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N_2 fixation impacted by carbon fixation via dissolved organic carbon in the changing Daya Bay, South China Sea

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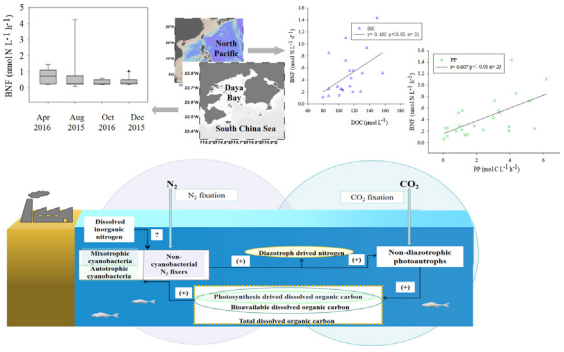
HIGHLIGHTS

- N_2 fixation was first measured in Daya Bay (South China Sea), indicating that N_2 fixation may actively occur under N-replete conditions.
- Non-diazotrophic phytoplankton may impact N_2 fixation by regulating the availability of 'fresh' dissolved organic carbon (DOC) here.
- This study provides implications for understanding the constraints of N_2 fixation in coastal settings experiencing increasing anthropogenic impacts.

GRAPHICAL ABSTRACT



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ABSTRACT

We present the first concurrent measurements of N_2 fixation rates ($^{15}N_2$ uptake), primary production (^{14}C uptake), dissolved organic carbon (DOC) concentrations, and diazotrophic community composition derived from nitrogenase (*nifH*) abundance in the subtropical Daya Bay (DB) of the coastal northern South China Sea (NSCS) from 2015 to 2017. N_2 fixation rates ranged from n.d. - 4.51 nmol N L^{-1} h^{-1}. Such values were generally higher than those reported in the neighbouring NSCS open waters and several well-studied oligotrophic waters, thereby suggesting that N-replete conditions do not prevent N_2 fixation in coastal waters. N_2 fixation rates were positively and significantly correlated with the primary production and the concentration of DOC in DB in the spring and summer. Combined with other lines of evidence, we suggest that N_2 fixation may be facilitated by non-diazotrophic phytoplankton via a probable regulation of the quantity and quality (bioavailability) of DOC in DB. Since DB represents a suitable site that has experienced dramatic human-induced changes in environmental conditions, our results likely provide insights in understanding how N_2 fixation and relevant biogeochemical processes may respond to intensified global anthropogenic forcing in similar coastal settings.

1. Introduction

N_2 fixation is one of the major sources of new nitrogen for the marine environment and plays a key role in carbon and nitrogen

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biogeochemical cycles, thus exerting a profound impact on global climate (Capone et al., 2005; Karl et al., 2002). To date, our knowledge of marine N_2 fixation is mainly obtained from the tropical/subtropical oligotrophic sea areas (Capone et al., 2005; Karl et al., 2002). Interestingly, there is increasing evidence indicating that N_2 fixation may be much more geographically widespread in marine environments than previously thought (Bombar et al., 2016; Farnelid et al., 2011; Jiang et al., 2018; Shiozaki et al., 2017). Recent studies in several 'atypical' regimes, such as large river plumes and coastal upwellings, suggest that the seemingly unfavourable N-replete conditions in coastal waters do not necessarily hinder N_2 fixation (Grosse et al., 2010; Mulholland et al., 2012; Subramaniam et al., 2008; Zhang et al., 2015). Non-cyanobacterial diazotrophs were suggested to be more abundant in coastal areas and play a role in sustaining marine N_2 fixation over a broad spatial range (Farnelid et al., 2013; Riemann et al., 2010; Shiozaki et al., 2017), thus implying that there are more complex regulations on N_2 fixation than previously thought (Benavides et al., 2016; Bombar et al., 2016; Rahav et al., 2013).

Organic carbon is a major growth-limiting factor for oceanic heterotrophic or mixotrophic bacteria (Kirchman and Rich, 1997; Wambeke et al., 2008). Several groups of heterotrophic diazotrophs (such as Gammaproteobacteria) have been reported to be linked to the excretion of organic carbon from phototrophs (Moisander et al., 2014; Zehr and Karl, 2007), thus highlighting the possible importance of dissolved organic carbon (DOC) in regulating the dynamics of N_2 fixation (Rahav et al., 2016). Even the globally important N_2-fixing autotrophic cyanobacteria *Trichodesmium* spp. have been recently observed in the uptake of dissolved organic matter in the field (Benavides et al., 2017). Considering that marine phytoplankton play a major role in shaping local nutrient fields and the distribution of organic matter by releasing a dramatic portion of photosynthetic carbon as DOC (Kirchman et al., 2001), one may reasonably speculate that C fixation may regulate non-cyanobacterial or mixotrophic cyanobacterial N_2 fixation in coastal waters via DOC. If this is the case, we must determine how these two processes (N_2 and C fixation) interplay (compete or/and facilitate) with one another. To date, such investigations have mainly focused on oligotrophic waters (Benavides et al., 2017; Berthelot et al., 2015; Karl et al., 2012; Rahav et al., 2013). In a word, our understanding of the interplay between these two processes in coastal waters, which is experiencing intensifying human stressors and experiencing non-negligible changes in recent decades, is generally scarce.

As an interface between terrestrial and marine ecosystems, coastal waters have an important impact on the nutrient cycles subject to intensifying human impact (Wysocki et al., 2007). Recent studies in several atypical regimes, such as large river plumes and coastal upwellings, suggest that the seemingly unfavourable N-replete conditions in coastal waters do not necessarily hinder N_2 fixation (Grosse et al., 2010; Mulholland et al., 2012; Subramaniam et al., 2008; Zhang et al., 2015). Daya Bay (DB, 114°E, 22°N) is a semi-closed subtropical embayment in the northern South China Sea covering an area of approximately 600 km^2. Its water depth ranges from 6 to 16 m (average 10 m). The largest river entraining into DB is the Danao River, with a freshwater discharge of approximately 1.6×10^8 m^3 a^{-1}. The runoff of other rivers is less than one-tenth that of the Danao River (Ren et al., 2013). Throughout the past three decades, the DB region has experienced rapid environmental changes. As a result of agricultural, industrial and urbanization activities, an increase in the dissolved inorganic nitrogen (DIN) level has continued since the late 1980s (Wu et al., 2017). In addition, the operation of the two nuclear power plants (the Daya Bay Nuclear Power Plant since 1993 and the Lingao Nuclear Power Plant since 2002) have caused water warming (approximately 0.07 °C yr^{-1}) in DB (Yu et al., 2010). Obvious shifts in the ecosystem, such as changes in the phytoplankton community structure, have been observed from available monitoring results (Wu et al., 2017). This highlights the necessity of incorporating N_2 fixation research to comprehensively understand the biogeochemical dynamics in the rapidly changing DB, which may also provide implications for coastal waters under similar anthropogenic pressures.

