reveal metabolic pathways active in the bloom. Highly expressed genes (HEGs) were grouped in functional pathways and compared among the three different time points during the bloom and with a previously published bloom (Zhang et al., 2019) as well as laboratory cultures (Shi et al., 2017). As microRNA regulation of gene expression appears to be important in dinoflagellates, which have limited transcriptional regulation (Lin et al., 2015), we also profiled microRNA from the samples. *P. donghaiense* microRNAs and their predicted target gene transcripts identified in the bloom were also compared with those from the previous results on cultures.

2. Methods

2.1. Samples collection and environmental measurements

Surface seawater (0–2 m) samples were collected from May 6 to May 7 in 2014 when a "Red Tide" bloom was visually observed at Baicheng Beach, Xiamen Harbor (24°25′ N, 118°6′ E, Fig. 1). Samples were collected at 11:00 pm on May 6 (night), 5:00 am (early morning, Morning) and 1:00 pm (midday) on May 7. For each time point, 500 mL seawater was prefiltered through a 200 μm nylon mesh to remove large particles and zooplankton, then filtered onto 3 μm pore-size 42 mm diameter polycarbonate membrane (Merck Millipore, MA, USA) using a vacuum pump under low vacuum pressure (<10 PSI). The filters were cut into four even pieces, and each was immediately transferred to a 2 mL tube; two tubes containing 1 mL TRI Regent buffer for RNA and two containing 1 mL DNA lysis buffer (100 mM Tris-Cl, 50 mM EDTA, pH = 8) for DNA. The samples for RNA were snapped frozen in liquid N_2 and then stored at −80 °C until RNA extraction. The samples for DNA were stored at −20 °C until DNA extraction.

Meanwhile, a set of other samples was collected for microscopic examination and measurements of nutrients and chlorophyll *a*. Fifty-mL seawater samples were collected and fixed in Lugol's solution (2%) for subsequent microscopic species identification and enumeration in Sedgwick-Rafter counting chamber. Fifty-mL seawater samples were filtered through 0.2 μm membrane and the filtrates were stored at −80 °C for subsequent nutrient measurements, which were actually carried out for the midday and night samples, but not for Morning due to accidental loss of the sample. Concentrations of inorganic nitrogen (nitrate and nitrite), silicate and phosphate were determined using continuous flow analyzer (San++, SKALAR, Holland) (Li et al., 2017). Another set of 50-mL samples was filtered onto GF/F membranes and

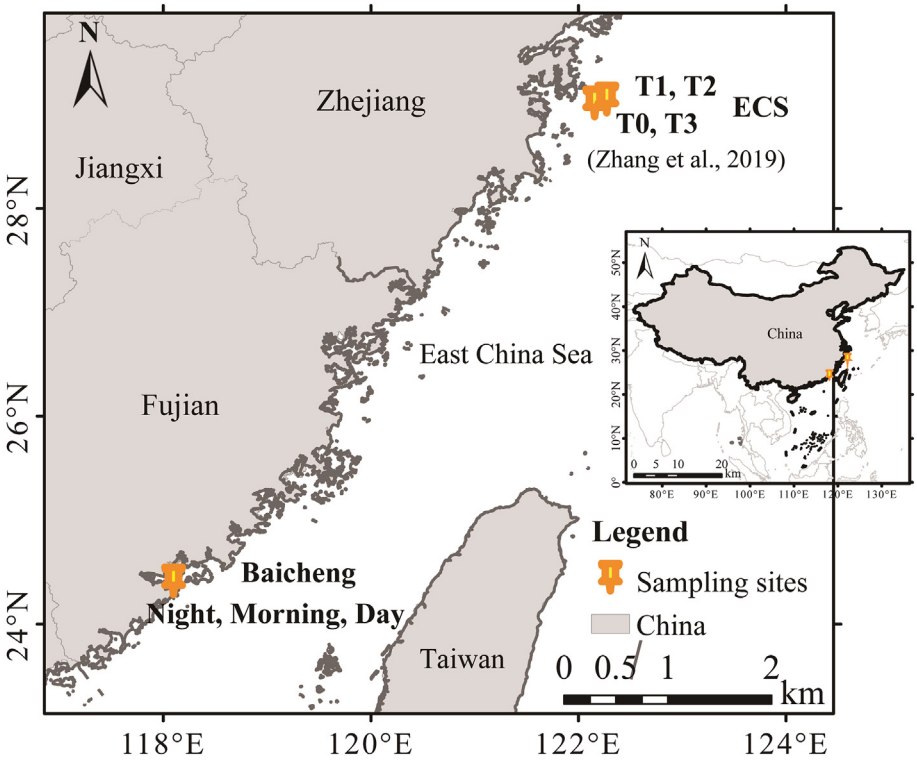

Fig. 1. Sampling sites of the study.

stored at −80 °C for chlorophyll *a* measurement using Trilogy Laboratory Fluorometer (Turner Designs, USA).

2.2. Nutrient addition bioassay

One liter of live bloom water was collected at 1:00 pm on May 7. Zooplanktons were removed from the water samples by filtering through a 200-μm mesh to minimize the effects of grazing (Paerl et al., 2011). The remaining water sample was brought to the laboratory immediately (45 min in air-conditioned car set at sea surface temperature) for nutrient enrichment incubation. Water sample was diluted five times using artificial seawater to avoid cell density saturation. Fifteen cultures of 100 mL volume contained in new sterilized NEST cell culture 200-mL flasks were set up, with triplicate for the control (no nutrient addition) and each of the four nutrient additions, phosphate (3.6 μM), nitrate (44 μM), phosphate + nitrate (3.6 μM P + 44 μM N) and trace metal (585 pM iron, 1.915 pM copper, 3.825 pM zinc and 45.5 pM manganese). Incubation was carried out for 2 days at 20 °C under a 14 h:10 h light dark cycle and a photon flux of 100 μE m^{-2} s^{-1}. Cell concentration was measured microscopically every day to follow the growth of dominant species *Prorocentrum* spp. using a previously reported method (Wang et al., 2016). Differences in growth among the nutrient addition groups and the control were analyzed using repeated-measures analysis of variance (RM ANOVA) (Winer et al., 1962). RM ANOVA analyses were followed by all pairwise multiple comparisons (*post hoc* testing), using the Holm-Sidak method.

2.3. DNA extraction and high-throughput 18S rDNA metabarcoding

DNA from the three time-point samples in duplicate was extracted following a recently improved protocol (Yuan et al., 2015). The DNA concentration and quality was determined using NanoDrop spectrophotometer (ThermoScientific, Wilmington, DE, USA). The DNA extracts were used as the templates in PCR. Primers 18SV4-F (5′-GGCAAGTCTGGTGCCAG-3′) and 18S V4-R (5′-GACTACGACGGTAT CTRATCRTCTTCG-3′) were used to amplify the V4 variable regions of the 18S rRNA gene (rDNA) (Brate et al., 2010). The PCR amplification was carried out in a total volume of 50 μL, containing 5 μL of DNA (5 ng), 2 μL of each fusion primers including 18S V4 primer sequence, sequencing adaptor and Illumina FC sequences (10 μM), and 25 μL Enzymatics Veraseq 2.0 Master Mix. Thermal cycle consisted of an initial denaturing step at 95 °C for 3 min; followed by 35 cycles of 95 °C for 30s, 60 °C for 45 s and 72 °C for 45 s; and a final extension at 72 °C for 10 min. From the PCR products, Illumina sequencing libraries (Miseq PE300) were prepared and sequenced. The sequencing output was analyzed using the Qiime2 pipeline. Dada2 was used to filter low quality reads and obtain features table. Features from two replicates were averaged as final quantification. Taxonomic assignment of the features was performed based on the SILVA 132 release and NCBI nucleotide database.

