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Low nitrate availability promotes diatom diazotroph associations in the marginal seas of the western Pacific

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ABSTRACT: Heterocystous cyanobacteria that form diatom diazotroph associations (DDAs) are important N_2-fixers in tropical and subtropical oceans, but factors affecting their symbioses and abundances are not well understood. We investigated the seasonal dynamics of diazotrophic cyanobacteria *Richelia* and *Calothrix* (cyanobionts) and their DDAs in the South China Sea (SCS) and the Kuroshio. Cyanobiont abundance and the proportions of diatoms forming DDAs, measured by symbiotic percentages (SPs), were determined, and a nitrate addition experiment was carried out to discern the effects of nitrate availability on DDAs. Vertically-integrated cyanobiont abundance was higher in summer than winter, higher in the Kuroshio than the SCS, and positively correlated with nitracline depth. On average, 83% of the cyanobionts were distributed above the nitracline. SPs were high when the nitracline was deep. Hemiauloid diatoms *Hemiaulus membranaceus* and *H. sinensis* were more likely to form DDAs than the rhizosolenoid diatom, *Rhizosolenia clevei.* Hemiauloids were abundant in the warm seasons when the SPs were high as the nitracline deepened; rhizosolenoids were abundant in winter when the SPs were low as the nitracline shoaled. Although enrichment with nitrate dramatically reduced the SP and increased the abundance of *R. clevei*, the abundances of *H. membranaceus* and *H. sinensis* were not affected, suggesting that hemiauloids were more dependent than rhizosolenoids on the nitrogen fixed by *Richelia*. Low nitrate availability increased the abundances of symbiotic cyanobionts and promoted their association with host diatoms. Nitrate depletion might thus drive the growth of hemiauloid or rhizosolenoid diatoms when they form DDAs with N_2-fixing cyanobacteria.

KEY WORDS: Nitracline · Symbiosis · Heterocystous diazotroph · Potential host diatom · Symbiotic percentage · Free-living cyanobionts · South China Sea · Kuroshio

INTRODUCTION

Fixation of atmospheric nitrogen (N_2) is an important process in the biogeochemical nitrogen cycle and contributes significantly to new production in nitrate-limiting tropical and subtropical ecosystems (Karl et al. 1997, Carpenter et al. 1999, Bonnet et al. 2011). Unicellular cyanobacteria (Montoya et al. 2004), the filamentous non-heterocystous cyanobacteria *Trichodesmium* spp. (Capone et al. 2005), and diatom diazotroph associations (DDAs) (Subramaniam et al. 2008) are the 3 most important groups of diazotrophic phytoplankton that fix nitrogen in the ocean. DDAs are formed when diazotrophic cyanobacteria form a symbiosis with diatom hosts. The heterocystous *Richelia intracellularis* (hereafter *Richelia*) and *Calothrix rhizosoleniae* (hereafter *Calothrix*) are the 2 most common symbiotic cyanobacteria (cyanobionts). *Richelia* are usually symbiotic inside the diatom genera *Rhizosolenia*, *Guinardia*,

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and *Hemiaulus*, while *Calothrix* are symbiotic epiphytically on diatom genera *Chaetoceros* and *Bacteriastrum* (Sundström 1984, Carpenter 2002, Foster et al. 2011).

Symbiotic percentage (SP) of a host diatom species, which is defined as the percentage of diatom cells that are symbiotic with cyanobionts, has been used frequently to quantify the symbiotic status of DDAs (Heinbokel 1986, Villareal 1994). In natural waters, studies of DDAs have dealt mostly with DDA blooms and associated environmental factors. In DDA blooms in the North Pacific Subtropical Gyre, abundances of cyanobionts could reach $>10^5$ heterocysts m^{-3} (Villareal et al. 2011). The availability of phosphorus and/or iron is often thought to be crucial to the occurrence of DDA blooms (Carpenter et al. 1999, Dore et al. 2008, Subramaniam et al. 2008, Kitajima et al. 2009). Studies on DDAs in non-blooming situations are very rare. In addition to iron, phosphorus, and silicon, the concentration of nitrogen has been linked to the occurrence of DDAs in non-blooming conditions. A study in a cold eddy in the North Pacific Subtropical Gyre reported that although $>7 \times 10^6$ heterocysts m^{-2} occurred outside the eddy, DDAs were not found inside the eddy, suggesting that increased availability of nitrate in the eddy might control the abundances of diazotrophs (Vaillancourt et al. 2003). In the eastern Mediterranean Sea, high abundances of *Richelia* associated with *Rhizosolenia* spp. and *Hemiaulus* spp. during summer–autumn were related to a high silicate to nitrate ratio, which suggested the depletion of nitrate (Bar-Zeev et al. 2008).

Although DDAs are distributed widely in the oceans, their occurrence in the northern South China Sea (SCS) and upstream Kuroshio Current has not been reported. We studied the spatial and temporal dynamics of *Richelia* and *Calothrix* in the northern SCS and the upstream Kuroshio, and examined the effect of nitrate on the formation of DDAs. To our knowledge, there have been no previous studies of DDAs under non-bloom conditions or on the SPs of *Chaetoceros* sp. or *Bacteriastrum* spp. in the open ocean.

MATERIALS AND METHODS

Study area

The field data from 29 cruises on the SCS and the Kuroshio were collected between March 2001 and May 2010 (Table 1). The cruises were classified by season as summer (June–August), autumn (September–November), winter (December–February), or spring (March–May). Among the 19 stations surveyed, 9 (Stns S1 to S9) were located between 18° N and 22° 10′ N in the SCS basin, and 10 (Stns K1 to K10) were located between 21° 25′ N and 22° 10′ N in the upstream Kuroshio Current of the West Philippine Sea (Fig. 1). Stn S8, the most frequently visited station in this study, is the SEATS (South East Asia Time Series Station). Bottom depths of all stations were greater than 2000 m.

Hydrographic observations

On all cruises, water samples for nutrient and biochemical measurements were collected from 7 to 9 depths between the surface and 200 m by 20 l Go-Flo bottles attached to a rosette frame. Seabird SBE 9 conductivity-temperature-depth (CTD) sensors and an underwater photosynthetically active radiation (PAR) sensor (OSP200L, Biospherical) were attached to the frame. The euphotic depth (D_E, m) was defined as the depth where irradiance was 0.8 % of that at the surface (Chen et al. 2008), as estimated by the light extinction coefficient (K, m^{-1}). The surface PAR influx (SPAR, mol quanta m^{-2} d^{-1}) was recorded continuously using a shipboard irradiance sensor (QSR-2200, Biospherical) during the cruises, and the mean daytime SPAR (µmol quanta m^{-2} s^{-1}) was calculated by averaging the trapezoidal-integrated SPAR between 6:00 and 18:00 h. The mixed layer depth (D_M, m) was the depth of upper layer water that had a density (σ_t) gradient less than 0.1 kg m^{-3} (Chen et al. 2008). The stratification index (SI, kg m^{-4}) was the

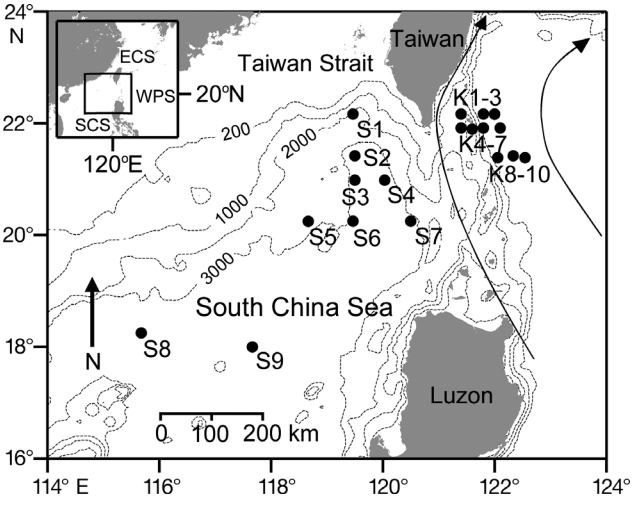

Fig. 1. Locations of sampling stations in the South China Sea (SCS, Stns S1 to S9) and the Kuroshio (Stns K1 to K10). Arrows east of 121° E indicate the Kuroshio path (after Liang et al. 2003). ECS: East China Sea; WPS: West Philippine Sea

Table 1. Sampling times of cruises from March 2001 to May 2010 in the South China Sea (Stns S1 to S9) and the Kuroshio (Stns K1 to K10). Abundances and symbioses of potential host diatoms were determined at the underlined stations