DB provides a suitable site to examine the possible link between N_2 fixation and carbon fixation and to evaluate the role of DOC regulation in coastal waters. DB has been generally assumed to be favourable for N_2 fixation, as indicated by its original low DIN concentration (early 1980s and before) and occasional *Trichodesmium* blooms (these have disappeared in the last decade, however) (Li et al., 2010), inspiring us to examine whether the N_2 fixation occurs in DB and how it may be regulated. Indeed, DB has been continuously impacted by human activities and has changed from an oligotrophic bay to a eutrophic bay (Wu et al., 2017). The nutrient contents and structures have substantially changed in the past 30 years because of intensifying human activities (Wu et al., 2017). The concentration of dissolved inorganic nitrogen (DIN) has increased over the past three decades, with the ratio of DIN to dissolved inorganic phosphate (DIP) increasing dramatically over time from 1.5 to >50 at present (Wu et al., 2017). Dramatic changes in nutrient structure and warming waters have been suggested as being largely responsible for shifts at the ecosystem level, and it may have caused the increase of DOC here (Jiang et al., 2009). DB in present day is characterized by a relatively high primary production (ranging from 14 to 214 mmol C m^{-2} d^{-1}) (Liu et al., 2012; Song et al., 2009; Song et al., 2004). In contrast, our knowledge of N_2 fixation in the rapidly changing DB is substantially lacking. There are few N_2 fixation rate data reported in the adjacent coastal upwelling and open waters of the NSCS (Chen et al., 2014; Hong et al., 2017; Zhang et al., 2015), while no such data are available for DB.

The aims of this study include the following: i. to obtain the spatial and temporal patterns of N_2 fixation and primary production in DB; ii. to evaluate the possible role of DOC in controlling N_2 fixation in DB; and iii. to examine the possible interactions between N_2 fixation and carbon fixation. Our results add important knowledge on N_2 fixation in a least-studied coastal waters area and provide insights to better understand coastal water biogeochemistry in the context of ongoing and intensifying human forcing.

2. Materials and methods

2.1. Sampling period

To achieve the objective of this study, five surveys were carried out in the summer (August 2015), winter (December 2015), spring (April 2016 and April 2017) and autumn (October 2016), respectively. DNA samples were only collected in April 2017. Sampling locations are shown in Fig. 1. We measured the N_2 fixation rate in parallel with the C fixation rate via an isotope tracer assay. Natural DOC concentrations were also measured. Seawater samples were collected using 5-L Niskin bottles from both the first 50 cm of the surface and approximately 0.5 m above the seafloor.

2.2. Physico-chemical parameters

Seawater temperature and salinity were measured using YSI 6600 multi-probe sensors (Yellow Springs Instrument Co., USA). Concentrations of nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_4^+), and silicate (SiO_3^{2-}) were analysed using a Lachat QuickChem 8500 autoanalyzer (Lachat Instruments, USA) after standard colorimetric methods. Phosphate (PO_4^{3-}) was measured using a spectrophotometer via the standard molybdenum blue method (Hansen and Koroleff, 1983). The nutrient detection limits were 0.05 μmol L^{-1} (NO_3^-), 0.02 μmol L^{-1} (NO_2^-), 0.03 μmol L^{-1} (NH_4^+), 0.02 μmol L^{-1} (PO_4^{3-}) and 0.45 μmol L^{-1} (SiO_3^{2-}), respectively. Other environmental parameters, such as chlorophyll *a*, were provided by the China-973 Bay program.

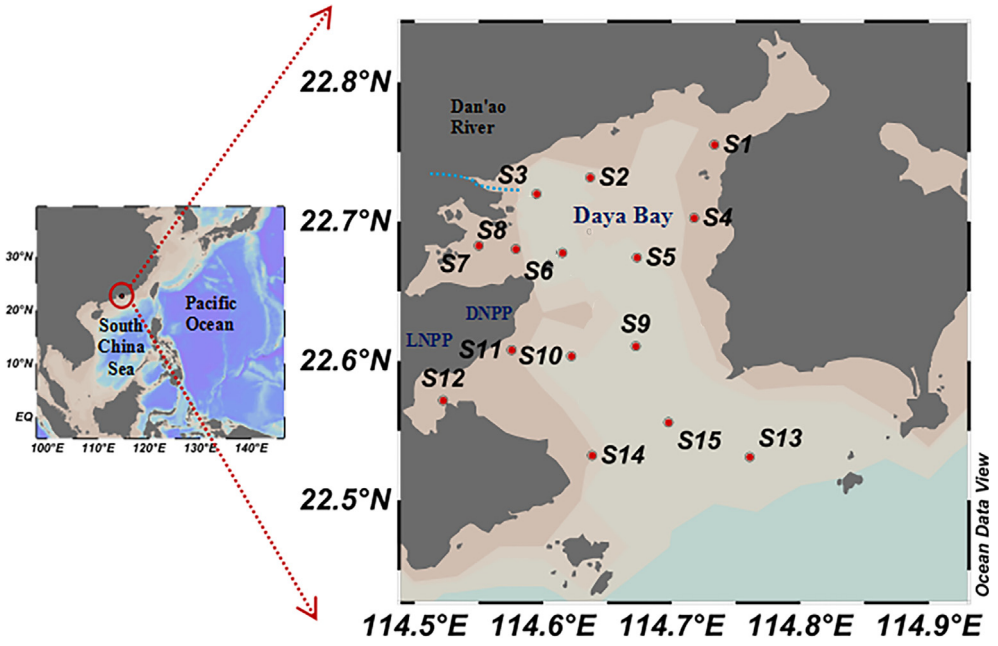

Fig. 1. Sampling locations in Daya Bay. DNPP and LNPP represent the Daya Bay Nuclear Power Plant and the Lingao Nuclear Power Plant, respectively.

2.3. Dissolved organic carbon (DOC)

After collection, duplicate seawater samples (30 mL) were immediately filtered onto pre-combusted (450 °C, 4 h) Whatman GF/F membranes (0.7 μm pore size) to remove particulate organic carbon (POC). Filtrates were then collected in pre-combusted (500 °C, 4 h) brown glass bottles and stored frozen (−20 °C) until measurement. DOC concentrations were measured using the high temperature catalytic oxidation (HTO) method on a Shimadzu TOC-VCPH analyzer following the procedures of Stubbins and Dittmar (2012). Standards were prepared to determine the content of DOC of samples by volumetric dilution of a stock solution (potassium hydrogen phthalate, Carl Roth, Germany). Aliquots of deep seawater reference material (from the Consensus Reference Material Project, CRM) were used to monitor the stability of the analyzer. Analyses of the CRM deviated by <5% from the reported value (http://hansell-lab.rsmas.miami.edu/_assets/pdf/copy-of-hansell-crms-products-table-1-lc-7.11.181.pdf).