2.4. RNA extraction and metatranscriptomic and microRNA sequencing

RNA was extracted as previously described (Zhang et al., 2019). Shortly, each of the samples was mixed with a 1:1 mixture of 0.5 mm and 0.1 mm-diameter zirconia/silica beads (Biospec, USA), and beat at the rate of 6 m per second on a FastPrep-24 bead mill (MP Biomedicals, USA) for three times to ensure complete cell breakage. RNA was extracted following the TRI Reagent protocol coupled with the Direct-zol™ RNA columns (which is designed for isolating both small RNA and total RNA), essentially as reported previously (Lin et al., 2010). RNA concentration was measured using a NanoDrop ND-2000 Spectrophotometer, while integrity was assessed using RNA 6000 Nano LabChip Kit in microcapillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Australia). Samples with the RNA integrity number (RIN) ≥ 6.0 were used for metatranscriptome sequencing. The same RNA samples were used for mRNA and microRNA (miRNA) sequencing. For Illumina metatranscriptomic sequencing, 1 μg total RNA from each sample was used to generate a paired-end RNA-Seq library by polyA mRNA workflow using a NEBNext® Ultra™ RNA Library Prep Kit (NEB, USA). Small RNA libraries were generated using NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB, Ipswich, MA, USA)

with the same amount of total RNA as used in RNA-seq. Sequence files of mRNA and microRNA have been deposited into NCBI Sequence Read Archive under the accession number SRR8881733, SRR8881734, SRR8881735, SRR8881736 and SRR8881737 for mRNA sequences of marine bloom community at Night, Morning and Day, and microRNA sequences at Night and Day, respectively.

2.5. Overall gene function profiling

Raw reads of mRNA sequencing were preprocessed by trimming adapter sequences and low quality reads (Q value ≤ 20 accounts for ≥40% of the read, and ≥10% N containing reads). The filtered high quality reads were then mapped to SILVA release 122 to remove the ribosomal RNA reads. The remaining clean reads were used for downstream analyses. To retrieve all expressed genes in the bloom community, RNA-Seq reads were assembled *de novo* into contigs using Trinity (r2013-02-25) (parameter: -default) (Haas et al., 2013). The number of reads in each sample mapped to each contig was counted to estimate expression level of the gene represented by the contig. Open reading frames in contigs were predicted using FragGeneScan (Rho et al., 2010). The resulting deduced protein sequences were searched against eggNOGV4.1 database (Roth et al., 2013) for COG annotation with a 1e-5 value cutoff by BLAST tool. The read counts of each COG were summarized. Unassigned reads were excluded from further functional analysis.

2.6. Identification of highly or differentially expressed genes

To annotate taxonomically and functionally, the clean reads were mapped to a local integrated database (combining MMETSP datasets with transcriptomes of *Amphidinium carterae*, *Karenia mikimotoi* and *P. donghaiense* transcriptomes (Shi et al., 2017) using Bwa 0.7.12-r1039 (parameters: -defalt) (Langmead and Salzberg, 2012). The mapped reads with quality ≥ 20 were used in further analysis. As *P. donghaiense* was shown to be the bloom species by the microscopic and metacoding data, further deeper analysis was focused on this species. Expression levels were quantified using RSEM, and normalized as RPKM value (Reads Per Kilobases per Million reads).

To obtain a robust active genes set associated with *P. donghaiense* bloom, we compared current transcriptomic data with that of another *P. donghaiense* bloom, which occurred in East China Sea (ESC; data covering pre-bloom sample of T0 and bloom period samples of T1, T2, T3) (Zhang et al., 2019) and that from *P. donghaiense* cultures (phosphate-replete and P-depleted conditions). RNA extraction and sequencing methods in the previous studies were the same as in the current study. Primary component analysis (Fig. S1) showed that the bloom samples from ESC (T1, T2, T3) and Baicheng (Morning, day, and night) clustered in a group, the pre-bloom from ESC (T0) and culture samples (P-replete, P-depleted) were separated from each other and both separated from the bloom samples. Further comparative analysis was based on this clustering pattern, with samples within each group treated as replicates. Highly expressed genes (HEGs), defined as RPKM > 75% quatile of expressed genes in each sample, were used to conduct KEGG enrichment in the Baicheng (night, Morning and midday) or ESC (T1, T2, T3) bloom samples. Genes classified into pathways of human disease and organismal systems were excluded for further analysis. HEGs were identified in the Baicheng bloom samples (night, Morning and midday) and the culture samples (P-replete and P depleted) (Shi et al., 2017), and differential expressed genes (DEGs) were identified between the Baicheng bloom and the culture samples. Differential expression was analyzed using edgeR Bioconductor package (Robinson et al., 2010) Genes with fold changes > 2 and FDR < 0.05 were defined as differentially expressed genes (DEGs) for *P. donghaiense*.

2.7. Trend analysis

For the three time points in the Baicheng bloom event, trend analysis was conducted with short time-series expression miner (STEM), resulting in gene expression profiles. As there were no replicates (samples from each time point were pooled for sequencing due to the high cost back in 2014), the analysis was limited to trend finding rather than quantifying differential expression of individual genes. The data was first log-transformed, the maximum unit change in model profiles between time points being set at 1, maximum number of model profiles set at 8, and other parameters at default. To investigate gene function distribution of the temporal profiles, genes in each profile were assigned to KEGG pathways. And KEGG pathways with ≥4% relative gene contribution (gene in KEGG pathway/total gene number in each profile) at least in one profile were used to further explore gene functions.

2.8. MicroRNA analysis

For miRNA raw dataset, low quality reads (>1 base with Q-score < 20 or N containing reads) were filtered. Sequences mapped with non-coding RNAs (rRNA, scRNA, snoRNA, snRNA and tRNA) from Genebank and Rfam 11.0 databases were also excluded. The remaining clean data was used to identify known miRNA against miRBase, and novel miRNA by MIREAP v0.2 with default parameters. To focus on miRNAs from *P. donghaiense*, miRNA data from *P. donghaiense* P-replete and P-depleted cultures (Shi et al., 2017) were used as the reference to identify *P. donghaiense* miRNA from our bloom samples (night and midday). miRNAs that matched those expressed in at least one cultured sample were kept for further analysis. We used patmatch v1.2 to predict target mRNAs of these miRNAs. The expression level of miRNA was normalized by TPM (Transcript Per Million). Differential expression analysis of miRNA was compared between culture samples and bloom samples using the DEGseq R package (Wang et al., 2010). The differentially expressed miRNAs were set as P value ≤ 0.05 and fold changes ≥ 2. GO or KEGG pathway enrichment was analyzed for the DEGs and the predicted target genes of microRNA (adjust P value, or Q value ≤ 0.01).