Season	Cruise no.	Dates	Stations	
			South China Sea	Kuroshio
Winter	780	23–30 Dec 05	S8	
	819	18–30 Dec 06	S2, S5	K9
	852	10–15 Dec 07	S2	K4, K6, K7
	886	08–14 Dec 08		K4, K6, K7
	821	12–19 Jan 07	S8	
	708	10–21 Feb 04	S2, S5, S8, S9	
Spring	606	20–31 Mar 01	S5, S8	
	638	7–15 Mar 02	S5	
	SC33	28 Mar–2 Apr 05	S8	
	1217	21–25 Apr 07		K1, K2, K3, K4
	1284	14–19 Apr 08	S2	K4
	717	3–8 May 04	S8	
	SKII	22 Apr–2 May 05	S2, S5, S6, S7	K9
	899	17–20 May 09		K4
	1455	12–17 May 10	S3, S4	K8, K10
Summer	722	24 Jun–6 Jul 04	S2, S5, S8	
	SC37	30 Jun–9 Jul 06	S8	
	1160	21–27 Jun 06		K9
	1234	7–10 Jul 07		K4, K6
	726	3–7 Aug 04	S8	
	763	8–18 Aug 05	S1, S2, S5, S6	K9
	1310	2–7 Aug 08		K4, K7
	910	14–20 Aug 09	S2, S3, S4	K5, K8, K10
Autumn	1316	5–10 Sep 08		K4, K7
	843	27 Sep–8 Oct 07	S1, S2	K4
	734	12–23 Oct 04	S2, S5	K3, K9
	812	18–24 Oct 06	S8	
	736	5–11 Nov 04	S8	
	773	7–15 Nov 05	S5, S8	

average density difference between 5 m and 100 m (Chen et al. 2008). The concentration of nitrate and nitrite (N+N) was measured either by the pink azo dye method adapted for a flow injection analyzer with a copper-coated cadmium reductor (Pai & Riley 1994) that had a detection limit of 0.1 µM, or by the chemiluminescent method (Garside 1982) with a detection limit of 1 to 2 nM. Soluble reactive phosphorus (SRP) concentrations were measured by the phosphomolybdate blue method (detection limit 0.2 µM; Strickland & Parsons 1972), or the modified magnesium-induced co-precipitation method with arsenate interference correction (detection limit 2 to 3 nM within 10 to 50 nM; Thomson-Bulldis & Karl 1998). The nitracline depth (D_N), defined as the depth at which N+N was 100 nM (Le Borgne et al. 2002), was used as an index of nitrate availability to phytoplankton in the upper water column. Silicate (SiO_2) concentration was measured by the silicomolybdenum blue method (Pai et al. 1990) adapted for a segmented flow analyzer. Chlorophyll *a* (Chl *a*) concentration was determined by fluorescence spectrophotometry (F3010, Hitachi) after acetone extraction (Strickland & Parsons 1972).

Estimation of dust influx

The influx of aeolian dust deposition (mg m^{-2} d^{-1}) was estimated by the Spectral Radiation-Transport Model for Aerosol Species (SPRINTARS) (Takemura et al. 2000). The flux at each station was the mean of daily estimates for 14 d before field sampling began (Hashihama et al. 2009).

Enumeration of diatom diazotroph associations

Specimens for identification and enumeration of the cyanobionts and their potential hosts were prepared on board by gentle filtration (pressure <100 mm Hg) of a 1.2 or 2.4 l water sample onto a 10 µm Nuclepore™ polycarbonate filter membrane (25 mm diameter) on top of a Whatman GF/C filter paper. The polycarbonate membranes were laid on a microscope slide and covered by a drop of immersion oil (Merck) and a cover slip. All specimens were kept in darkness at −20°C until microscopic examination. All cyanobionts were identified and counted at 400× magnification using a Zeiss epifluorescence microscope with blue excitation (BP 450-490, FT 510, LP 520) and bright field in alternation so that the associated diatoms of cyanobionts could be identified. The filaments of cyanobionts showed orange–yellow fluorescence under blue excitation.

A diatom cell that contained symbiotic cyanobionts was called a 'host' and a diatom that could be symbiotic with *Richelia* or *Calothrix* was called a 'potential host' whether or not it was symbiotic with cyanobionts (e.g. Villareal 1992). The identification of DDAs was carried out based on the morphology of the host diatoms following Janson (2002). All endosymbiotic cyanobionts were treated as *Richelia* and all epiphytic cyanobionts were treated as *Calothrix*. The potential host diatoms were identified following the descriptions of Sundström (1984, 1986) and Hasle & Syvertsen (1997). For the endosymbiotic potential

host species (*Rhizosolenia clevei*, *Guinardia cylindrus*, or *Hemiaulus* spp.), symbiotic percentage (SP) was the percentage of diatom cell symbiotic with *Richelia* (Heinbokel 1986). For the potential host species (*Chaetoceros compressus* or *Bacteriastrum* spp.) on which *Calothrix* were epiphytically symbiotic, SP was the percentage of diatom chains attached epiphytically with *Calothrix*. The whole diatom chain was treated as symbiotic, not just the cells that were in direct contact with the cyanobionts. The specific SP at a station was calculated by dividing the depth-integrated abundance of hosts with that of the potential hosts. The number of *Richelia* per host cell (heterocysts cell^{-1}) was the mean of *Richelia* filaments inside all cells of a host diatom species. The number of *Calothrix* per host cell (heterocysts cell^{-1}) was the mean of the number of *Calothrix* filaments divided by the total number of cells in the chain of a host species. The cyanobionts that were not symbiotic with diatoms were treated as 'free-living cyanobionts' (FL) and were not distinguished as *Richelia* or *Calothrix*.

Cell densities of the potential hosts were determined every season at 2 to 4 stations in the SCS and the Kuroshio (Table 1). All potential host diatoms with and without cyanobionts were identified and counted as individual cells for endosymbiotic DDAs and as chains and cells per chain for epiphytic DDAs. Depth-integrated abundances of cyanobionts and potential hosts were calculated by trapezoidal integration from the surface to 100 m depth, and expressed as heterocysts m^{-2} and cells m^{-2}, respectively. The vertical distributions of cyanobiont abundance were calculated by pooling all stations within a season and were used to compare the seasonal differences in abundance. Comparisons of the vertical distributions between diazotrophic diatoms and non-diazotrophic diatoms were performed at the stations where the potential hosts were examined (Table 1).

Nitrate enrichment experiment

An on-board nitrate enrichment experiment was conducted at Stn K10 on one additional cruise (3 to 9 Dec 2010). The surface water temperature was 25.9 ± 0.2°C. Seawater was collected from 5 m depth and placed in four 20 l acid-washed polycarbonate carboys, 2 each for the nitrate-enrichment and control groups. Sodium nitrate solution in excessive amount was added to 2 carboys to a final concentration of 10 µM on Days 0, 2, and 4 to ensure nitrate repletion after continuous sampling. All carboys were incubated for 7 d under natural light inside a large plastic container with continuous flow of *in situ* surface seawater. On Days 0, 2, 4, and 6, a 2.4 l aliquot of the incubation water was sampled and filtered gently (<100 mm Hg) onto a 47 mm, 5 µm Nuclepore™ polycarbonate filter membrane, and kept in darkness at −20°C until microscopic examination.

Statistical analysis

The seasonal and spatial differences in cyanobiont abundance were tested by 2-way analysis of variance and Duncan's multiple range test. Pearson's correlation coefficient (r) was used to examine the relationships between cyanobiont abundance and environmental variables as well as between any 2 of the 13 environmental variables, including surface seawater temperature (SST), surface salinity, surface N+N, surface SRP, surface SiO_2, surface Chl *a*, D_M, SI, depth of Chl *a* maximum (D_{CM}), D_N, D_E, SPAR, and dust influx. To elucidate key environmental variables relating to the dynamics of cyanobiont abundance, principal component analysis was performed using a correlation-standardized data matrix from the 13 environmental variables and the abundance of all cyanobionts (Jolliffe 2002). Stepwise multiple regression analysis (Draper & Smith 1981) was performed to determine the most important environmental variable related to the abundance of cyanobionts. Relationships between SPs, potential host abundances, and environmental variables also were tested with Pearson's correlations. The values of cyanobiont abundance, potential host abundances, and dust influx (after log transformation) as well as SPs (after logit transformation) were found to meet the normality assumption after the Shapiro-Wilk test (Royston 1992), except for the SP of *Hemiaulus membranaceus* ($W = 0.816$, $p < 0.01$). The SAS program (SAS Institute) was used for all statistical procedures.

RESULTS

Water properties

Being located at the low latitudes, the upstream Kuroshio and the northern SCS are generally very oligotrophic and are influenced strongly by the SW and NE monsoons. Unlike the SCS, water in the Kuroshio is more uniform spatially and less variable seasonally in temperature and biochemical properties. The maximum and minimum SSTs measured during the 29 cruises were 30.6 and 23.0 °C, respec-

tively (Table 2). Winter, when the NE monsoon prevailed, was the season with distinctive water properties in both locations. Mean SSTs were not different for the SCS and the Kuroshio in summer, but were lower in the SCS than in the Kuroshio in winter. Surface Chl *a* in the SCS was generally higher and more variable seasonally than in the Kuroshio. The water column of the Kuroshio had lower SI and deeper D_M than the SCS. The SPAR was lower in winter than the other 3 seasons in both the SCS and the Kuroshio. The D_E, which was shallower in winter, was always deeper in the Kuroshio.

Nutrient availabilities suggested that diatoms were limited by nitrogen, but not by silicate or phosphorus, in both the Kuroshio and the SCS. The D_N was deeper and less variable in the Kuroshio than in the SCS (Table 2). Similarly, surface N+N and SRP varied little seasonally in the Kuroshio, but were low in summer and high in winter in the SCS. Surface SiO_2 (Table 2) was above the half-saturation constant of 0.02 to 0.5 µM for growth of marine diatoms (Carlsson & Granéli 1999). The surface N+N to SRP ratios were below the N:P Redfield ratio of 16, and the surface SiO_2 to N+N ratios were above the Si:N Brzezinski ratio of 1 (Brzezinski 1985) (Table 2).