2.4. N_2 fixation rate

Biological N_2 fixation rate (BNF) data were obtained via $^{15}N_2$ tracer assay (Mohr et al., 2010). $^{15}N_2$ gas (98% atom ^{15}N) was ordered from Cambridge Isotope Laboratories Inc., USA. A simplified purifying pathway ($^{15}N_2$ gas stock cylinder → degassed sulfuric acid trap → potassium permanganate trap) was applied to efficiently remove any possible residual contamination of the ^{15}N labelled fixed-N (Dabundo et al., 2014). The isotope tracer was then prepared by adding 10 mL $^{15}N_2$ gas (98% atom ^{15}N) to 1 L prefiltered (0.2 μm pore size) degassed seawater in transparent Nalgene polycarbonate bottles at 5 °C and then shaken vigorously until bubbles disappeared (Mohr et al., 2010). The bottles were then stored at 5 °C. After collection, duplicate seawater samples were filled bubble-free into pre-cleaned (soaked in 0.1 M HCl for 24 h and washed with Milli-Q water) 1.2 L clear Nalgene polycarbonate bottles along with 40 mL $^{15}N_2$-enriched tracer and then sealed. The bottles were then shaken for approximately 5 min before being placed in a deck incubator with flowing seawater pumped from the surface of the sea. The incubation was carried out between 9:00 am and 17:00 pm during the daytime and lasted for about 6 h. The start time of the incubation at each station differed by approximately 1 h. After incubation, N_2 fixation samples were filtered onto pre-combusted (450 °C, 4 h) Whatman GF/F membranes (0.7 μm, 25 mm), then dried (60 °C) and stored frozen (−20 °C) until measurement. Natural suspended particulate organic matter samples were also collected for background ^{15}N analysis by filtering 4 L seawater onto GF/F filters. Samples were thawed and dried (60 °C) again before analysis. Particulate organic nitrogen (PON) content and its ^{15}N abundance ($\delta^{15}N$, ‰ vs air) were measured on a Finnigan Delta V Advantage isotope ratio mass spectrometer interfaced with a Carlo Erba NC 2500 elemental analyzer (EA-IRMS). BNF was calculated after Montoya et al. (1996). Reproducibility for $\delta^{15}N$ measurements was no >0.2‰. Our sampling possibly represents an underestimated result for the non-cyanobacterial N_2 fixation rate, as suggested by Bombar et al. (2018).

2.5. Primary production

Primary production (PP) was determined by a ^{14}C uptake assay (Wolfe and Schelske, 1967). After collection, seawater samples (100 mL) were immediately transferred into three 125 mL pre-cleaned Nalgene polycarbonate bottles, and then 1 μCi $NaH^{14}CO_3$ was added to each bottle. Two bottles were assigned as light, and one as dark. Bottles were fitted to simulate light densities and were placed in the deck incubator with flowing surface seawater. After incubation, PP samples were filtered onto mixed cellulose ester membranes (0.2 μm, 25 mm) and then stored frozen (−20 °C). After removing inorganic carbon on the membranes by acid (HCl) fuming, the assimilated ^{14}C radioactivity was measured on a liquid scintillation counter (Perkin-Elmer TriCarb 2900TR) in the land laboratory.

2.6. DNA sampling, extraction, *nifH* amplification and sequencing

A total of 8 stations were sampled in April 2017 (S1, S3, S9, S10, S11, S12, S13, and S14). To collect DNA samples, the particulate organic matter (POM) in 2 L seawater was filtered with Millipore filters (with a pore size of 0.2 μm). The filters were stored in liquid nitrogen for further processing. In the laboratory, the filters were cut into small pieces. A Qiagen DNeasy Plant extraction kit was used to extract DNA according to the manufacturer's instructions. By using the nested PCR approach (Zehr and Turner, 2001), approximately 360 bp fragments of *nifH* were amplified. In brief, the 20 μL PCR mixture contained 1 μL DNA, 10 μl 2 × Taq

Plus PCR MasterMix (TIANGEN), 0.5 μL each of the forward and reverse primers (250 nM), and 8 μL nuclease-free water. The thermal cycling conditions were set as follows: 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The second nested PCR was carried out with 1 μL product from the first round as the template and using the same conditions as mentioned above except for the primers *nifH*1 and *nifH*2. Amplified products were checked via electrophoresis on a 1% agarose gel and purified using a Gel DNA Recovery Kit™ (Zymoclean). For all the PCR products, paired-end PCR-free libraries were constructed and were sequenced on an Illumina HiSeq2500 platform, and 250-bp paired-end reads were generated (Novogene Bioinformatics Technology Co. Ltd.).

2.7. Sequences quality control and analysis

Low quality reads and bases were removed following Sogin et al. (2006). Barcodes and primer sequences were trimmed from the 5′ end and reads lacking exact matches to a barcode were discarded (Bolger et al., 2014). We used FLASH to stitch paired-end reads together by finding the correct overlap between them (Magoč and Salzberg, 2011). Given that our PCR product length is 362 bp, sequences shorter than 330 bp were removed. The chimaeras were removed using usearch6 and repetition sequences were discarded by RDPTools to make sure every sequence was unique in each sample (Edgar et al., 2011). Using Framebot, all nucleotide sequences were translated into amino acid sequences and aligned against the *nifH* family proteins (PF00142), which were retrieved from the UniProt database (Wang et al., 2013). Framebot is a Java-based tool that uses frameshift correction and nearest-neighbour identification to accurately detect and correct frameshifts caused by indel sequencing errors (Wang et al., 2013). The identity of all sequences must be >95%. All the sequences used in this study are available in the NCBI Sequence Read Archive under accession ID SRP133726 (http://www.ncbi.nlm.nih.gov/Traces/sra).

2.8. Statistical analysis

Pearson's correlation analysis was conducted using the Statistical Package for Social Sciences program (version 19.0) to reveal the relationships among nitrogen fixation rates, primary production, DOC and environmental factors.

3. Results

3.1. Physico-chemical parameters

Sea surface temperatures (SST) ranged from 17.1 to 31.1 °C. The maximum SST was observed at station S1 (31.1 °C) in the summer and the minimum SST was observed at station S3 (17.1 °C) in the spring. The discharge of the nuclear power plants caused a visible increase in SST near station S11, which was 1–2 °C higher than the SST of ambient waters in both the spring and winter. In general, the SST in the western coastal waters were higher than those in the eastern waters (Fig. 2a). Sea surface salinity (SSS) ranged from 19.4 to 36.1 during our sampling period. The maximum and minimum SSS were observed at station S13 and S3, respectively, during summer. In the summer, due to the influence of river discharge, the SSS at station S3 (near the Danao River estuary) was significantly lower than that at other stations. The difference was small for the other seasons (Fig. 2b).

The concentrations of DIN, DIP and SiO_3^{2-} for the four seasons span a relatively wide range: 1.1–65.98 μmol L^{-1}, 0.01–8.10 μmol L^{-1} and 0.11–57.23 μmol L^{-1}, respectively. Surface concentrations of DIN, DIP, and SiO_3^{2-} were generally highest at station S3, which was close to the mouth of the Danao River and decreased outward from the inner bay in general (Fig. 3). Ammonium was a non-negligible species of DIN in the autumn, while nitrate was generally the major species of DIN in the other seasons. A notable feature of nutrient stoichiometry is that the ratios of DIN: DIP were significantly higher than the Redfield value (67.4 ± 80.1, $n = 53$) during all seasons.