3. Results

3.1. Environmental conditions and community structure in the Baicheng bloom

A Red-Tide bloom was visually observed on May 6, 2014 at Baicheng Beach, Xiamen Harbor (Fig. 1). The weather during bloom was mostly cloudy and occasionally rainy, resulting in a low salinity concentration (17 practical salinity unit). The pH of seawater was 7.89. Seawater samples collected at Night, Morning and Day covered different times of the day. Chl *a* concentration we measured was 184.55 (Day) to 322.86 μg/L (Night) (Table 1), indicating an overwhelming bloom condition. Microscopic analysis showed that the bloom species was *P. donghaiense* and its concentration was 1.96×10^7 cell/L in the Day sample. 18S rDNA metabarcoding analysis showed that *Prorocentrum* accounted for 97.10%, 72.59% and 91.69% in Night, Morning and Day samples (Fig. 2), consistent with the microscopic cell count result. The other consituents of the phytoplankton community were mainly Syndiniales (1.56%, Alveolata), Gonyaulacales (0.63%, Alveolata), and Mediophyceae (0.49%, Stramenopiles).

Table 1
Environmental parameters measured when Day and Night samples were collected.

Sample	Chl *a* (μg/L)	PO_4^{3-} (μM)	NO_2^- (μM)	NO_3- (μM)	SiO_3^{2-} (μM)
Night	322.86	0.63	11.34	55.27	66.26
Day	184.55	0.22	8.11	42.75	72.23
Average	253.71	0.43	9.73	49.01	69.25

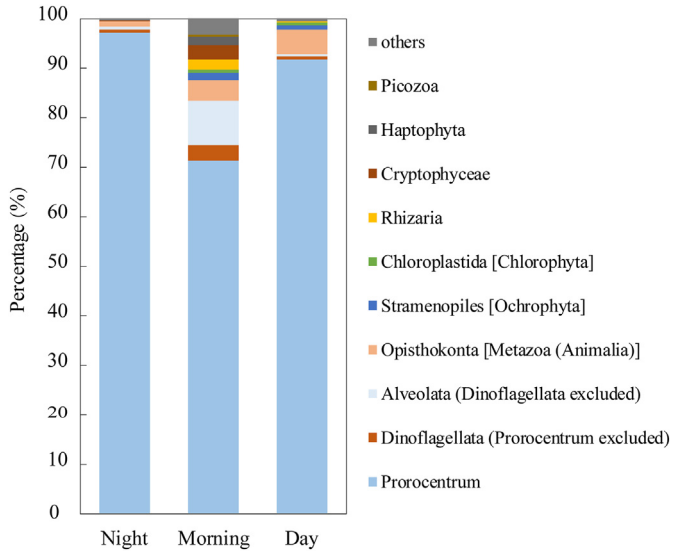

Fig. 2. General plankton community structure based on 18S rDNA metabarcoding. The square bracket in the legend indicates the most dominant contributor in the category.

Concentrations of nutrients, including phosphate, nitrite and nitrate, averaged 0.43, 9.73 and 49.01 μM, respectively, and overall were higher at Night than Day, indicating rapid consumption early at day and abundant resupply at night. Silicate concentration was 66.26 and 72.23 μM at Night and Day, respectively, showing the opposite trend compared with the other nutrients (Table 1).

3.2. Nutrient-addition bioassay results

Cell concentrations of the control and the nutrient-amended (phosphate, nitrate, phosphate + nitrate, trace metal) cultures all increased over the two-day incubation. However, none of the nutrient additions enhanced growth compared to the control ($P > 0.05$, RM ANOVA, Fig. S2). This result indicated that none of these nutrients were limited for the growth of phytoplankton including the bloom species, *P. donghaiense*.

3.3. General functional gene profiles in the Baicheng bloom assemblage

The reads assignment result showed that the expressed genes were from 8 phyla of organisms (Fig. S3A) besides the unclassified organisms. The most highly represented was dinoflagellates, in which *P. donghaiense* accounted for 92.78%, 50.20, and 85.52% at Night (May 6), Morning (May 7), and Day (May 7), respectively. Ranked second was diatoms, which contributed about 1.05–14.66% of the total metatranscriptomic reads (with *Skeletonema dohrnii* as the dominant species). The most strongly expressed genes in *S. dohrnii* were NADH dehydrogenase and ras-related protein (Supplementary dataset 1).

To determine the overall functional distribution of expressed genes in the three bloom samples, reads were classified into clusters of Orthologous Groups (COG). Totally, 23 KOG categories were identified, with similar landscapes in three samples from Baicheng bloom (Fig. S3B). The top three COGs were "post-translational modification, protein turnover, chaperones" (average 4.33%), "cytoskeleton" (3.82%) and "translation, ribosomal structure and biogenesis" (3.18%), representing the essential processes of cell formation. The fourth was "signal transduction mechanisms", accounting for 2.58% reads. The energy and nutrient related orthologous groups also exhibited high expression, with energy production and conversion accounting for 2.46% of total reads (fifth), and inorganic ion transport and metabolism accounting for 1.05%. The sixth, carbohydrate transport and metabolism, contributed 1.86% of reads. The "cell cycle control, cell division, chromosome partitioning" displayed a median expression level (0.67%) in the community, while "defense mechanisms" was the second lowest (0.10%).

3.4. Gene expression in P. donghaiense during the Baicheng bloom

We first attempted to identify genes that were very highly expressed (RPKM ≥ 5000) at least at two time points in Baicheng bloom. The top five so identified included ribosomal proteins (comp99336_c1, comp99336_c1), choloroplast peridinin-colorophyll a-binding protein (PCP, comp100620_c0), rhodopsin (comp101143_c2), nuclear protein (comp98756_c0) and photosystem II D1(comp53527_c0) (Fig. 3). Then, we tried to find temporal trends of all expressed genes. Four different diel expression patterns (A–D) were observed (Fig. S4A). Groups A and B exhibited the highest expression at Night or Day, and the others showed the lowest (C) or highest (D) expression in Morning. Profile A contained the greatest number of genes among the four groups. The majority of the genes in the above profiles were distributed in 25 KEGG pathways (Fig. S4B). Among these abundant pathways, genes in 22 pathways were clustered more in profile group A than B, indicating that more genes in these pathways showed higher expression at Night than Day. These included two cell growth and death (oocyte meiosis and gap junction), two cellular community (gap junction and focal adhesion), four transport and catabolism (endocytosis, phagosome, peroxisome, lysosome), potentially associated with grazing, four signal transduction (MAPK, cGMP-PKG, PI3K-Akt and cAMP) and among others. Besides, photosynthesis and ribosome were much more abundant in profile group B than A, indicating that gens in photosynthesis and ribosome pathways were more highly expressed at Day than Night.

Cell proliferation plays essential roles in bloom development, which is expected to result from mitosis. In our expressed gene dataset, we detected 150 mitotic (Supplementary dataset 2) genes. Among these genes, genes encoding proliferating cell nuclear antigen (PCNA) and 14-3-3 were most highly expressed in Morning. Genes coding for wee1-like protein kinase, cell cycle checkpoint protein, structural maintenance of chromosome, elongation factor 3, replication factor C subunit, cell cycle 14 and cyclin-dependent kinase 2, were most highly expressed at Night. And *defective entry into mitosis 1* (DIM1) and DIM2 were most highly expressed at Day.