The deposition of aeolian dust in the SCS and the Kuroshio indicated significant seasonal and spatial variations, being the highest in summer during the SW monsoon and lowest in winter during the NE monsoon (Table 2). The summertime dust influx was higher in the SCS than in the Kuroshio.

Many environmental variables were correlated; in particular, SST was positively correlated with D_N, D_{CM}, D_E, SPAR, and dust influx, and negatively correlated with surface Chl *a*, N+N, SRP, and salinity (see Table S1 in the Supplement at www.int-res.com/articles/suppl/a073p135_supp.pdf). The dust influx, D_N, D_E, and D_{CM} were positively correlated with each other and negatively correlated with surface Chl *a*, N+N, and SRP (Table S1).

Abundances of cyanobionts

Depth-integrated abundances of all cyanobionts (host-associated plus FL) were significantly higher in summer than winter ($F_{3,63} = 4.07$, $p < 0.05$) (Table 3) and in the Kuroshio than in the SCS ($F_{1,63} = 5.66$, $p < 0.05$). The difference between maximum and minimum abundances was 9 fold in the SCS and 2 fold in the Kuroshio, respectively (Table 3). These abundances were generally much lower than those reported in bloom conditions.

The principal component analysis clearly differentiated the regional and seasonal dynamics of cyanobiont abundance and their corresponding environ-

Table 2. Seasonal means of environmental variables in the South China Sea and the Kuroshio from March 2001 to May 2010. Data indicate mean ± SE; (n = no. of stations). SST, surface seawater temperature; N+N, nitrate plus nitrite; SRP, soluble reactive phosphorus; N:P, ratio of N+N to SRP; Si:N, ratio of SiO_2 to N+N; Chl *a*, chlorophyll *a*; Dust, dust influx; D_N, nitracline depth; D_{CM}, depth of Chl *a* maximum; SI, stratification index; D_M, mixed layer depth (layer of water with a density gradient less than 0.1 kg m^{-3}); SPAR, surface photosynthetically active radiation; D_E, euphotic depth (0.8% of surface photosynthetically active radiation)

Variable	South China Sea				Kuroshio			
	Winter (n = 9)	Spring (n = 12)	Summer (n = 12)	Autumn (n = 8)	Winter (n = 7)	Spring (n = 9)	Summer (n = 9)	Autumn (n = 5)
SST (°C)	24.1 ± 0.4	26.6 ± 0.4	29.2 ± 0.2	27.9 ± 0.3	26.2 ± 0.3	27.7 ± 0.3	29.6 ± 0.3	28.1 ± 0.7
Salinity	34.2 ± 0.1	34.1 ± 0.1	33.8 ± 0.1	33.8 ± 0.1	34.3 ± 0.1	34.4 ± 0.1	34.1 ± 0.1	34.4 ± 0.1
N+N (nM)	372 ± 273	106 ± 82	22 ± 7	43 ± 12	28 ± 8	18 ± 4	27 ± 9	27 ± 12
SRP (nM)	74 ± 15	47 ± 10	19 ± 2	15 ± 1	43 ± 12	41 ± 5	26 ± 4	18 ± 5
SiO_2 (µM)	1.9 ± 0.3	2.0 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	0.8 ± 0.1	1.2 ± 0.1	1.1 ± 0.0	0.9 ± 0.1
N:P	3 ± 2	1 ± 1	2 ± 1	3 ± 1	1 ± 0	1 ± 0	1 ± 0	3 ± 2
Si:N	37 ± 16	127 ± 29	149 ± 28	62 ± 15	67 ± 29	83 ± 15	83 ± 28	80 ± 29
Chl *a* (mg m^{-3})	0.39 ± 0.04	0.17 ± 0.03	0.15 ± 0.03	0.12 ± 0.02	0.20 ± 0.05	0.10 ± 0.01	0.12 ± 0.02	0.12 ± 0.03
Dust (mg m^{-2} d^{-1})	0.3 ± 0.1	0.8 ± 0.1	6.0 ± 0.5	1.1 ± 0.3	0.3 ± 0.1	2.9 ± 0.5	4.4 ± 0.8	1.4 ± 0.3
D_N (m)	20 ± 8	39 ± 6	55 ± 5	52 ± 8	66 ± 6	68 ± 9	70 ± 6	69 ± 10
D_{CM} (m)	32 ± 6	59 ± 6	69 ± 4	80 ± 10	63 ± 12	91 ± 7	88 ± 9	88 ± 10
SI (×10^{-2} kg m^{-4})	1.0 ± 0.2	2.8 ± 0.1	3.0 ± 0.2	2.2 ± 0.4	0.8 ± 0.3	1.2 ± 0.1	1.6 ± 0.2	0.9 ± 0.3
D_M (m)	79 ± 9	27 ± 3	57 ± 5	73 ± 11	119 ± 13	119 ± 17	78 ± 17	117 ± 17
SPAR (mol quanta m^{-2} d^{-1})	35.1 ± 2.9	55.4 ± 5.4 (n = 10)	45.1 ± 7.0 (n = 10)	50.6 ± 5.3	38.6 ± 3.3	67.4 ± 8.5 (n = 5)	54.9 ± 7.9 (n = 7)	55.3 ± 6.2
D_E (m)	75 ± 4	83 ± 6 (n = 11)	105 ± 6	115 ± 5	111 ± 5 (n = 4)	122 ± 10 (n = 7)	113 ± 4 (n = 6)	132 ± 10

Table 3. Mean seasonal abundance of cyanobionts (×10^3 heterocysts m^{-2}) in different diatom diazotroph associations in the South China Sea and the Kuroshio from March 2001 to May 2010. SYM, symbiotic *Richelia* plus *Calothrix*; *R-R*, *Rhizosolenia clevei-Richelia*; *G-R*, *Guinardia cylindrus-Richelia*; *Hm-R*, *Hemiaulus membranaceus-Richelia*; *Hs-R*, *Hemiaulus sinensis-Richelia*; *C-C*, *Chaetoceros compressus-Calothrix*; *Bc-C*, *Bacteriastrum comosum-Calothrix*; *Bd-C*, *B. delicatulum-Calothrix*; *Be-C*, *B. elongatum-Calothrix*; *Bh-C*, *B. hyalinum-Calothrix*; FL, free-living *Richelia* plus *Calothrix*. Data indicate mean ± SE (n = no. of stations). Seasonal means with different superscript letter are significantly different ($p < 0.05$) after 2-way ANOVA and Duncan's multiple range test. Asterisks indicate significant difference between the SCS and the Kuroshio; $^{*}p < 0.05$; $^{**}p < 0.01$. Interactions are not significant

Diatom-diazotroph associations	South China Sea				Kuroshio			
	Winter (n = 9)	Spring (n = 12)	Summer (n = 12)	Autumn (n = 8)	Winter (n = 7)	Spring (n = 9)	Summer (n = 9)	Autumn (n = 5)
SYM*	231 ± 58^{a}	708 ± 319^{ab}	1800 ± 395^{b}	1705 ± 727^{b}	1410 ± 264^{a}	1636 ± 542^{ab}	2350 ± 607^{b}	1857 ± 290^{b}
*R-R***	44 ± 28^{a}	208 ± 95^{a}	213 ± 59^{a}	162 ± 29^{a}	575 ± 133^{a}	581 ± 206^{a}	466 ± 212^{a}	317 ± 75^{a}
G-R	5 ± 5^{a}	92 ± 53^{a}	215 ± 74^{a}	257 ± 108^{a}	46 ± 29^{a}	191 ± 73^{a}	153 ± 61^{a}	98 ± 61^{a}
Hm-R	141 ± 63^{a}	130 ± 51^{a}	472 ± 137^{ab}	1029 ± 598^{b}	275 ± 148^{a}	105 ± 59^{a}	599 ± 205^{ab}	619 ± 114^{b}
Hs-R	28 ± 19^{a}	119 ± 71^{a}	424 ± 237^{a}	152 ± 47^{a}	159 ± 92^{a}	342 ± 140^{a}	473 ± 158^{a}	335 ± 137^{a}
C-C	12 ± 8^{a}	159 ± 71^{ab}	445 ± 110^{b}	117 ± 48^{a}	187 ± 59^{a}	390 ± 208^{ab}	437 ± 170^{b}	122 ± 23^{a}
Bc-C	0^{a}	0^{a}	6 ± 5^{a}	0^{a}	67 ± 67^{a}	16 ± 11^{a}	0^{a}	169 ± 169^{a}
*Bd-C** (p = 0.055)	0^{a}	0^{a}	0^{a}	0^{a}	81 ± 81^{a}	0^{a}	18 ± 12^{a}	132 ± 132^{a}
Be-C	0^{a}	0^{a}	0^{a}	0^{a}	11 ± 11^{a}	0^{a}	0^{a}	0^{a}
*Bh-C**	0^{a}	0^{a}	0^{a}	0^{a}	4 ± 4^{a}	0^{a}	92 ± 61^{a}	71 ± 71^{a}
FL	6 ± 3^{a}	61 ± 32^{a}	288 ± 125^{a}	105 ± 47^{a}	110 ± 31^{a}	245 ± 74^{a}	229 ± 76^{a}	239 ± 86^{a}
All cyanobionts*	236 ± 59^{a}	769 ± 340^{ab}	2089 ± 402^{c}	1810 ± 710^{bc}	1519 ± 276^{a}	1881 ± 609^{ab}	2579 ± 667^{c}	2096 ± 320^{bc}