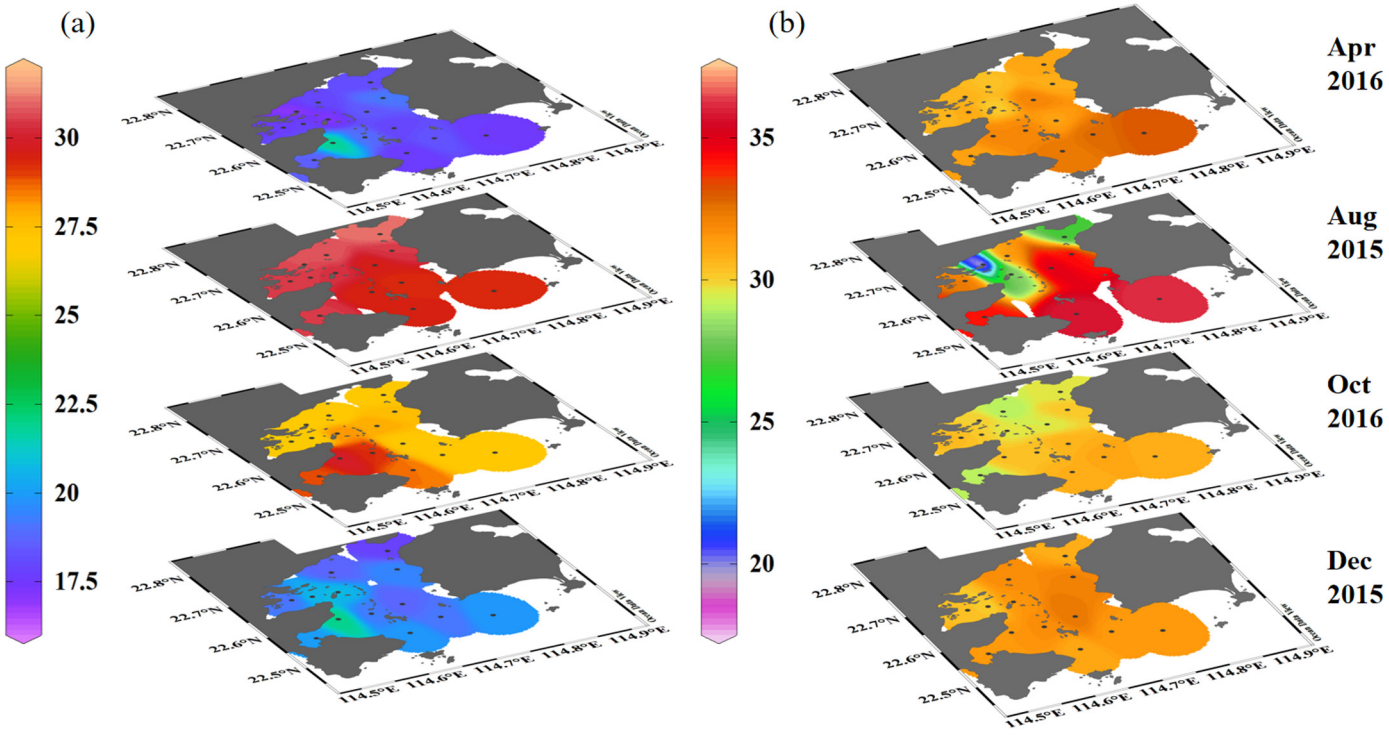

Fig. 2. Distribution of (a) sea surface temperature (°C) and (b) surface sulfate in Daya Bay.

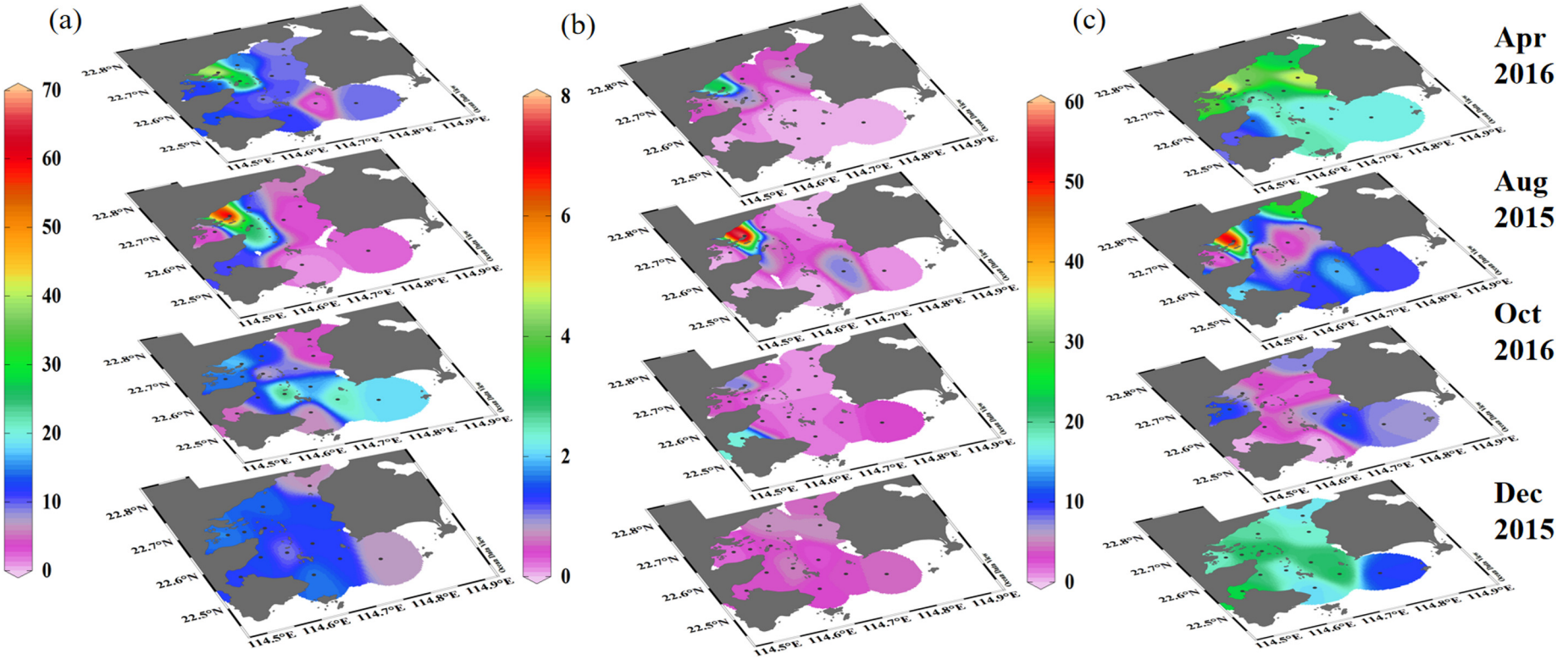

Fig. 3. Distribution of (a) dissolved inorganic nitrogen (μmol L^{-1}), (b) dissolved inorganic phosphate (μmol L^{-1}) and (c) dissolved silicate (μmol L^{-1}) in Daya Bay.

3.2. N_2 fixation rate

The surface biological N_2 fixation rate (BNF) ranged from n.d. to 4.51 nmol N L^{-1} h^{-1}. Surface BNF in the summer (0.91 ± 1.48 nmol N L^{-1} h^{-1}, n = 8) and spring (0.80 ± 0.44 nmol N L^{-1} h^{-1}, n = 6) were higher than in the autumn (0.35 ± 0.16 nmol N L^{-1} h^{-1}, n = 7) and winter (0.43 ± 0.31 nmol N L^{-1} h^{-1}, n = 9) (Fig. 4a). We observed a diatom (*Thalassiosira minima*) bloom at station S6 in summer, where the surface BNF was 4.51 nmol N L^{-1} h^{-1}, which was one order of magnitude higher than those measured at the non-bloom stations. If we do not take the bloom station into account, BNF in summer were similar to that in autumn. During the spring, surface biological BNFs were only detected in five stations, and BNF in the northeastern areas of the bay (station S1) were higher than those in other areas (Fig. 5). BNFs in autumn were generally lower than that in other seasons and had little spatial variation. In winter, the maximum BNF was observed at station S11 (1.02 nmol N L^{-1} h^{-1}), which was located near the thermal discharge from the nuclear power plant. In general, biological BNFs in the inner bay were higher than those in the outer bay during all four cruises. BNF slightly decreased with depth at most stations (Fig. 6a).