3.5. Highly expressed gene shared between the Baicheng and ESC blooms

To obtain a robust set of *P. donghaiense* genes associated with the bloom of this species, common HEGs (with RPKM > 75% quartile of expressed genes) from our samples (46.5 Gbp in total, Table 2) and previously reported ECS bloom samples (42.1 Gbp, Table 2) (Zhang et al., 2019) were identified. There were 14,348 and 13,914 HEGs from the two bloom sample sets, respectively, and 8813 were shared in all the six bloom samples (Fig. 4A, Supplementary dataset 3). Among these common HEGs were 7 nitrate, 2 ammonium, 3 inorganic phosphate transporters, an alkaline phosphatase and a dependent methionine synthase genes (Fig. 4B, supplementary dataset 4). A total of 22 pathways were enriched by annotated common HEGs, which were functionally distributed in three sections: 1) genetic information processing, 2) metabolism, and 3) cellular processing (Fig. 4C), with the first two accounting for most of the pathways. Besides, several additional pathways were related to human and other animal's disease (Supplementary dataset 3); however, as their functions in phytoplankton are unknown, no further discussion will be given to these genes. The genetic information processing section included three sub-section: transcription (spliceosome), translation (ribosome, RNA transport, and mRNA surveillance pathway), and folding, sorting and degradation (protein processing in endoplasmic reticulum, proteasome and protein export), composing essential processes of protein synthesis. And the top two abundant sub-sections in the metabolism section were energy and carbohydrate metabolism. Energy metabolism comprised of oxidative

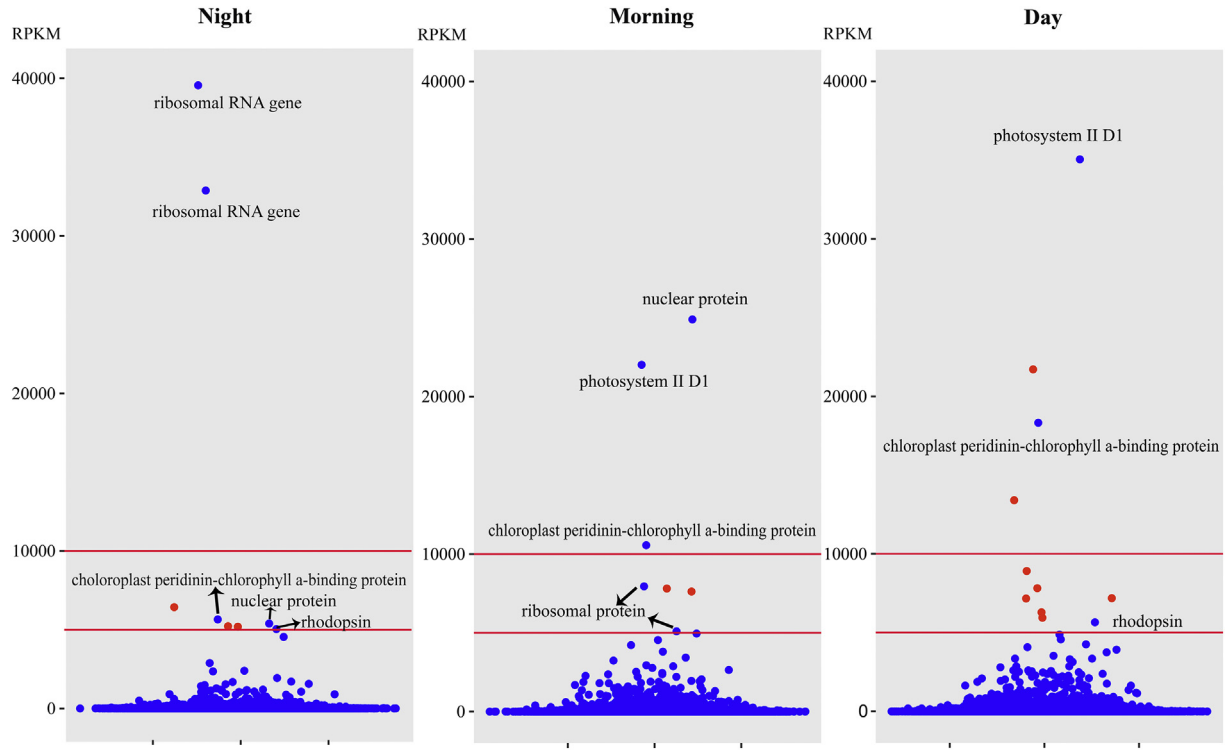

Fig. 3. Gene expression of *P. donghaiense* in the three bloom samples. Genes with RPKM ≥ 5000 are labeled with names except the orange dots, which either have no match or are matched to unknown genes.

phosphorylation, photosynthesis, and carbon fixation. The oxidative phosphorylation contained 39 active genes, among which 9 were V-type H^+-proton ATPase and 12 were F-type H^+-ATP synthase. The carbohydrate metabolism contained glycolysis/gluconeogenesis, TCA cycle and pentose phosphate pathways. The glycolysis/gluconeogenesis pathway consisted of many genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose-bisphosphate aldolase. TCA cycle was enriched of highly expressed malate dehydrogenase (MDH) and citrate synthase (CIS) genes. The cellular processing section included lysosome, phagosome and oocyte meiosis pathways.

3.6. Active expression of P. donghaiense energy and nutrient acquisition genes as well as microbial defense genes in the bloom compared to cultures

Comparisons of HEGs and DEGs were conducted between the Baicheng bloom and laboratory cultures (24.50 Gbp, Table 2). There

Table 2
Data used for comparative metatranscriptomic analysis.

Sample	Source	Condition	Data volume (Gbp)	Reference
Normal		L1 medium with 36 μM P	12.40	Shi et al., 2017
P depleted	Culture	L1 medium with 1 μM P	12.10	
T0		Pre/non-bloom (9:25, 30th April 2014)	8.56	
T1		Bloom (11:40, 13th May 2014)	10.92	
T2		Bloom (11:40, 15th May 2014)	10.72	
T3	ECS	Bloom (15:00, 20th May 2014)	11.89	Zhang et al., 2019
Night		Bloom (23:00, 6th May 2014)	15.60	
Morning		Bloom (5:00, 7th May 2014)	14.30	
Day	Baicheng	Bloom (13:00, 7th May 2014)	16.60	This study
Total	–	–	113.09	

were 14,348 common HEGs in our three bloom samples (Fig. S5A) and 40,837 in two culture ssamples, which composed 30 and 47 pathways, respectively (Fig. S5B). Of these, oxidative phosphorylation and pentose phosphate pathway were particularly enriched in both the bloom samples (Fig. S4B), which were also highly represented by common HEGs in Baicheng and ECS bloom sample sets, indicating a higher activity in energy and carbohydrate metabolism, respectively.

DEGs analysis indicated 2116 up- and 3578 down-regulated genes in *P. donghaiense* under the bloom compared to the culture conditions (Supplementary dataset 5). We detected a large number of transcripts encoding proteins that play roles in energy acquisition, nutrient transport and defense (Fig. 5, Supplementary dataset 6). In particular, photosynthesis genes PsbJ, PsbP, photosystem I psaA/psaB proteins, chloroplast ferredoxin, pohotosystem II reaction centre I proteins, and most of F-type ATP synthase (ATPase) genes, which are involved in photoenergy harvesting and conversion, exhibited higher expression in the bloom. Furthermore, genes encoding nutrient transporters, including nitrate, zinc, bidirectional sugar and sulfate transporters, were also up-regulated in the bloom. In addition, a total of 8 genes annotated as defense response to bacterium was up-regulated in the bloom, including pleurocidin, cyclotide, gallidermin, liver-expressed antimicrobial peptide 2 (LEAP-2) and antimicrobial peptides.