mental variables (Fig. 2). Three principal components (PCs), all with eigenvalues >1 (Kaiser's rule, Jolliffe 2002), explained 68.7% of the system variance (Table S2 in the Supplement). PC1 (39.4%) mainly described the optimal environmental conditions for the abundance of cyanobionts. The abundance was higher when SST was high, D_N, D_{CM}, and D_E were deep, and N+N, SRP, SiO_2, and Chl *a* were low (Fig. 2, Table S2), corresponding to the water properties of a warm season. The positive correlation of dust influx and PC1 (Table S2) suggested that aeolian iron, which was higher in summer than winter, was also a possible driving factor. PC2 (19.8%) mainly described the degree of water column mixing and water property factors (surface salinity and SiO_2 concentration) that differentiate the Kuroshio and the SCS and the seasons in the SCS (Table S1). The plot of PC1 vs. PC2 (Fig. 2) clearly showed that water properties of the Kuroshio varied little and fell mostly in Quadrant IV, indicating that the cyanobiont abundance was higher, D_N, D_{CM}, D_E, and D_M were deeper, and surface salinity was greater in the Kuroshio than the SCS. The SCS data scattered in the other 3 quadrants, reflecting the rather broad and dynamic seasonal variations of its water properties. While summer and autumn data fell mostly in Quadrant I, winter data occupied Quadrant III, which was characterized by high surface nutrients and Chl *a* concentrations, but low cyanobiont abundance, temperatures, dust influx, and SPAR, and shoaled D_N, D_E, and D_{CM} (Fig. 2). The spring data occupied Quadrant II (Fig. 2).

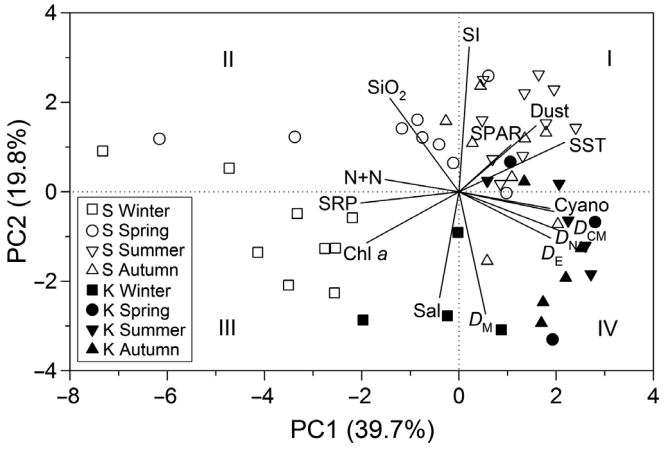

Fig. 2. Relationship between the first (PC1) and the second (PC2) principal components (59.5% of the total variance explained) with 53 stations and eigenvectors of 14 variables. Abbreviations: Cyano, abundance of all cyanobionts; SST, surface seawater temperature; D_N, nitracline depth; N+N, surface nitrate plus nitrite; SRP, surface soluble reactive phosphorus; SiO_2, surface silicate; Chl *a*, surface chlorophyll *a*; D_{CM}, depth of Chl *a* maximum; D_E, euphotic depth; SI, stratification index; D_M, mixed layer depth; Sal, surface salinity; SPAR, surface photosynthetically active radiation; Dust, dust influx. Open symbols indicate the South China Sea (S) and filled symbols indicate the Kuroshio (K). Roman numerals denote the quadrants. Percentages of system variance explained by each principal component are in parentheses

Multiple regression analysis showed that both SST (partial $t = 4.64$, $p < 0.0001$) and D_N (partial $t = 2.63$, $p < 0.05$) were significantly associated with abundances of all cyanobionts. The regression equation was $Log_{10}(abundance) = -2.636 + 0.186\ SST + 0.008\ D_N$ ($R^2 = 0.42$, $p < 0.0001$, $n = 71$). SST varied seasonally in the SCS and the Kuroshio (Fig. 3A, Table 2) and was highly correlated with many other environmental variables (Table S1), so we treated it as a proxy for seasonality. D_N was treated as a proxy for seasonality in the SCS (Fig. 3B1, Table S1) and also for regional differences between the SCS and the Kuroshio (Fig. 3B3, Table 2).

Vertical distributions of the cyanobionts

The variation of cyanobiont abundance was related to D_N only in the SCS, not in the Kuroshio. On average, 83% of all cyanobionts were above the D_N (Fig. 4). The distribution of cyanobionts seemed vary with the depth of D_N, which varied with seasons in the SCS (Fig. 4A), but varied little in the Kuroshio (Fig. 4B). Similar to cyanobionts, DDAs were distributed mainly above the D_N. In contrast, 56% of non-diazotrophic diatoms was distributed below the D_N. They concentrated abundantly at a depth much deeper than the DDAs (Fig. S1 in the Supplement).

The vertical distribution of the cyanobionts did not seem to be related to light level. In the SCS, the mean depth of cyanobiont abundance maximum (Fig. 4A) was 30 m in autumn and about 5 m in the other seasons. The light setting in the depth of the autumn maximum was 19% of light penetration depth (LPD), or 218 µmol quanta m^{-2} s^{-1} in terms of daytime average. In the other seasons, it was 72 to 79% of LPD or 584 to 945 µmol quanta m^{-2} s^{-1}. In the Kuroshio, the abundance maximum occurred at 5 m in spring (82% of LPD, 1272 µmol quanta m^{-2} s^{-1}), but deepened to 20 m in winter (42% of LPD, 371 µmol quanta m^{-2} s^{-1}) and summer (43% of LPD, 538 µmol quanta m^{-2} s^{-1}), and moved to 60 m in autumn (11% of LPD, 140 µmol quanta m^{-2} s^{-1}) (Fig. 4B).

Symbiotic patterns of potential hosts

There were 2 distinctive groups of endosymbiotic DDAs: the rhizosolenoids (*Rhizosolenia clevei*; Fig. S2A,B in the Supplement) and the hemiauloids (*Hemiaulus membranaceus*, Fig. S2E,F; *H. sinensis*, Fig. S2G,H). SPs varied with season (Table 4) and were inversely related to the abundances of rhizosolenoid diatoms. In the SCS, the SPs of *R. clevei* increased to 30.8 ± 5.9 (SE) % in the warm seasons when these diatoms were scarce, but decreased to almost zero in winter when the diatoms were abundant. Its seasonal variations in the Kuroshio were less dynamic (Table 4). When data from both regions were pooled, the SPs of *R. clevei* were positively correlated with D_N (Fig. 5A) and negatively correlated with its own cell abundances (Fig. 5B). Each host *R. clevei* cell contained an average of 1.2 to 1.6 *Richelia* heterocysts (Table 4).

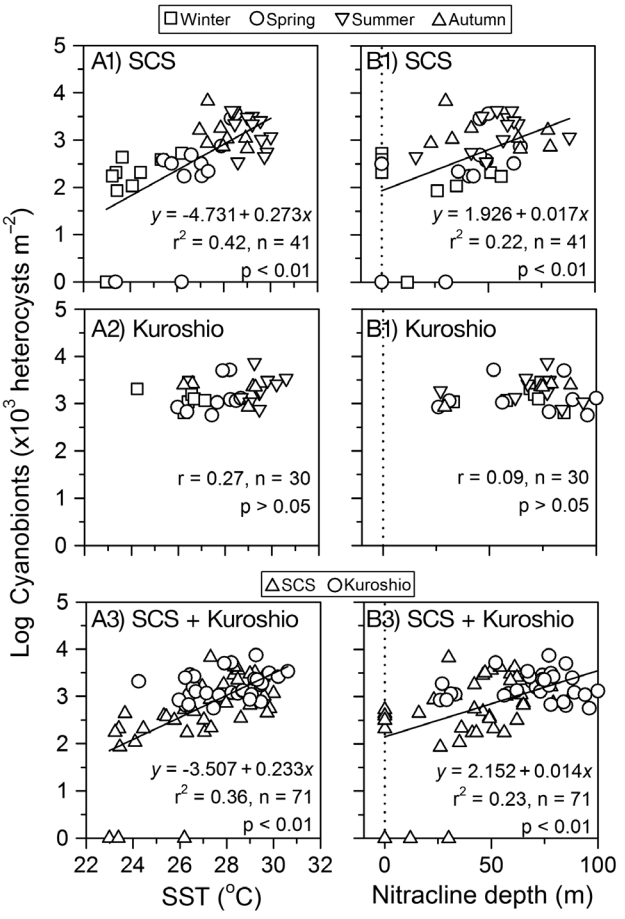

Fig. 3. Relationships between abundances of all cyanobionts (symbiotic and free-living) and (A) surface seawater temperature (SST) and (B) nitracline depth in (A1,B1) the South China Sea (SCS), (A2,B2) the Kuroshio, and (A3,B3) the SCS plus the Kuroshio with all seasons pooled; n = no. of stations