3.3. Primary production and Chl a

Surface PP in the spring (5.95 ± 6.73 mmol C m^{-3} h^{-1}, n = 15) and summer (5.44 ± 6.37 mmol C m^{-3} h^{-1}, n = 10) were much higher than in the autumn (2.21 ± 1.19 mmol C m^{-3} h^{-1}, n = 15) and winter (0.26 ± 0.21 mmol C m^{-3} h^{-1}, n = 13). Similar to surface BNF, surface PP in the spring was higher in the western area of the bay, with a maximum at station S4 (29.2 mmol C m^{-3} h^{-1}) (Fig. 4b). At station S6, where *Thalassiosira minima* bloomed during the summer cruise, surface PP reached a peak value of 18.32 mmol C L^{-1} h^{-1}. PP in the inner bay was notably higher than that at the bay mouth. In both the autumn and winter, the maximum surface PP was observed at station S15 (4.28 mmol C L^{-1} h^{-1} and 0.76 mmol C L^{-1} h^{-1}, respectively), and surface PP near the coast was higher than at the centre of DB. The contents of Chl *a* in the spring (32.2 ± 44.7 μg L^{-1}, n = 15) and summer (9.6 ± 11.7 μg L^{-1}, n = 9) were significantly higher than in autumn (4.9 ± 4.4 μg L^{-1}, n = 15) and winter (1.4 ± 1.2 μg L^{-1}, n = 13) (Fig. 4d). During our investigation, the maximum of PP (29.2 mmol C m^{-3} h^{-1}) and Chl a (184 μg L^{-1}) were both observed at station S4 in spring. The distribution of Chl *a* was similar to that of PP. PP and Chl a contents have no apparent correlation with the water depth.

3.4. Dissolved organic carbon (DOC) concentration

The surface concentration of DOC ranged from 73.5 to 242.8 μmol L^{-1}, with an average of 113.4 ± 46.2 μmol L^{-1} (n = 52). Higher seasonal average concentrations were observed during summer sampling (158.5 ± 40.6 μmol L^{-1}, n = 9 in August 2015, 128.2 ± 41.2 μmol L^{-1}, n = 15 in April 2016, 106.4 ± 11.1 μmol L^{-1}, n = 15 in October 2016, and 85.3 ± 10.1 μmol L^{-1}, n = 13 in December 2015). The highest DOC concentration was observed at station S3 near the mouth of the Danao River during spring sampling in April 2016 (Fig. 4c). DOC concentrations at the stations near the coast of DB are generally higher than those in the middle or the mouth of the bay. The DOC concentration slightly decreased with depth at most stations (Fig. 6b).

3.5. Taxonomic composition of diazotrophs

A total of 1,131,232 robust reads and 1,021,885 unique sequences were obtained based on the *nifH* genetic fragments from the 13 samples. The sequence numbers in all the 13 samples ranged from 8433 to 168,271, resulting in near-saturation for most rarefaction curves. Diazotrophic proteobacteria were dominant, but neither *Trichodesmium* nor heterocystous cyanobacterial diatom symbionts were observed (Fig. 7). The proteobacteria *nifH* phylotype accounted for 94.5% of the total *nifH* amplicons and was widely distributed and detected at all sites. More specifically, the most abundant sequences, with 62.8% reads, were clustered as Deltaproteobacteria, whereas the second most abundant, with 8.8% reads, was associated with *Nitrospira*. In addition, 6.9% was clustered as Gammaproteobacteria, 5.1% of reads were Alphaproteobacteria, and 5.5% of reads were diazotrophic cyanobacteria. The N_2-fixing cyanobacteria were only rich in the surface

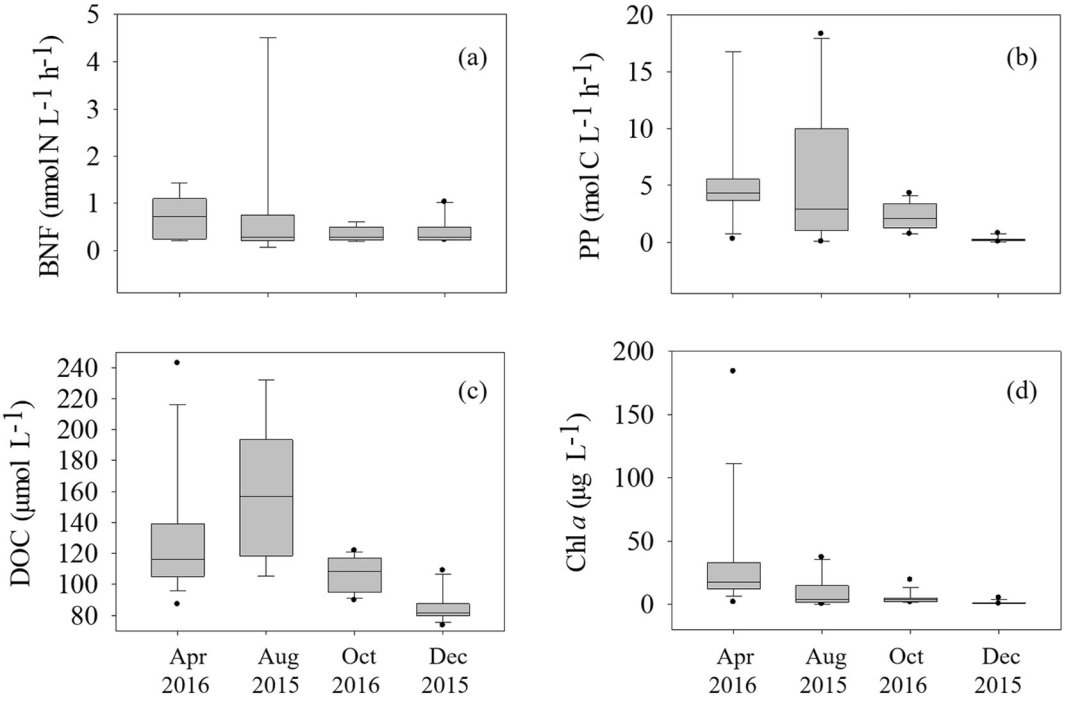

Fig. 4. Seasonal variations of (a) N_2 fixation rates (BNF), (b) primary production (PP), (c) dissolved organic carbon (DOC) and (d) Chl *a*.