3.7. MicroRNA expression and their target genes in the Baicheng P. donghaiense bloom

To ensure *P. donghaiense* origin, only miRNAs that matched those that had been detected in *P. donghaiense* cultures were selected for further analysis. This screening yielded a total of 76 miRNAs from the two bloom (Night and Day) and two culture samples (Supplementary dataset 7), with 1 to 7458 target genes for each (Supplementary dataset 8). Of these miRNAs, 37 were novel while 39 matched previously documented miRNAs in miRBase database. KEGG enrichment comparison of the predicted target genes showed that 9 pathways were common in all four samples, such as protein processing, biosynthesis of amino acids,

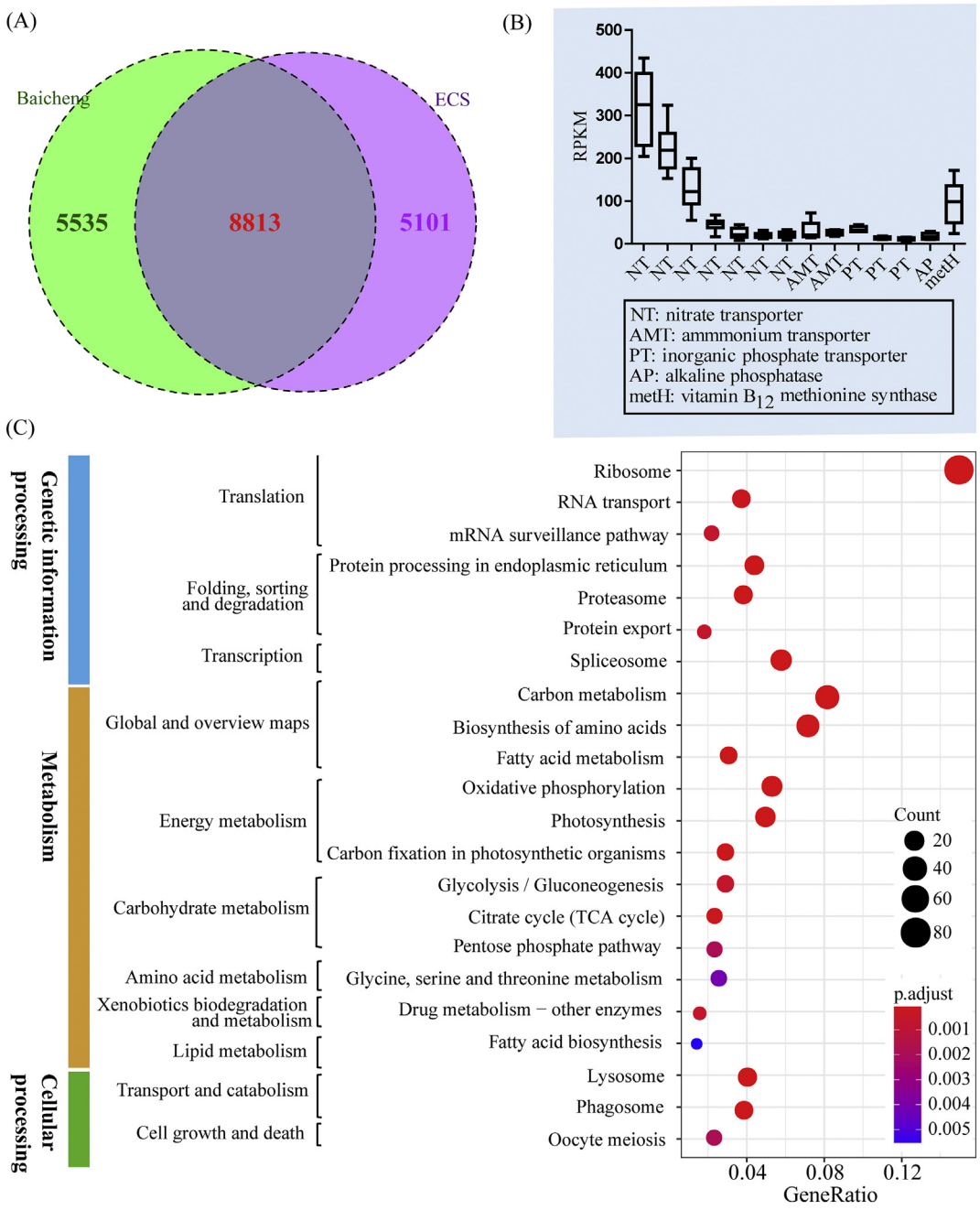

Fig. 4. Highly expressed genes in Baicheng bloom and ECS bloom of *P. donghaiense* samples. (A) Venn diagram showing high number (8813) of common and similar numbers (5535, 5101) of unique HEG in Baicheng and ESC blooms. (B) Common HEGs related to nutrient uptake. (C) KEGG enrichment of common HEGs from two bloom samples. Genes in each sample with RPKM >75% quartile of expressed genes were defined as highly expressed genes.

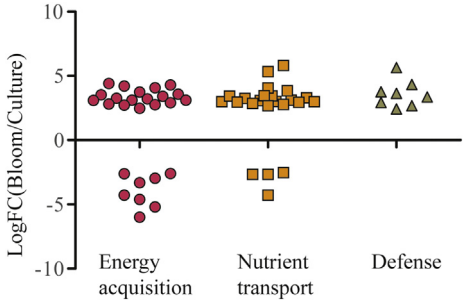

Fig. 5. Differentially expressed genes between Baicheng bloom and laboratory cultures for energy acquisition, nutrient transport and defense.

lysosome, mRNA surveillance, and spliceosome (Fig. 6A). In addition, alanine, aspartate and glutamate metabolism pathways were specifically enriched in the bloom. While glycine, serine and threonine metabolism, glutathione metabolism and olfactory transduction were specific in P-replete cultures.

Differential expression analysis indicated that 13 miRNAs, totally targeting 6858 genes, were differentially expressed between bloom and culture conditions (Fig. 6B). Among them, three top highly expressed microRNAs in the bloom included MIR396-x, MIR166-y and novel-m0045-3p. MIR396-x, predicted to regulate peroxidase and nucleotidyltransferase genes, displayed nearly opposite expression pattern (expression changes among bloom *vs.* culture samples) to the two target genes. MIR166-y was predicted to regulate calpain and xyloglucan galactosyltransferase genes, and exhibited similar