The symbiotic pattern of the hemiauloids was very different; specifically, their SPs were high when the host diatom abundances were high (Table 4). In the SCS during the warm season when the hosts were abundant, the SPs of *H. membranaceus* and *H. sinensis* were 97.4 ± 2.6 and 18.6 ± 9.4%, respectively, but in the spring SPs for both were 0%. The SPs of *H. membranaceus* and *H. sinensis* were significantly higher and displayed less seasonal variation in the

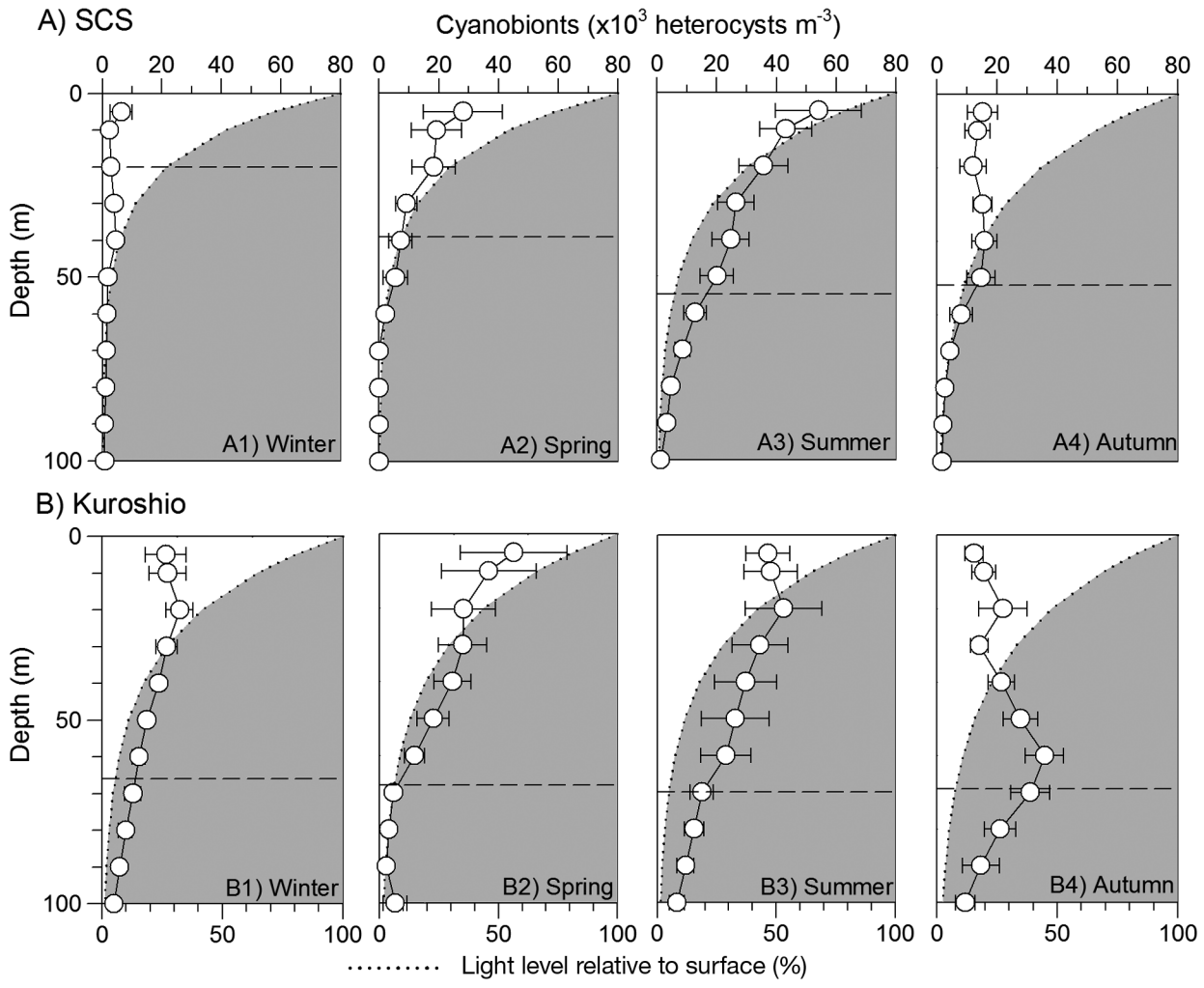

Fig. 4. Seasonal vertical profiles of the abundances of all cyanobionts (symbiotic and free-living) and light level relative to surface in (A) the South China Sea (SCS) and (B) the Kuroshio. Dashed lines indicate mean seasonal nitracline depths (D_N). Error bars are standard error of the mean (SE)

Kuroshio than in the SCS (for *H. membranaceus*: $F_{1,9}$ = 4.72, p = 0.058, n = 17; for *H. sinensis*: $F_{1,6}$ = 6.32, p < 0.05, n = 12). When data from both regions were pooled, the SPs of *H. membranaceus* were positively (p = 0.06) correlated with D_N (Fig. 5C). In the SCS, the SPs of *H. membranaceus* were positively correlated with its cell abundances (Fig. 5D). The significance, however, could be driven by 1 point in this relatively small data set. Hemiauloid diatoms hosted fewer *Richelia* in the cold season than in the warm season, as did the rhizosolenoid group in the SCS. The hemiauloids hosted about 2 *Richelia* cell^{-1} and slightly more (t = 7.17, p < 0.0001) in the Kuroshio (2.13 ± 0.02 [SE] heterocysts cell^{-1}, n = 1327) than in the SCS (1.92 ± 0.02 heterocysts cell^{-1}, n = 1715).

The numbers of *Richelia* filaments in each *Guinardia cylindrus* (Fig. S2C,D) were greater in the spring and summer than the winter and autumn in the SCS ($F_{3,475}$ = 10.92, p < 0.0001), and were greater in the spring than the summer in the Kuroshio ($F_{3,267}$ = 17.79, p < 0.0001) (Table 4). But the SP of *G. cylindrus* was not related to its own cell abundance or environmental variables. The SP of *G. cylindrus* (73.3 ± 8.9%, n = 18) was significantly higher (t = 4.48, p < 0.0001) than that of *R. clevei* (24.0 ± 4.5%, n = 21).

The symbiotic pattern of epiphytic DDAs was generally similar seasonally and spatially to that of the rhizosolenoid group. The SPs of *Chaetoceros compressus* tended to be lower in winter and spring than in summer and autumn in the SCS, and varied little seasonally in the Kuroshio. The SPs of *C. compressus* were significantly greater in the Kuroshio ($F_{1,12}$ = 5.94, p < 0.05, n = 20) than in the SCS (Table 4). The SPs of *C. compressus* were positively correlated with D_N (Fig. 5E) and negatively correlated with its cell abundance (Fig. 5F) in the pooled data of the SCS

Table 4. Seasonal means of potential host abundances (×10^3 cells m^{-2}), symbiotic percentage (SP, %), numbers of endosymbiotic *Richelia* per host cell (heterocysts $cell^{-1}$), and number of epiphytic *Calothrix* per host cell (heterocysts $cell^{-1}$) in diatom diazotroph associations in the South China Sea and the Kuroshio. Data indicate mean ± SE; (n = no. of stations; m = number of endosymbiotic host cells or epiphytic host chains examined)