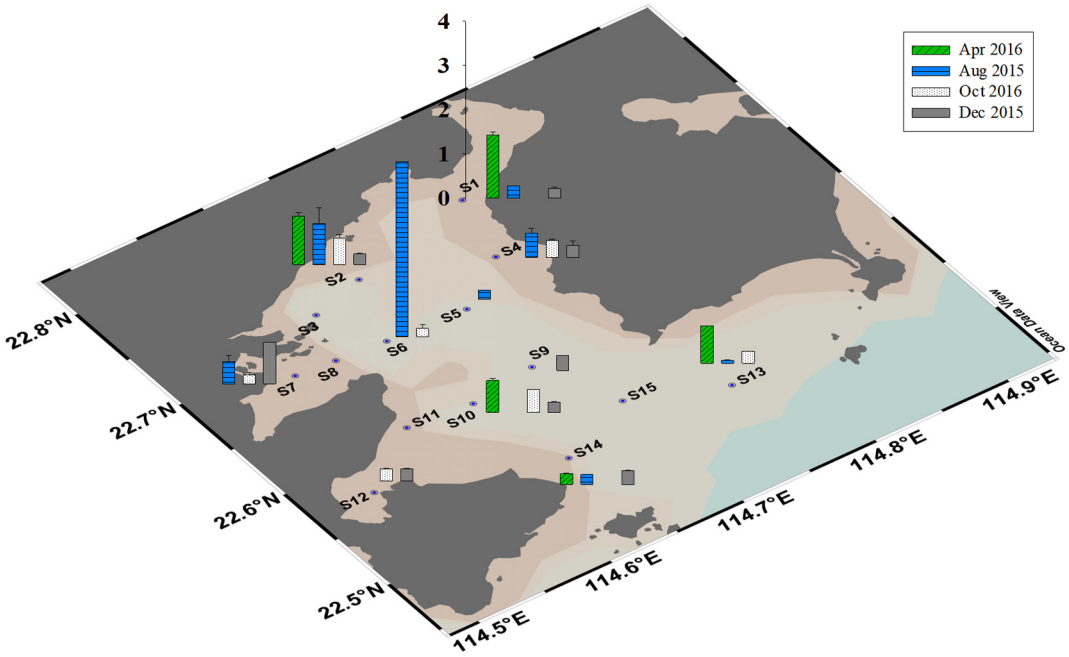

Fig. 5. Distribution of the logarithm of N_2 fixation rates (nmol N L^{-1} h^{-1}). All stations have the same scaling of Y-axes as S1.

waters of station S11 (96.9%), which showed a 100% sequence similarity with Cyanobacterium sp. NBRC 102756, a unicellular diazotrophic cyanobacteria (Fig. 7a). In addition to the Deltaproteobacteria (including Desulfuromonadales), *Nitrospira* was also abundant in the Danao River (station S1 and station S3) compared with other stations (Fig. 7b).

3.6. Statistical analysis

Significant seasonal heterogeneity of BNF was observed in the study area (Levene's t-test, $p < 0.05$). Pearson's correlation analyses indicate that BNF are correlated significantly and positively with primary production ($r = 0.522$, $p < 0.01$), DOC ($r = 0.424$, $p < 0.05$), and Chl a ($r = 0.770$, $p < 0.01$) in spring and summer (Table 1). BNF in autumn and winter has no statistical correlation with PP, DOC and Chl *a*.

4. Discussion

4.1. Correlations of BNF, PP and DOC

BNF in DB under non-bloom conditions, if scaled to daily rates, were in the range of the values reported (n.d. to 7.6 nmol N $L^{-1}d^{-1}$) in the neighbouring NSCS open waters (Lin et al., 2013; Liu et al., 2016; Wu et al., 2018; Zhang et al., 2015). Our results confirm that the N_2 fixation can actively occur under relatively N-replete conditions at least in the subtropical coastal waters of the NSCS. We observed a positive correlation between the N_2 fixation rate and DOC in DB during spring and summer (Table 1). Even without the bloom station in summer (BNF: 4.51 nmol N L^{-1} h^{-1}), a positive correlation still hold (Fig. 8b). This is the first time that such a relationship (among BNF, PP and DOC) has been concurrently reported in the SCS, to our knowledge. We suggest

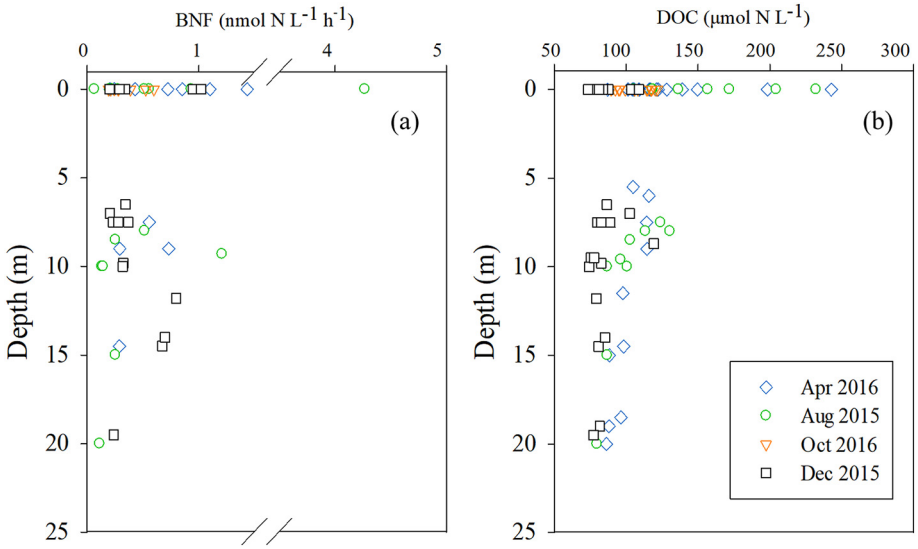

Fig. 6. Vertical distribution of (a) BNF and (b) DOC concentrations.