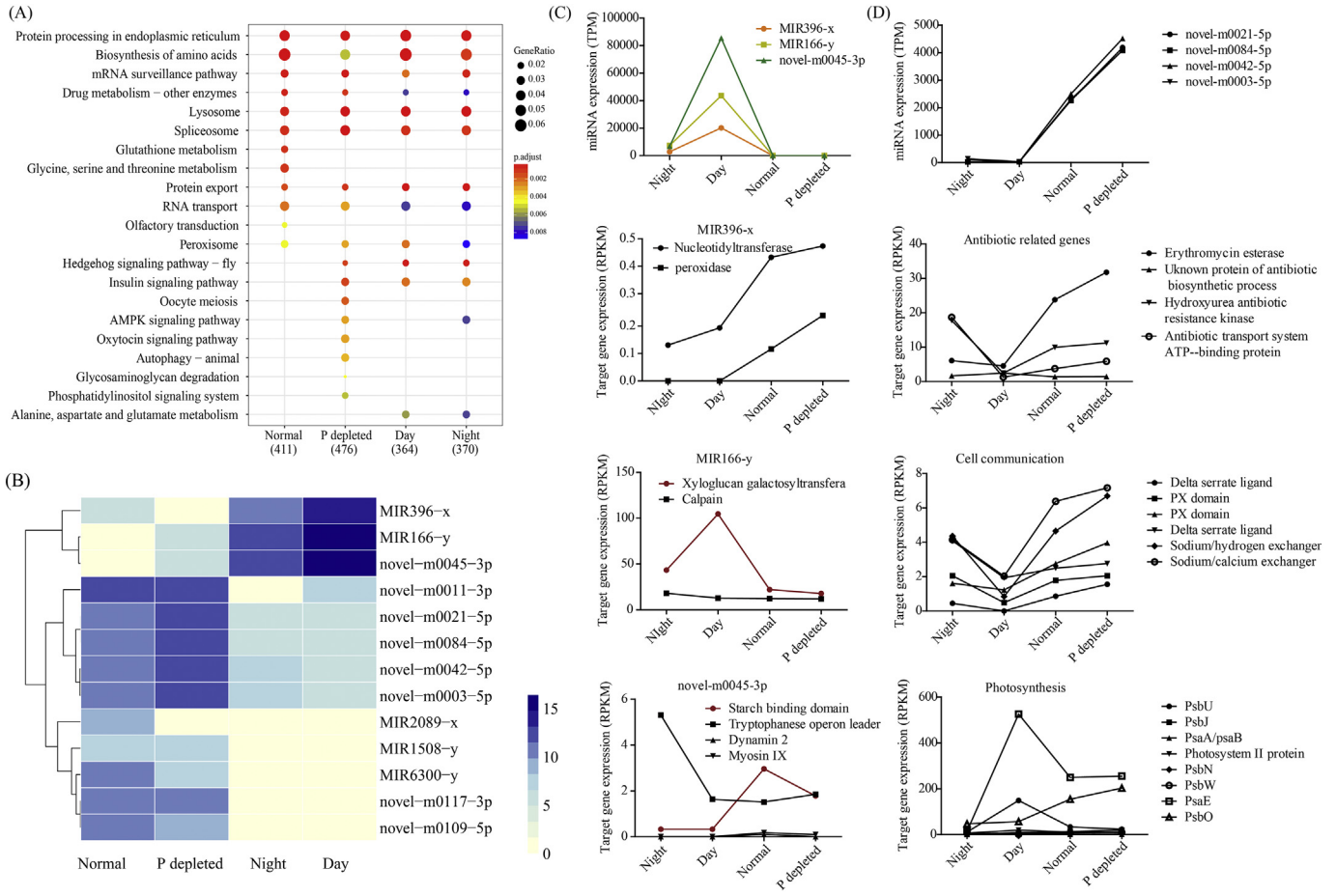

Fig. 6. Comparison between the current bloom and previous culture samples on expression levels of target genes and microRNA in *P. donghaiense*. (A) KEGG enrichment for miRNA target genes expressed in cultures (Normal and P-depleted) and current bloom (Day and Night). (B) Differential expression of miRNAs between culture and bloom conditions. (C) The top three most abundant differentially expressed miRNA in the bloom condition and their target gene expression. (D) The top four most abundant differentially expressed miRNA in culture conditions and their target gene expression.

expression profile xyloglucan galactosyltransferase gene. Novel-m0045-3p, predicted to regulate four genes encoding starch binding domain-containing protein, tryptophanese operon leader peptide, dynamin 2 and myosin IXB proteins, showed opposite expression pattern to the starch binding domain-containing protein (Fig. 6C). Additionally, four miRNAs were highly expressed in culture conditions, including novel-m0021-5p, novel-m0084-5p, novel-m0042-5p and novel-m0003-5p. Their target genes functionally distributed on antibiotic, cell communication and photosynthesis. Expression profiling showed that these four miRNAs displayed similar expression patterns to antibiotic and cell communication related genes, but opposite to most of photosynthetic genes (Fig. 6D).

4. Discussion

In situ physiological and molecular studies of harmful algal blooms are still sparse because of the difficulty of catching a bloom in a timely fashion. Here we were able to study a bloom because it occurred right off our campus, allowing us to respond immediately and collect samples at three time points for physiological and metatranscriptomic analyses. However, accompanying the emergency prompted study was the limitation in the lack of enough samples for tracking changes (especially those before the bloom) in bloom physiologies and environmental conditions, and thus the inability to interrogate linkages between the metatranscriptomic signals detected and environmental variables. Nevertheless, by comparing our metatranscriptomic data generated in the bloom to that of another bloom and that of laboratory cultures (representing a non-bloom condition) of the same species, bloom-specific signals can be detected to help understanding bloom drivers. Furthermore, as an algal bloom is a complex and poorly understood phenomenon, critical factors contributing to the bloom formation may be beyond the commonly measured environmental parameters, which can potentially be unveiled by metatranscriptomic analysis. Therefore, we seized this opportunity and conducted metatranscriptomic study to characterize highly expressed metabolic pathways that are potentially important in driving or maintaining the bloom. By comparing bloom samples sequenced in this study with those recently sequenced for another bloom (Zhang et al., 2019) as well as culture samples previously sequenced (Shi et al., 2017), genes and metabolic pathways putatively driving or associated with bloom formation of this species were identified, and the potential roles of these genes in the formation and maintenance of *P. donghaiense* bloom can be discussed.

4.1. Nutrient condition: potential roles of DOP utilization, vitamin-auxotrophy and phagotrophy in P. donghaiense blooms

Nutrients are believed to be one of the most important driving factors of HABs, implicating eutrophication as a major culprit (Anderson et al., 2008; Heisler et al., 2008). Results of the nutrient addition experiment in this study indicated that *P. donghaiense* (and co-existing phytoplankton species) was not N, P, or trace metal limited. Our measurement of nutrients in the ambient environment during the bloom also indicated high levels and rapid supply of major nutrients. The up-regulation of nitrate transporters compared with their

expression levels in the cultures suggests that *P. donghaiense* probably more actively used nitrate as the source of N-nutrient during the bloom, although both nitrate and ammonium transporters were highly expressed. Previous studies have documented that *P. donghaiense* is able to use both nitrate and ammonium (Hu et al., 2012). A recent culture study also showed that *P. donghaiense* grows as well on urea as on nitrate (Jing et al., 2018), but urea transporter and urease (enzyme required for utilizing urea) were not highly represented in our metatranscriptome, suggesting that urea was unlikely to be the major source of N-nutrient driving the bloom. This reiterates the increasing recognition that what is observed in the laboratory may not necessarily reflect what occurs in nature, underscoring the need for *in situ* studies.

Alkaline phosphatase (AP, comp95052_c0), an indicator of P-stress (Anderson et al., 2008; Lin et al., 2016), exhibited lower expression in *P. donghaiense* during the bloom (11–17 RPKM) than that in P-depleted cultures (20 RPKM) reported in Shi et al. (2017), a trend agreeable with the result of nutrient addition bioassay indicating no phosphorus-nutrient limitation. The active expression of AP, however, suggests that dissolved organic phosphate (DOP) utilization might still contribute to meeting P-nutrient demand. Besides, inorganic phosphate transporter genes were highly expressed in the bloom conditions, further suggesting that the bloom species probably heavily invested in the nutrient transporters to efficiently uptake P-nutrient from the environment.