Variable	South China Sea				Kuroshio			
	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
Rhizosolenia clevei								
Abundance	16606 ± 11674	736 ± 314	499 ± 238	524 ± 94	405 ± 127	1106 ± 273	1270 ± 387	714 ± 147
	(n = 3)	(n = 2)	(n = 3)	(n = 3)	(n = 2)	(n = 2)	(n = 4)	(n = 2)
SP	1.0 ± 0.8	17.4 ± 3.8	27.2 ± 5.2	30.8 ± 5.9	43.9 ± 8.7	10.3 ± 1.1	31.6 ± 15.8	28.9 ± 16.3
	(n = 3)	(n = 2)	(n = 3)	(n = 3)	(n = 2)	(n = 2)	(n = 4)	(n = 2)
Richelia per host	1.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.1	1.2 ± 0.0	1.2 ± 0.0	1.6 ± 0.0	1.5 ± 0.1	1.3 ± 0.1
	(m = 40)	(m = 291)	(m = 92)	(m = 127)	(m = 442)	(m = 508)	(m = 372)	(m = 136)
Guinardia cylindrus								
Abundance	302 ± 302	43 ± 26	139 ± 127	458 ± 137	62 ± 53	180 ± 133	135 ± 88	47 ± 19
	(n = 3)	(n = 2)	(n = 3)	(n = 3)	(n = 2)	(n = 2)	(n = 4)	(n = 2)
SP	0	85.9 ± 14.1	100 ± 0	62.6 ± 25.8	83.0 ± 17.0	100 ± 0	73.7 ± 20.2	50.0 ± 50.0
	(n = 1)	(n = 2)	(n = 2)	(n = 3)	(n = 2)	(n = 2)	(n = 3)	(n = 2)
Richelia per host	1.0 ± 0.0	1.5 ± 0.1	1.5 ± 0.1	1.2 ± 0.0	1.3 ± 0.1	2.3 ± 0.1	1.9 ± 0.1	1.1 ± 0.1
	(m = 7)	(m = 195)	(m = 113)	(m = 164)	(m = 27)	(m = 118)	(m = 79)	(m = 47)
Hemiaulus membranaceus								
Abundance	128 ± 70	40 ± 40	71 ± 55	349 ± 156	331 ± 127	101 ± 88	372 ± 218	793 ± 474
	(n = 3)	(n = 2)	(n = 3)	(n = 3)	(n = 2)	(n = 2)	(n = 4)	(n = 2)
SP	20.9 ± 20.9	0	34.7 ± 34.7	97.4 ± 2.6	94.0 ± 6.0	96.8 ± 3.2	68.3 ± 22.4	64.4 ± 35.6
	(n = 2)	(n = 1)	(n = 2)	(n = 3)	(n = 2)	(n = 2)	(n = 3)	(n = 2)
Richelia per host	1.8 ± 0.0	1.6 ± 0.0	2.1 ± 0.0	2.0 ± 0.0	2.5 ± 0.1	1.8 ± 0.1	2.3 ± 0.1	2.0 ± 0.0
	(m = 199)	(m = 183)	(m = 314)	(m = 453)	(m = 105)	(m = 107)	(m = 276)	(m = 163)
H. sinensis								
Abundance	0	42 ± 42	0	1072 ± 653	128 ± 128	322 ± 266	400 ± 206	169 ± 28
	(n = 3)	(n = 2)	(n = 3)	(n = 3)	(n = 2)	(n = 2)	(n = 4)	(n = 2)
SP		0		18.6 ± 9.4	94.9	87.5 ± 12.5	48.2 ± 26.3	52.7 ± 1.9
	(n = 0)	(n = 1)	(n = 0)	(n = 3)	(n = 1)	(n = 2)	(n = 3)	(n = 2)
Richelia per host	1.4 ± 0.1	1.6 ± 0.0	2.3 ± 0.1	1.9 ± 0.1	2.4 ± 0.2	1.9 ± 0.0	2.2 ± 0.1	2.4 ± 0.1
	(m = 77)	(m = 180)	(m = 243)	(m = 66)	(m = 54)	(m = 263)	(m = 288)	(m = 69)
Chaetoceros compressus								
Abundance	87962 ± 52594	97128 ± 97128	15168 ± 11067	3060 ± 399	949 ± 55	4186 ± 544	3185 ± 1437	2984 ± 1744
	(n = 3)	(n = 2)	(n = 3)	(n = 3)	(n = 2)	(n = 2)	(n = 4)	(n = 2)
SP	0	0.1	14.3 ± 14.3	11.6 ± 8.4	16.4 ± 13.4	59.4 ± 9.4	52.3 ± 18.8	27.4 ± 15.3
	(n = 3)	(n = 1)	(n = 3)	(n = 3)	(n = 2)	(n = 2)	(n = 4)	(n = 2)
Calothrix per host	0.25 ± 0.07	0.30 ± 0.03	0.32 ± 0.02	0.31 ± 0.04	0.25 ± 0.02	0.53 ± 0.08	0.44 ± 0.03	0.24 ± 0.02
	(m = 3)	(m = 80)	(m = 93)	(m = 16)	(m = 58)	(m = 87)	(m = 72)	(m = 16)
***Bacteriastrum* spp.**								
Abundance	212750 ± 133558	25727 ± 25620	7712 ± 6352	3902 ± 1482	7196 ± 5729	4682 ± 3133	13348 ± 9745	2589 ± 317
	(n = 3)	(n = 2)	(n = 3)	(n = 3)	(n = 2)	(n = 2)	(n = 4)	(n = 2)
SP	0	0	0	0	0	1.5 ± 1.5	7.0 ± 6.5	28.7 ± 0.6
	(n = 3)	(n = 2)	(n = 3)	(n = 3)	(n = 2)	(n = 2)	(n = 4)	(n = 2)
Calothrix per host			0.57 ± 0.08		1.44 ± 0.12	0.86 ± 0.38	1.39 ± 0.23	1.20 ± 0.22
	(m = 0)	(m = 0)	(m = 8)	(m = 0)	(m = 31)	(m = 3)	(m = 4)	(m = 7)

and the Kuroshio. *Bacteriastrum* spp. rarely formed DDAs in the SCS (Tables 3 & S3, the latter in the Supplement). In the Kuroshio, the SPs of *Bacteriastrum* spp. showed a tendency to be higher in summer and autumn than in winter and spring (Table 4), but were not related to its cell abundance or environmental variables.

Attachments of *Calothrix* to *Chaetoceros* and *Bacteriastrum* were oriented very differently. In *Chaetoceros* DDAs, most *Calothrix* filaments were attached to the diatoms' apertures and oriented transversely to the long axis of the diatom cell chain (Fig. S2I,J). By contrast, *Calothrix* on *Bacteriastrum* DDAs were usually attached to the diatom setae and mostly oriented parallel to the axis of the diatom chain (Fig. S2K,L).

Bacteriastrum chains carried more *Calothrix* filaments than did *Chaetoceros* chains (Fig. S2I–L, Table 4). Since the numbers of *Calothrix* per host cell were not significantly different ($F_{3,49} = 1.36$, $p > 0.05$) among the 4 species of *Bacteriastrum* (Table S3), the

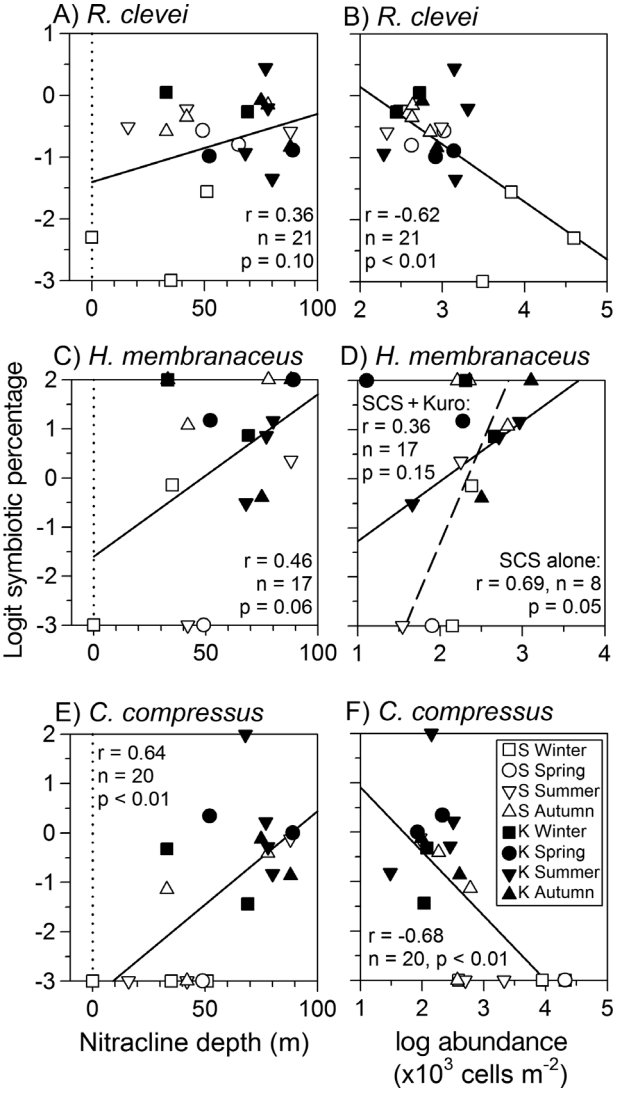

Fig. 5. Relationships between nitracline depth and symbiotic percentages of (A) *Rhizosolenia clevei*, (C) *Hemiaulus membranaceus*, and (E) *Chaetoceros compressus*, and between symbiotic percentages and abundances of (B) *R. clevei*, (D) *H. membranaceus*, and (F) *C. compressus*. Open symbols indicate the South China Sea (S) and filled symbols indicate the Kuroshio (K); n = no. of stations. Solid line indicates combined data of the South China Sea and Kuroshio, and dash line indicates the SCS alone

4 species were combined as *Bacteriastrum* spp. and compared with *C. compressus*. The numbers of *Calothrix* per host cell were greater in the Kuroshio than the SCS for both hosts of *Chaetoceros* ($t = 3.74$, n = 425, $p < 0.01$) and *Bacteriastrum* ($t = 3.45$, n = 53, $p < 0.01$) (Table 4). The overall average (the Kuroshio and the SCS) for *Bacteriastrum* (n = 53) was 1.24 ± 0.09 heterocysts cell^{-1}, and was 0.36 ± 0.01 heterocysts cell^{-1} for *Chaetoceros* (n = 425).

Nitrate addition experiment

The N+N, SRP, and SiO_2 concentrations of the water initially collected from Stn K10 were 10 nM, 27 nM, and 1.1 µM, respectively. The initial seawater contained an average of 3790 diatoms l^{-1}, 49 coccolithophores l^{-1}, and 19 heterocysts l^{-1} of cyanobionts. The cyanobionts were composed of 66% *Hemiaulus membranaceus-Richelia* (*Hm-R*) DDA, 25% *Rhizosolenia clevei-Richelia* (*R-R*) DDA, and 9% FL filaments. The initial water also contained 7 *H. membranaceus* cells l^{-1} and 6 *R. clevei* cells l^{-1}. No DDAs of *G. cylindrus*, *H. sinensis*, *C. compressus*, or *Bacteriastrum* spp. were found.