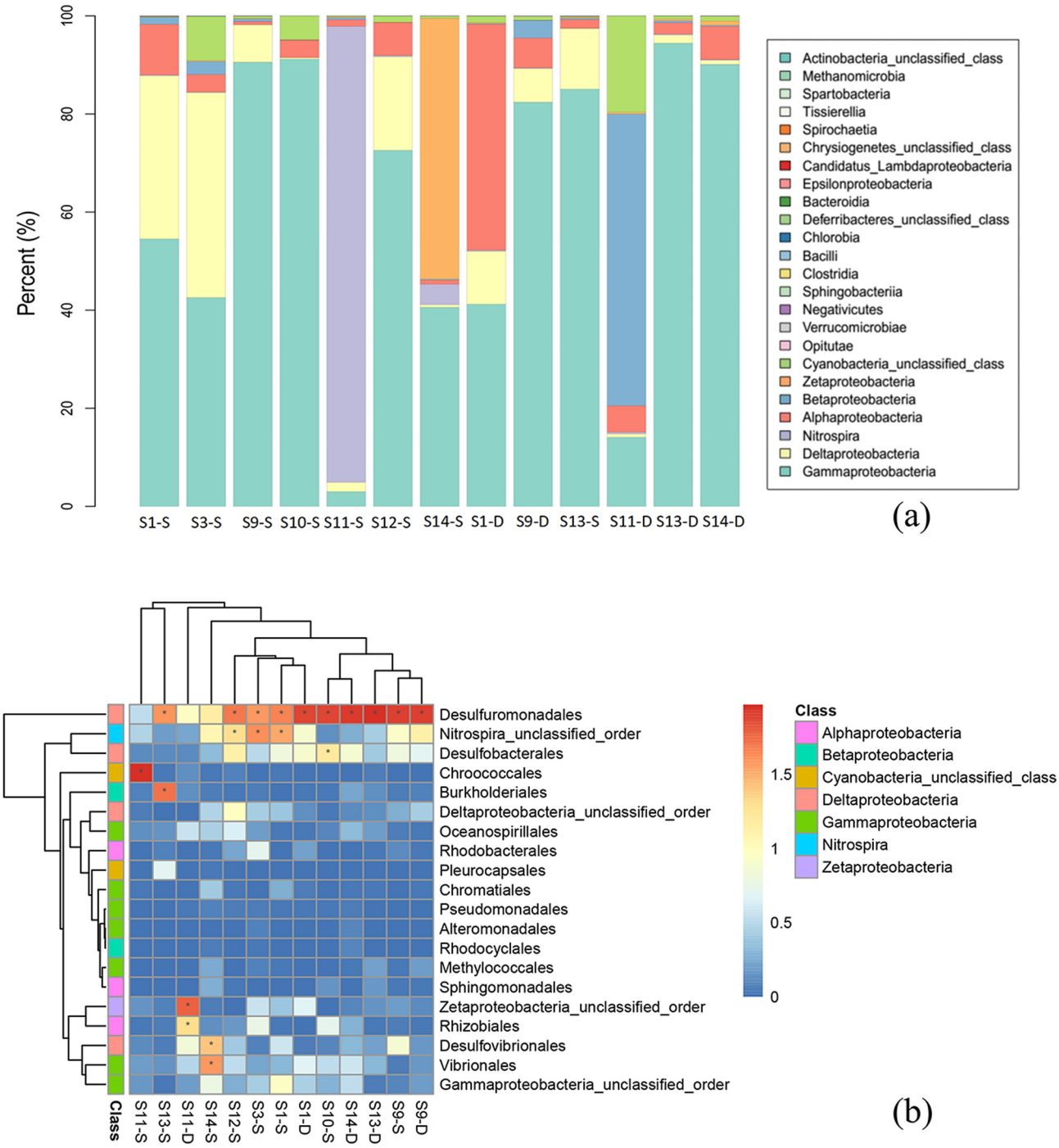

Fig. 7. (a) Relative abundances of the diazotroph community at class level (S and D represent the surface and the bottom of the station, respectively). (b) Heatmap of relative abundance of order in samples. Raw values of the relative abundance (log((1 + X)/(sum(X + 1)) are colour-coded in the corresponding heatmap legend (* denotes a relative abundance > 16%).

that the correlation between the N_2 fixation rate and DOC probably implies the importance of heterotrophic and/or mixotrophic N_2 fixers in DB, while more spatially and temporally resolved investigations are needed for further validation of the N_2 fixation in different microbial fractions. Studies in the Baltic estuary, the North Atlantic, and the South Pacific tropical/subtropical open waters have suggested that the activity of heterotrophic N_2 fixers may be closely related to the availability of DOC (Benavides et al., 2015; Berthelot et al., 2015; Farnelid et al., 2013; Halm et al., 2012; Moisander et al., 2014). Interestingly, the spatial-temporal distribution of N_2 fixation observed in this study (Fig. 5) is similar to reported patterns of either bulk heterotrophic bacteria abundance (Du, 2013) or bacteria production in DB (Wang, 2012). This is also in broad agreement with the results of the *nifH* abundance-based diazotrophic structure, which confirms that diazotrophic proteobacteria were the dominant N_2 fixers in DB in April 2017. More spatially and temporally resolved samplings are called for in understanding the diazotrophic community in coastal regimes, as they have great variability.

The positive correlation between BNF and PP in spring (April 2016) and summer (August 2015) where both BNF and PP were relatively high among the four seasons was observed (Fig. 8a, without the bloom station S6), indicating a coupling for the processes of N_2 fixation and primary production. In contrast, an uncoupling of BNF and PP in the eastern Mediterranean Sea was reported (Rahav et al., 2014), and the

Table 1
Pearson's correlations of nitrogen fixation rates (NFR) with primary production (PP), content of dissolved organic carbon (DOC) and environmental factors.

			T	S	$[NO_2^-]$	$[NO_3^-]$	$[NH_4^+]$	[DIP]	$[SiO_3^{2-}]$	pH	DO	BNF	[DOC]	PP	[Chl *a*]
Spring and summer	BNF	Coefficients	−0.37	−0.633**	0.542**	0.274	0.223	0.299	0.122	0.189	0.376	1	0.424*	0.522**	0.770**
		N	25	25	25	25	25	25	25	25	25	25	22	25	25
	DOC	Coefficients	−0.036	−0.344*	0.04	0.089	0.087	0.142	0.203	0.504**	0.478**	0.522**	0.439**	1	0.795**
		N	48	48	48	48	48	48	48	48	48	25	41	48	48
	PP	Coefficients	0.125	−0.633**	0.755**	0.641**	0.589**	0.734**	0.441**	0.352*	0.255	0.424*	1	0.439**	0.374*
		N	41	41	41	41	41	41	41	41	41	22	41	41	41
Autumn and winter	BNF	Coefficients	−0.032	0.125	−0.014	0.089	−0.076	−0.016	0.032	0.053	−0.027	1	0.052	−0.091	0.121
		N	29	29	28	28	28	28	28	29	29	29	29	29	29
	DOC	Coefficients	0.613**	−0.655**	−0.564**	−0.339*	0.448**	−0.075	−0.707**	−0.082	−0.401**	0.052	1	0.593**	0.425**
		N	44	44	43	43	43	43	43	44	44	29	44	44	43
	PP	Coefficients	0.703**	−0.582**	−0.564**	−0.296	0.478**	−0.064	−0.679**	−0.105	−0.344*	−0.091	0.593**	1	0.551**
		N	44	44	43	43	43	43	43	44	44	29	44	44	43

Note: BNF (nmol N L^{-1} h^{-1}), PP (mol C L^{-1} h^{-1}), Chl a (μg L^{-1}), DO (mg L^{-1}), T (°C), DOC and nutrients concentrations (μmol L^{-1}).
* $p<0.05$.
** $p<0.01$.

competition of the two pathways (i.e., N_2 fixation vs PP) for limited nutrient resources (Fe, P) in the ultra-oligotrophic environment was suggested to be the main cause. However, this is unlikely to be applicable to Daya Bay, where nutrients (Fe, P) are non-limiting in general (mesotrophic). Therefore, the relationship of N_2 fixation and primary production will likely vary greatly over regimes. Nevertheless, it is worth noting that the two types of regimes (ultra-oligotrophic eastern Mediterranean vs mesotrophic Daya Bay) also share a similarity, i.e., the non-cyanobacterial (heterotrophic specially) fraction can contribute significantly to N_2 fixation.

N_2 fixation makes a minor contribution to the ^{14}C-based PP N demand (0.3% after Redfield stoichiometry C:N of 106:16) in DB. This is not surprising, as DB is characterized by a relatively high photosynthetic phytoplankton (diatom-dominated) abundance (Wu et al., 2017), although the information (i.e., abundance) regarding *nifH*-bearing microorganisms are unknown. Such contributions of N_2 fixation in supplying primary production N demand are similar to those reported in the NSCS (Chen et al., 2014; Zhang et al., 2015) but are somewhat lower than those in the Mekong River plume (southern SCS) and the Amazon River plume, where diatom-diazotroph associations (DDAs) are quite abundant (Grosse et al., 2010; Subramaniam et al., 2008). Thus, the positive correlation between BNF and PP probably implies a regulation of primary production on N_2 fixation in the spring and summer in DB.

4.2. N_2 fixation impacted by carbon fixation via DOC

The most likely candidate explanation for the correlation is that carbon fixation (by non-diazotrophic phytoplankton) may impact N_2 fixation by changing the quantity and bio-availability of DOC in DB, especially during the spring and summer, when primary production is higher. Carbon is a major growth-limiting factor for oceanic heterotrophic or mixotrophic bacteria (Kirchman and Rich, 1997; Wambeke et al., 2007). Since N_2 fixation is an energy-demanding pathway, abundant bio-available DOC represents a vital source of energy that fuels heterotrophic or mixotrophic diazotrophs, although its exact mechanisms are not yet clear (Langlois et al., 2015; Moisander et al., 2014); this dynamic likely provides them with a competitive advantage. Moreover, some autotrophic N_2-fixing cyanobacteria are also capable of utilizing DOC (Benavides et al., 2017).