Many algal species are vitamin auxotrophic, requiring different combinations in vitamin B_{12} (cobalamin), B_1 (thiamine) and B_7 (biotin) (Croft et al., 2006). Approximately one-half of microalgal species require cobalamin as a growth supplement (Andersen, 2005), and much higher proportion of harmful algal species requires B_{12} and B_1 (Tang et al., 2010). Although B_{12}-independent methionine synthase (*metE*) enables growth without cobalamin, as shown in *Chlamydomonas* and *Cyanidioschizon* (Helliwell et al., 2014), algae (*e.g. Chlamydomonas*) may preferentially use vitamin-B_{12}-dependent methionine synthase gene (*metH*) over *metE* when B_{12} is available. *Thalassiosira*, which requires cobalamin (Guillard and Ryther, 1962), only possesses *metH* gene. For *P. donghaiense*, no *metE* gene was detected but a *metH* exhibited high expression in the Baicheng bloom, and our search in the previously reported ESC bloom metatranscriptomic data (Zhang et al., 2019) also revealed high expression of *metH*. This suggests that *P. donghaiense* is probably a vitamin B_{12} auxotroph, as suggested previously (Tang et al., 2010). Moreover, *metH* seems to be expressed at higher levels in bloom samples (avg. RPKM of 95.95) than laboratory cultures (47.79 RPKM) (Shi et al., 2017) and pre-bloom samples (11.70 RPKM) (Zhang et al., 2019), suggesting stronger B_{12}-dependent metabolism in *P. donghaiense* under natural bloom conditions.

Most HAB species are known to be mixotrophic, even in eutrophic waters (Burkholder et al., 2008). *P. donghaiense* also has been reported to be able of phagotrophy (Jeong et al., 2005a; Jeong et al., 2005b). In this study, we found active expression, especially at nighttime, of genes associated with endocytosis, phagosome, peroxisome, and lysosome, features characteristic of phagotrophy (Cavalier-Smith, 2009). It is evident that active phagotrophy might have been one of the promoting factors of population growth and bloom formation for *P. donghaiense*.

4.2. Most active metabolic pathways in P. donghaiense during blooms: energy and carbohydrates

Our microscopic, 18S rDNA and mRNA sequencing results indicated that *P. donghaiense* was dominant both in abundance and metabolic activities during the bloom. In contrast, diatoms, the second most highly represented lineage in the metatranscriptomes, displayed very low expressed gene percentage overall, with NADH dehydrogenase and Ras-related protein genes being the most highly expressed genes in these diatoms. NADH dehydrogenase is a key component of NADH enzyme complexes, which connects electron transfer and proton translocation to drive ATP synthesis (Weiss et al., 1991). Ras superfamily has diverse roles, including cell growth and division regulation (Yang and Watson, 1993). The strong expression of NADH dehydrogenase and Ras-related protein in *S. dohrnii* in the bloom suggests that the co-existing diatoms probably heavily invest in energy metabolism and reproduction despite low abundance during a dinoflagellate bloom. This implies that *S. dohrnii* population might be under the control of grazing or other cell loss processes.

As energy metabolism is fundamental to phytoplankton growth, active expression of photosynthesis, energy conversion and oxidative phophorylation genes is cucial for the formation and maintenance of an algal bloom. This is ranked the fifth most abundantly expressed COG in the bloom community. PCP is a light harvesting component in dinoflagellates and related to circadian rhythm (Nassoury et al., 2001; Shi et al., 2018). The photosystem II D1 was also a light response protein in PSII reaction center and controlled by endogenous circadian rhythm (Nassoury et al., 2001). Both were among the top five highly expressed genes in the community and their expression levels increased from Night to Morning to Day. Furthermore, diverse DEGs in the photosynsthetic system are indicative of progressively elevated photosynsthesis during the bloom.

Proton pump rhodopsin (Béjà et al., 2000; Lin et al., 2010; Marchetti et al., 2015) functions independently of photosynthetic apparatus as an alternative way of capturing light energy to produce ATP (Shi et al., 2015). The rdohopsin gene in *P. donghaiense* (comp101143_c2) was also in the top five most highly expressed geneset in the present bloom, and was up-regulated in the present and a previously reported bloom with an average RPKM of 3283, compared to cultures with RPKM of 1571 and to pre-bloom period with RPKM of 131 (Zhang et al., 2019). These photoconversion related genes with high expression levels and up-regulation in the bloom (compared to cultures) imply that the bloom species utilizes a “dual engine” to efficiently capture photoenergy to promote population growth.

Oxidative phosphorylation is one of the major pathways to generate ATP (Bazil et al., 2016), and is controlled by cytoplasmic inorganic phosphate (Pi), the most important feedback signal (Bose et al., 2003). H-type ATPase contributes to production of ATP, while V-type ATPases, on the contrary, is a proton pump that takes the energy from ATP hydrolysis to produce a proton gradient driving ion uptake (Graham et al., 2003). More HEGs of H-type than V-type ATPase detected in the bloom suggests that ATP production rate through oxidative phosphorylation might be enhanced in the bloom. The actively expressed and specific up-regulation of oxidative phosphorylation under bloom conditions indicated that it might further add to energy production pathways for *P. donghaiense* to maintain dominance in the natural environment.

Carbohydrate transport and metabolism, the sixth most abundantly expressed pathway group in the bloom community, was represented by significantly enriched highly expressed genes in TCA cycle, glycolysis/gluconeogenesis and pentose phosphate in *P. donghaiense*. These pathways are essential for energy generation in all eukaryotes (Fernie et al., 2004). Malate dehydrogenase (MDH) is important for plant survival (An et al., 2016) for example, its expression was found to increase 5- to 6- fold in the light (Gotow et al., 1985). In the green alga *Chlamydomonas reinhardtii,* a study showed that the mutation of MDH dramatically influenced photosynthesis, and led to 1.5-time more lipid and 2-fold more starch accumulation under nitrogen deprivation (Kong et al., 2018). Citrate synthase (CIS) has been suggested to function in regulating the flow of fixed carbon into lipids in blue-green algae (Taylor, 1973). The high expression and up-regulation of MDH and CIS in TCA cycle might improve the algal survival. Glycolysis and gluconeogenesis are two opposing metabolic pathways involved in the degradation and synthesis of carbohydrates to maintaining glucose homeostasis (Metón et al., 2003). GAPDH has been shown to be involved in many cellular processes in addition to glycolysis (Tristan et al., 2011). Fructose-bisphosphate aldolase catalyzes an aldol cleavage

of fructose-1,6-bisphosphate to dihydroxyacetone-phosphate and glyceraldehyde 3-phosphate and a reversible aldol condensation (Marsh and Lebherz, 1992). Highly expressed GAPDH and fructose-bisphosphate aldolase might increase glycolysis for energy supply. Pentose phosphate pathway, highly active and up-regulated under bloom conditions, plays a major role in producing NADPH that contribute to fatty acid synthesis and scavenging of reactive oxygen species (ROS) (Patra and Hay, 2014). Highly active carbohydrate metabolism probably benefits *P. donghaiense* to outcompete co-existing species and form a bloom.