Throughout the incubation period, the density of symbiotic *Richelia* in the control bottles increased steadily and reached a maximum of 171 heterocysts l^{-1} on Day 6. In contrast, the density of symbiotic *Richelia* in the treatment bottles reached a maximum of 74 heterocysts l^{-1} on Day 4 (Fig. 6A1). The *Richelia* density in the control and treatment bottles became significantly different on Day 4 ($p < 0.05$) and Day 6 ($p < 0.01$). By contrast, FL filaments increased rapidly in the treatment bottles (Fig. 6A2). Densities of non-diazotrophic diatoms (Fig. 6A3) and coccolithophores (Fig. 6A4) dramatically increased in the treatment bottles.

Hm-R, *H. sinensis-Richelia* (*Hs-R*), and *R-R* were the most common DDAs in the experiment. *Hm-R* and *Hs-R* responded similarly to the nitrate addition; densities of *Richelia* in the control bottles increased steadily and were significantly higher than in the treatment bottles beginning on Day 2 (Fig. 6B1,C1). By contrast, densities of diatoms did not respond to the nitrate addition until Day 6, when *H. membranaceus* cells in the control bottles became significantly higher than in the treatment bottles ($p < 0.05$) (Fig. 6B2). The *H. sinensis* density was slightly, although not significantly higher in the control than in the treatment (Fig. 6C2). The numbers of cyanobionts per host were significantly higher in the control than in the treatment (Fig. 6B3,C3). *H. membranaceus* and *H. sinensis* in the control bottles were almost 100% symbiotic, but those in the treatment bottles had much lower SP (74.4 ± 9.0 to 83.5 ± 1.1% for *H. membranaceus*, Fig. 6B4; 35.8 ± 7.2 to 77.5 ± 2.5% for *H. sinensis*, Fig. 6C4).

In contrast to *Hm-R* and *Hs-R*, *Richelia* of *R-R* did not respond to the nitrate addition (Fig. 6D1). The density of *R. clevei* did not vary between the treatment and the control (Fig. 6D2). The number of *Richelia* per host *R. clevei* also did not differ significantly between the control and the treatment, except on Day 4 (Fig. 6D3). SP was higher in the control than the treatment since Day 4 (Fig. 6D4).

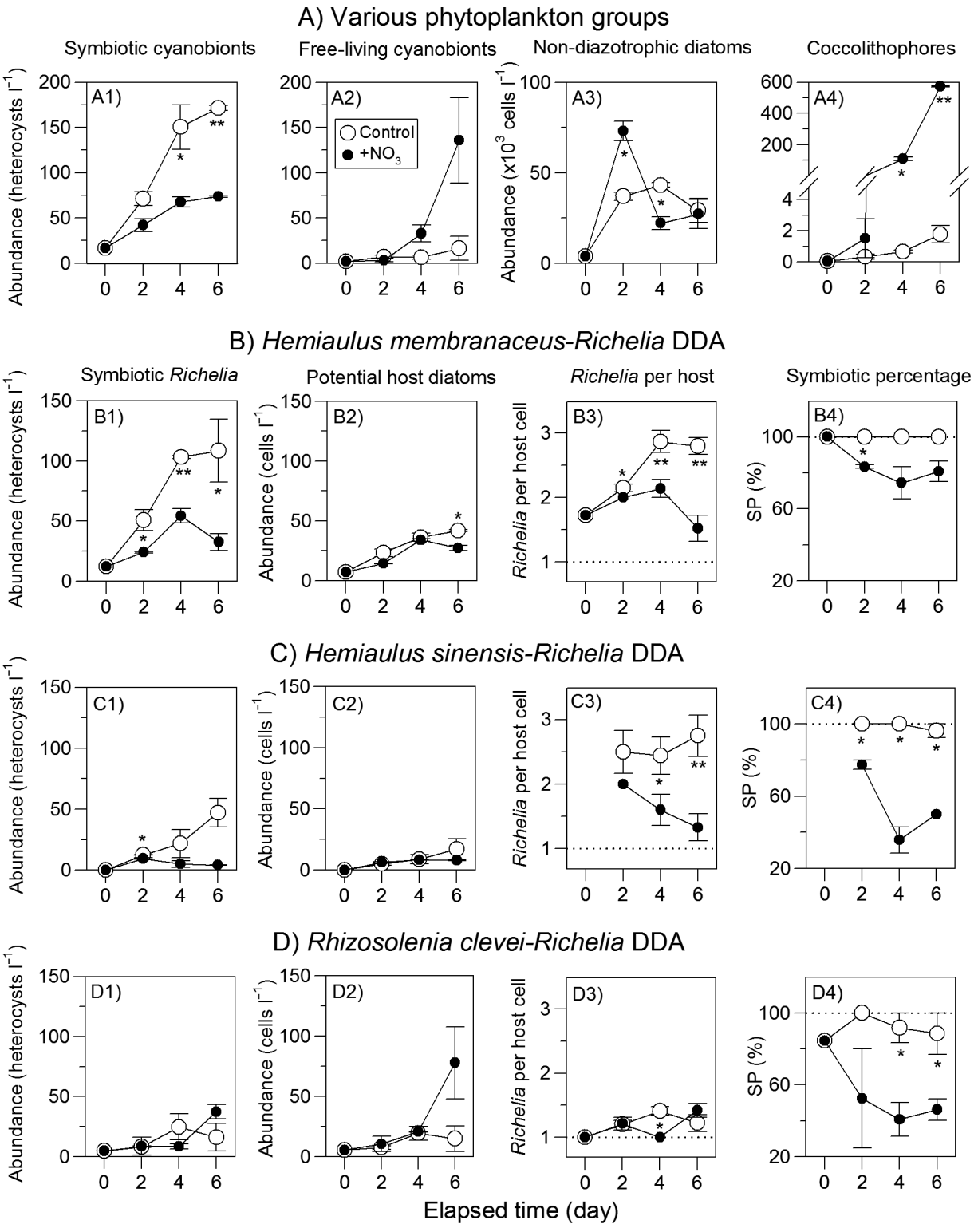

Fig. 6. Time-series changes in the abundances of (A) various phytoplankton groups including (A1) symbiotic cyanobionts, (A2) free-living cyanobionts, (A3) non-diazotrophic diatoms, and (A4) coccolithophores, as well as (B–D) 3 prevalent diatom diazotroph associations (DDAs) in response to nitrate enrichment in the on-board incubation experiment. The DDA abundances are depicted by the abundances of (B1–D1) symbiotic *Richelia*, (B2–D2) abundances of potential host diatoms, (B3–D3) number of *Richelia* per host cell, and (B4–D4) symbiotic percentage (SP). *$p < 0.05$ and **$p < 0.01$, indicate significant differences between enrichment and control

DISCUSSION

Nitrate availability

The combination of field results and the responses from the enrichment study illustrated that depletion of nitrate promoted the formation of more DDAs. More than 80% of the cyanobionts (and also their host diatoms) occurred above D_N, but less than half of non-diazotrophic diatoms occurred above D_N. In contrast to the diatoms that form DDAs, non-diazotrophic diatoms take up nitrate from the seawater (Chen et al. 2011). The enrichment study showed that a high concentration of nitrate promoted a flourishing of non-diazotrophic diatoms and coccolithophores (Fig. 6A3,4) but not DDAs (Fig. 6A1). D_N appears to be a good proxy to differentiate diazotrophic diatoms from non-diazotrophic diatoms in the vertical distribution.

Nitrate availability was a key factor influencing the spatial and seasonal variations of DDAs. The cyanobiont abundance, SPs, and number of *Richelia* per host cell of DDAs in the SCS fluctuated seasonally with changes in D_N. Their differences between the SCS and Kuroshio were also related to D_N. The multiple regression analyses indicated that D_N and SST were both related to the abundance of cyanobionts. This, together with our finding that DDAs responded strongly to the nitrate addition, as well as the strong relationships between D_N and SPs, and the strong relationship between D_N and vertical distributions of DDAs clearly indicate that independent of water temperature, nitrate availability alone plays an important role in affecting the DDAs. In addition to the formation of DDAs, the effects of increased nitrate availability on N_2 fixation of DDAs could be an important issue in oceanic ecosystems. Laboratory studies have indicated that high concentrations of nitrate hinder N_2 fixation rates of diazotrophic cyanobacteria, such as *Trichodesmium* (Holl & Montoya 2005) or the unicellular diazotroph *Crocosphaera watsonii* (Knapp et al. 2012). How high concentrations of nitrate affect N_2 fixation rates of DDAs remains to be studied.

Excess nitrate did not seem to adversely affect the growth of FL. In fact, the inconsistency arose from the rare occurrence of FL in the field samples, especially those collected when surface nitrate was replete. Unlike those in the enrichment experiment, FL filaments in nature could sink rapidly out of the euphotic zone. Janson et al. (1995) suggested that free-living *Richelia* are rare because they possess no gas vesicles. Our unpublished data also suggested that FL cyanobionts sank more rapidly than potential host diatoms, although diatoms in general are considered to sink fast (Scharek et al. 1999a,b). The hypothesis of rapid sinking could explain why FL constituted only a small fraction of natural cyanobiont populations in the Indian Ocean (Karsten 1907), the Sulu Sea (Gómez et al. 2005), and the Gulf of California (White et al. 2007). Their presence in the SCS and the Kuroshio (3 to 14%, Table 4) was similarly rare. The finding that FL flourished in response to nitrate enrichment in the experiment suggests a tendency toward disassociation of cyanobionts and their hosts when environmental nitrate is abundant.