Although there are undoubtedly other DOC sources (terrestrial, for instance) in DB, non-diazotrophic phytoplankton may have played an important role in supporting the correlation between N_2 fixation and DOC during the growth season. This is supported by significant correlations between DOC and some key biological parameters (Table 1 & Fig. 9). Most of the seawater DOC pool is characterized by relatively low bio-availability (Hansell, 2013). In DB, the most likely autochthonous source of bioavailable DOM is exudates from pelagic photosynthetic phytoplankton, which are known to be highly bioavailable because of their high monomeric and combined carbohydrate content and low C:N ratios (Bertilsson and Jones 2003). This is consistent with the observation that Sta. S3 at the mouth of the Danao River had the highest DOC concentration (>200 μmol C L^{-1} in spring and summer) during the sampling period, while N_2 fixation rates were not detected at this site. Thus, the quality of DOC, rather than solely its quantity, regulates N_2 fixations. A large portion of newly fixed carbon by photosynthesis can be released as DOC (Karl et al., 1998). A study in DB's neighbouring waters showed that a significant fraction (~20%) of the photosynthetic extracellular release is DOC (Liu, 2012). Our finding of

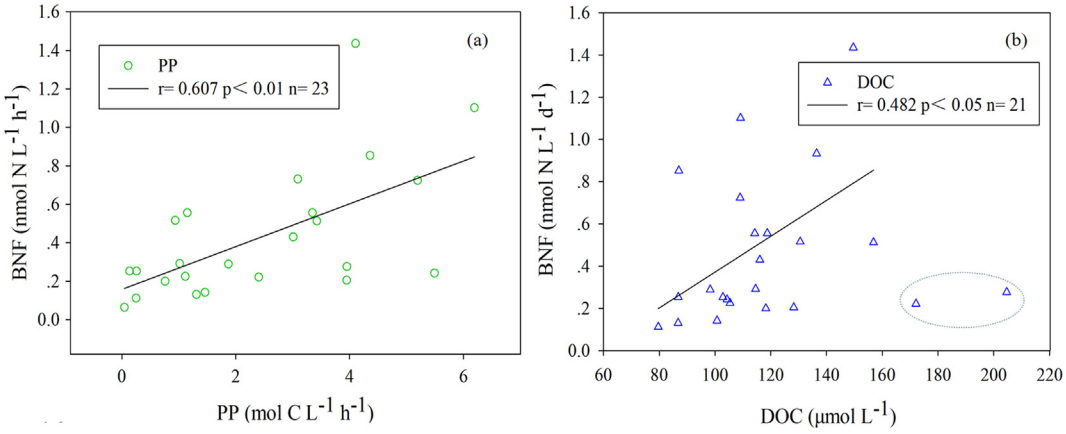

Fig. 8. Correlation between N_2 fixation rates (BNF), primary productivity (PP) and concentration of dissolved organic carbon (DOC) in Daya Bay during spring (April 2016) and summer (August 2015): (a) BNF vs PP, (b) BNF vs DOC. The outliers in the circle were observed at the surface of station S1 and the bottom of station S12 in summer.

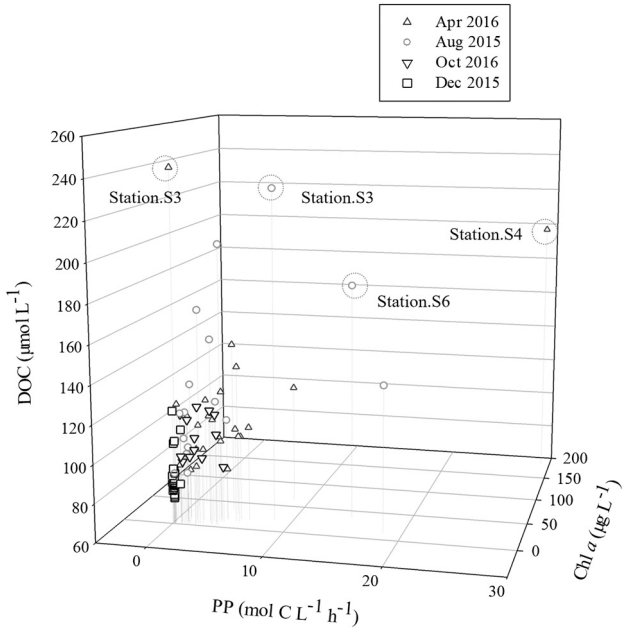

Fig. 9. Primary productivity, concentration of dissolved organic carbon and Chl *a* in spring (April 2016) and summer (August 2015).

a correlation between the BNF and DOC concentrations is in broad agreement with field studies in the coastal waters of the southern SCS (Moisander et al., 2008), Northern Pacific subtropical circulation (Zehr and Karl, 2007), and South Pacific (Moisander et al., 2014), which suggests that the excretion of organic carbon from phototrophs produce important heterotrophic N_2-fixing bacteria groups and in situ *nifH* expression. Thus, the correlation between the N_2 fixation and DOC probably reflects the correlation between C fixation and N_2 fixation, which means that the process of C fixation (or PP) regulates the process of N_2 fixation in coastal waters via DOC. Our results imply a potential relationship between N_2 fixation and carbon fixation, and such regulation needs to be investigated in different coastal regimes in future studies.

There is a possibility that shifts in phytoplankton, which have been taking place in the past decades in DB (Wu et al., 2017), may have exerted influence in regulating the N_2 fixation-DOC relationship in DB. This is because both the quantity and bioactivity of autochthonous DOC are largely dependent on phytoplankton production and community structure (Biddanda and Benner, 1997; Kirchman and Rich, 1997). Evident ecological changes have been observed in the past three decades (such as the drastic increase in Chl *a* concentration, miniaturized trend of phytoplankton, changed dominant species of phytoplankton, shift of alga blooms from diatoms-dominated to dinoflagellates-dominated, etc.) and changing nutrient field and seawater warming have been suggested to be the main causes (Li et al., 2015; Wu et al., 2017). Unfortunately, no historical data on N_2 fixations are available, and how this process may have responded to environmental changes in DB cannot be assessed here. However, there is no doubt that the interplay between N_2 and C fixation is complex and needs to be investigated further in future studies in coastal waters that have been exposed to an intensifying anthropogenic influence.

5. Conclusions

We find that N_2 fixation actively occurs under relatively N-replete conditions in DB in the coastal northern South China Sea. We first observed a tight link between N_2 fixation and C fixation here, as indicated by the positive correlation between the N_2 fixation rate and DOC concentration and primary productivity. We propose that such DOC regulation may also exist in similar tropical/subtropical coastal waters, where heterotrophic N_2 fixation may be important. We propose that non-diazotrophic phytoplankton play an important role in regulating N_2 fixation by changing the quality and bio-activity of DOC for diazotrophs in coastal waters. Our study adds knowledge on N_2 fixation dynamics in these under-sampled subtropical coastal waters and provides insights on constraints on N_2 fixation over a broad spatial range in the global ocean.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.04.176.

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