4.3. Active expression of anti-microbial mechanism in P. donghaiense during blooms

In bloom development, microbial defense may be important in bloom species survival of bacterial infection. It is thus no surprise to observe the active expression of a score of microbial defense genes in the Baicheng *P. donghaiense* bloom, as reported previously for the ECS bloom (Zhang et al., 2019). Pleurocidin is responsible for pore formation in the membrane of bacteria leading to lysis and therefore death, which has a strong anti-microbial activity and act as roles in innate host defense (Saint et al., 2002). Cyclotide has been reported as antibiotics of microbial origin (Craik et al., 1999). Gallidermin is a type of lanthionine-containing polypeptide antibiotic (Kellner et al., 1988). Antimicrobial peptide acts as antimicrobial agents by disrupting the membrane integrity of invading microbes (Bao et al., 2006). The up-regulation of these 8 defense genes indicated potential importance of microbial defense in bloom development.

4.4. Nocturnally active genes in in P. donghaiense blooms: with emphasis on phagotrophy and mitosis

Due to the lack of biological replicates in the diel sample set for transcriptome sequencing, differential gene expression among the three time points need to be taken with caution, and we limit our discussion to general trend of gene groups rather than quantitative analysis of differential expression of individual genes. First, the greatest number of genes exhibited the highest expression at night (Profile D), suggesting generally nocturnal gene expression activation in *P. donghaiense*. Several molecular functions that followed this diel mode of gene expression are worth noting. The majority of endocytosis genes exhibited the nocturnal mode of expression, suggesting that *P. donghaiense* might be grazing at nighttime, as discussed above. Gap junctions allow cell communication between adjacent cells in multicellular organisms (Giepmans, 2004). Cell modulates cell behavior through modifying focal adhesion to better adapt extracellular matrix environment (Wozniak et al., 2004). Most of genes in these two cellular community pathways and four signal transductions displayed the nocturnal mode of expression, implicating active communication with the environment at night.

Mitosis, a critical stage of the cell cycle, is an essential step of cell proliferation and population growth. Similar to observations in the ECS bloom (Zhang et al., 2019), numerous cell cycle genes were detected in the present bloom metatranscriptomes (Supplementary dataset 4), indicating an active cell division activity. Phase transition protein genes including 14-3-3 and PCNA increased expression from Night to Morning and decreased at Day, indicating a transition form G1-S-G2 transition. 14-3-3 proteins plays keys roles in G1/S- and G2/M-transition via binding to regulatory proteins to regulate their function (Hermeking and Benzinger, 2006). PCNA is a central component acting on DNA replication and cell division(Celis and Celis, 1985); its expression increases from G0- to G1- to S-phase, but is reduced in G2-M cells compared to S-phase cells in mammalian cells(Kurki et al., 1986), and its expression in dinoflagellates has been found to be associated with population growth(Zhang et al., 2006). In *Xenopus*, wee1-like protein is proven to delay mitotic initiation by phosphorylating Cdc2, and its down-regulation marks the successful completion of S phase (Mueller et al., 1995). Reduced wee1-like protein from Night to Morning in this study is indicative of active S phase completion for mitosis, which is evidenced by the active expression of anaphase-promoting complex, which hydrolyzes cyclin B and promotes entry into cell division anaphase (Lehner and O'Farrell, 1989). Cell division cycle 14 is responsible to developmental arrest of cell division or mitotic exit (Saito et al., 2004). The decreased expression of cell division 14 suggests active cell division from Night to Morning time points. Dim1 is a small protein (15 kDa) initially identified in fission yeast as an essential protein for cell cycle regulation and for chromosome segregation during mitosis (Berry et al., 1999). Dim1 is extraordinarily well conserved throughout the eukaryotic kingdom, with ~80% sequence identity (Zhang et al., 1999). The deletion of dim1 in Schizosaccharomyces pombe arrests the cell into G2 phase and prevent entry into mitosis(Berry and Gould, 1997). Enhanced expression of DIM1 gene from night to day in this study further suggests that there might be mitosis during in this period, in agreement to our previous finding that cell mitosis started at late night (Li et al., 2015). The active expression of the large number of cell cycle genes suggests that rapid cell proliferation was likely a major contributor to the P. donghaiense bloom, as was also the case for the ECS bloom (Zhang et al., 2019).

4.5. MicroRNA regulation

Recent studies suggest that microRNA may be the major gene regulation mechanism in dinoflagellates (Lin et al., 2015). A silencing RNA study provided initial evidence that the microRNA mechanism is functional in dinoflagellates (Zhang and Lin, 2019). No effort has been reported to investigate the role of microRNA in regulating gene expression in an algal bloom or natural phytoplankton assemblage. In the first attempt of this kind, we found 76 miRNAs with 17,623 target genes, implicating a broad range of biological processes that these miRNAs regulate. Some of these showed stronger regulation on alanine, aspartate and glutamate metabolism under bloom conditions, and others exhibited stronger control on glutathione, glycine serine and threonine metabolism in culture normal condition, suggesting a regulatory role of miRNAs in amino acid metabolism. The three abundant miRNAs were significantly more highly expressed in the bloom than the cultures, and their predicted target genes are functionally distributed in cellular components and response to environmental. These include peroxidase involved in oxidizing reactive oxygen species, xyloglucan galactosyltransferase that takes part in cell wall biosynthesis and actin microfilament organization (Li et al., 2013), dynamin 2 and myosin genes, which are components of cytoskeleton, and nucleotidyltransferase, which is an exonuclease that provides energy for cell growth. The relationship between microRNA expression and target gene expression, though expected to be inverse because microRNA is a negative regulator of gene expression, appears to be complex for *P. donghaiense* in the bloom. For instance, MIR166-y showed a positive correlation with its target xyloglucan galactosyltransferase in expression level, whereas novel-m0045-3P exhibited negative correlation with its target. Furthermore, four differently expressed miRNAs with lower expression under bloom conditions exhibited positive correlation with their targets that were related to antibiotic and cell communication genes, but negative correlation with photosynthetic genes. Therefore, the physiological roles of these miRNAs still remain to be experimentally demonstrated.

5. Concluding remarks

In summary, this study documents molecular characteristics of the harmful algal bloom of the dinoflagellate *Prorocentrum donghaiense* using ecological and metatranscriptomic analyses. By comparing with datasets from another bloom event and laboratory cultures of this species, our results revealed that genes related to N- and P-nutrient uptake,

phagotrophy, energy metabolism, carbohydrate metabolism, microbial defense and cell division were highly expressed during bloom, suggesting that these processes are important towards bloom formation of *P. donghaiense*. A strong nocturnal gene expression pattern was noted for many genes in *P. donghaiense*, indicative of potential increased activities of phagotrophy and environmental communication at night. MicroRNA sequencing and expression analyses revealed marked differences in amino acid metabolism between the bloom and cultures, and differentially expressed miRNAs showed positive correlation with their targets related to cellular components, antibiotic defense and cell communications, and negative correlation with photosynthetic genes. These provide clues for future experimental interrogation of bloom causing biological processes and environmental variables, which should examine enzyme activities, interactions of the bloom dinoflagellate with ambient prokaryotes, and relationships between environmental factors and molecular regulation in the bloom community.

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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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