Other environmental factors

In addition to nitrate availability, temperature, light intensity and availabilities of phosphate and iron might relate to DDA abundance. In the present study, in addition to SST correlating positively with cyanobiont abundance, SST was also positively correlated with D_N, D_{CM}, D_E, and dust influx, and negatively correlated with surface Chl *a*, N+N, SRP, and salinity. SST was thus a good proxy for seasonality. *Richelia* is distributed commonly in warm oceans where SST is above 18.0°C (e.g. Gómez et al. 2005), and so are diazotroph-associated diatoms (Hasle & Syvertsen 1997). Although light intensity controls phytoplankton photosynthesis and N_2 fixation, our study did not find a significant relationship between the vertical distribution of cyanobionts and light levels. Most DDAs were distributed above D_N, which fluctuated seasonally. The absolute PAR influx above D_N seemed sufficient to support DDAs. The abundance maxima of cyanobionts were concentrated at depths between 5 and 60 m, with PAR ranging between 140 and 1272 µmol quanta m^{-2} s^{-1}, a range much higher than light provision in most laboratory settings — such as 30 to 40 µmol quanta m^{-2} s^{-1} for *Trichodesmium* (Mulholland et al. 2001), 40 to 100 µmol quanta m^{-2} s^{-1} for *Calothrix rhizosoleniae* (Foster et al. 2010), or 70 to 80 µmol quanta m^{-2} s^{-1} for a Group C unicellular diazotrophic cyanobacterium (Taniuchi et al. 2012). For diatoms, photoinhibition occurs at about 204 µmol quanta m^{-2} s^{-1} (Ryther 1956). The near-surface distribution of DDAs, on the other hand, might facilitate DDAs with the benefit of aeolian iron flux. Diazotrophs require more iron than non-diazotrophic phytoplankton for the synthesis of nitrogenase and N_2 fixation (Paerl et al. 1994, Karl et al. 2002). The dust influx, a proxy for aeolian iron input, was higher in the SCS than the Kuroshio. Cyanobiont abundance, however,

was lower in the SCS than the Kuroshio. The seasonal variations of cyanobiont abundance are similar to those of *Trichodesmium* in the SCS and the Kuroshio (Chen et al. 2008). In addition to iron, phosphorus is an element related to the dynamics of DDAs. Various studies have identified the positive connection of phosphate abundances and N_2 fixation by *Rhizosolenia* spp.-*Richelia* (Mague et al. 1974), diazotroph abundances (Kitajima et al. 2009), and summertime blooms of *Trichodesmium* and/or diazotrophic diatoms (Dore et al. 2008). Our study, however, showed a negative relationship between cyanobiont abundance and surface SRP. This negative relationship could be due to the influences of other ecological factors, including D_N or SST, that co-varied with SRP (Chen et al. 2008).

Dependence on symbiosis of potential host diatoms

The potential hosts we studied differed significantly in the degree of dependency on their symbiosis with cyanobionts: strongly-dependent hemiauloid diatoms and weakly-dependent rhizosolenoid diatoms. Weakly-dependent *Rhizosolenia clevei* was more abundant in winter than summer in the SCS, which relates to the high nitrate availability in winter. This result is similar to a study in a cold eddy, in which the rhizosolenoid diatoms *R. bergonii* and *R. hebetata* were more abundant and did not contain endosymbionts inside the nitrate-replete eddy, while outside the eddy the rhizosolenoids contained symbiotic *Richelia* (Vaillancourt et al. 2003). In the present study, the highest population of *R. clevei* in the SCS occurred when its SP was the lowest, suggesting that the abundance of *R. clevei* might depend more on nitrogen from the environment than from symbiotic cyanobionts.

In contrast to the rhizosolenoid diatoms, the abundances of the hemiauloids *Hemiaulus membranaceus* and *H. sinensis* were highest in the warm season when the cyanobiont abundance, SPs, and numbers of *Richelia* per host cell were highest. Foster et al. (2011) suggested that the host diatom *Hemiaulus* enhances the growth and N_2 fixation of their associated *Richelia* in the oligotrophic open ocean. Different mechanisms of symbiosis in the hemiauloids and rhizosolenoids may explain the observation of Vaillancourt et al. (2003) in the cold eddy.

Hemiauloid and rhizosolenoid DDAs responded differently to the nitrate addition. The rhizosolenoid diatoms quickly outgrew their associated cyanobionts, which resulted in a decrease in SP. In the enrichment bottles, as *R. clevei* grew rapidly, the abundance of their *Richelia* and numbers of *Richelia* per host remained unchanged. In laboratory cultures, the maximal growth rate of *R. clevei* (1.04 d^{-1}) was shown to be higher than that of the symbiotic *Richelia* (0.80 d^{-1}) when nitrate is replete, whereas the growth rates are almost equal when nitrate is depleted (Villareal 1990). With an average of only 1 *Richelia* filament in 1 DDA, *R. clevei* could easily outgrow *Richelia*, resulting in a rapid decrease in SP (as shown by a drop of SP to 50% on Day 2 in the enrichment experiment). Our results are consistent with those of Villareal (1990), who reported that 12 d after addition of 45 µM nitrate in batch cultures of *R. clevei*, SP did not change from the initial value of 10 to 15%, whereas SP increased to 65% without nitrate addition. By contrast, the hemiauloid diatoms showed a drop in SPs, as the diatom cells divided faster than did *Richelia*. Although the numbers of *Richelia* in *H. membranaceus* and *H. sinensis* decreased following the nitrate addition, the abundances of hemiauloid diatoms in the enrichment bottles were not lower than the control until Day 6. Nitrogen provision from *Richelia* would be reduced as the number of *Richelia* in each host cell declined, and adversely affect the growth of the potential hosts. A similar mechanism could also explain the very low abundances of the strongly-dependent DDA diatoms in the winter in the SCS (Table 4). The fact that the SP and number of *Richelia* per *Hemiaulus* cell were both lower in the SCS than in the Kuroshio could also be caused by differences in nitrate availability between the 2 regions.

Epiphytic DDAs

The present study, to our knowledge, is the first to report the SPs of epiphytic *Chaetoceros compressus*- and *Bacteriastrum-Calothrix* (*B-C*) associations. Previous investigations on DDAs have only focused on endosymbiotic *Richelia* for *R-R*, *Guinardia cylindrus-Richelia*, or *Hemiaulus-Richelia* (Venrick 1974, Heinbokel 1986, Villareal 1994). Similar to endosymbiotic DDAs, SPs of *C. compressus* in the SCS varied seasonally and were relatively high in the warm season. The pattern of their symbiosis was similar to that of rhizosolenoid diatoms.

Little is known about the *B-C* association, with only one report in the North Pacific Subtropical Gyre (Venrick 1974); but that author did not identify the *Bacteriastrum* species and considered the association atypical. In the present study, 4 species of *Bacteria-*

strum were found to form symbiosis with *Calothrix*. Our records, though few, showed an apparently low SP (0 to 29.3%). Among the 21 cases examined, only 5 cases had a SP greater than 0. We found that *Bacteriastrum* carried more *Calothrix* per cell but had a lower SP than *Chaetoceros.* This seems contradictory considering their dependency on diazotrophs.

The SPs of *Bacteriastrum* spp. and *C. compressus* we reported might be somewhat underestimated. *Calothrix* filaments could have been detached from the diatom hosts during sample collection or preparation, although precautions were taken. Conversely, some abundances of free-living cyanobionts could have been overestimated. Nevertheless, the numbers of *Calothrix* in each *Chaetoceros* cell in the present study (0.36 ± 0.01 heterocysts cell^{-1}, n = 425) did not differ significantly (t = 0.82, p = 0.43) from the limited data of Gómez et al. (2005) (0.49 ± 0.16 heterocysts cell^{-1}, n = 11).

Guinardia-Richelia symbiosis

The current understanding of the *Guinardia cylindrus-Richelia* (*G-R*) association is very limited. Venrick (1974) reported a summer bloom with *G. cylindrus* at 1 × 10^7 cells m^{-3} and *Richelia* at 1.5 × 10^7 heterocysts m^{-3}. Heinbokel (1986) reported a SP of 100% for *G-R*. We report for the first time that the number of *Richelia* per cell in each *G-R* association was about 1 heterocyst cell^{-1} in the winter and autumn, and 2 heterocysts cell^{-1} in the spring and summer. There was, however, no distinct seasonal pattern in abundance or SP in the *G-R* association.

CONCLUSIONS

Nitrate availability was a key environmental factor influencing the abundance of DDAs. A decrease in symbiotic *Richelia* abundance and SP of potential host diatoms following the nitrate enrichment were in line with field observations that DDA abundance and SP were low when the nitracline was shallow. Potential host diatoms differed in their degrees of dependency on the cyanobionts; hemiauloid diatoms were more dependent on nitrogen from their symbiosis with *Richelia* than were rhizosolenoid diatoms. Environmental factors other than nitrate availability that often correlated with each other could also be important in affecting the abundance of DDAs. More monitoring experiments are needed to elucidate their individual effects.

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