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ORIGINAL ARTICLE

Seasonal dynamics of the endosymbiotic, nitrogen-fixing cyanobacterium *Richelia intracellularis* in the eastern Mediterranean Sea

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Biological nitrogen fixation has been suggested as an important source of nitrogen for the ultra-oligotrophic waters of the Levantine Basin of the Mediterranean Sea. In this study, we identify and characterize the spatial and temporal distribution of the N-fixing (diazotrophic) cyanobacterium *Richelia intracellularis*. *R. intracellularis* is usually found as an endosymbiont within diatoms such as *Rhizosolenia* spp and *Hemiaulus* spp. and is an important diazotroph in marine tropical oceans. In this study, two stations off the Mediterranean coast of Israel were sampled monthly during 2005–2007. *R. intracellularis* was identified by microscopy and by reverse transcribed-PCR which confirmed a 98.8% identity with known *nifH* sequences of *R. intracellularis* from around the world. The diatom–diazotroph associations were found throughout the year peaking during autumn (October–November) at both stations. Abundance of *R. intracellularis* ranged from 10 to 55 heterocysts l^{-1} and correlated positively with the dissolved $Si(OH)_4/(NO_3+NO_2)$ ratio in surface waters. Although the rates of nitrogen fixation were very low, averaging ~1.1 nmol N l^{-1} day^{-1} for the *R. intracellularis* size fraction (>10 μm) from surface waters, they correlated positively with heterocyst counts during thermal stratification. The lack of large-scale diatom–diazotroph blooms and the low rates of nitrogen fixation by these diazotrophs may result from the P-starved conditions affecting the Levantine basin.

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Introduction

Surface oceanic waters around the tropical and subtropical latitudes are commonly oligotrophic. In such nutrient-poor environments, symbiotic relationships are commonly observed and enhance survival via mutual benefits for both partners. Nitrogen is the most abundant substance (~78%) in the atmosphere and is essential for creating and sustaining life on earth. Yet, its inert form (N_2) is metabolically unavailable to most organisms, making the role of nitrogen-fixing organisms (diazotrophs) crucial in N-limited environments.

Many symbiotic associations are known between diazotrophs and other organisms from terrestrial symbioses between *Rhizobium* and legumes to aquatic symbioses between cyanobacterial diazotrophs and phytoplankton such as *Richelia intracellularis* and diatoms. *R. intracellularis* is a small (~10 μm in diameter) nitrogen-fixing cyanobacterium spatially segregating nitrogen fixation from photosynthesis and thus fixing atmospheric N during daytime. It consists of one polar heterocyst (site of nitrogen fixation) and three to 10 vegetative cells (Geitler, 1932). It is usually found as an endosymbiont within diatoms such as *Rhizosolenia* spp, *Hemiaulus* spp, or as an episymbiont attached to *Chaetoceros* spp. (Venrick, 1974; Taylor, 1982; Sundstrom, 1984; Gomez *et al.*, 2005). Diatom–diazotroph associations containing the cyanobiont *R. intracellularis* and the filamentous non-heterocystous *Trichodesmium* spp., were traditionally

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considered the dominant nitrogen-fixing plankton in marine tropical oceans. The 'New N' supplied by these organisms, and a variety of small unicellular diazotrophs described recently (Zehr *et al.*, 2001; Montoya *et al.*, 2004; Zehr *et al.*, 2007), contribute substantially to these systems (Venrick, 1974; Mague *et al.*, 1977; Carpenter and Romans, 1991; Capone *et al.*, 1997; Carpenter *et al.*, 1999; Arrigo, 2005). In the tropical North Atlantic, extensive N_2 fixation by blooms of diatoms and *R. intracellularis* produced nearly 70% of total N demand in surface waters (Carpenter *et al.*, 1999; Janson *et al.*, 1999).

The eastern Mediterranean Sea is extremely oligotrophic due to a general west to east anti-estuarine circulation, which exports nutrients from the Mediterranean into the North Atlantic (Bethoux *et al.*, 1998). The oligotrophic nature results in a high water clarity, low values of chlorophyll *a* (Chl *a*), low rates of primary production (Berman *et al.*, 1984; Azov, 1986; Li *et al.*, 1993; Yacobi *et al.*, 1995; Psarra *et al.*, 2000; Tanaka *et al.*, 2007), and dominance by small-sized phytoplankton (Li *et al.*, 1993; Yacobi *et al.*, 1995; Ignatiades *et al.*, 2002). The Levantine basin, located in the easternmost section of the Mediterranean, is the most oligotrophic region within the Mediterranean Sea. Its characteristics are a prominent deep chlorophyll maximum (DCM) at 100–130 m depth (Li *et al.*, 1993; Yacobi *et al.*, 1995), low phytoplankton biomass ($<$50 ng Chl *a* l^{-1}) in the surface layers (Li *et al.*, 1993; Vidussi *et al.*, 2001), an average salinity of $\sim$39 psu, and sea-surface temperatures between 16–28 °C.

Studies using geochemical and stable isotopic ($\delta^{15}N$) mass balances have suggested that nitrogen fixation supplies a significant source of new nitrogen to the Mediterranean (Bethoux, 1986; Bonin *et al.*, 1989; Bethoux *et al.*, 1992, 2002; Pantoja *et al.*, 2002). Yet, only recently have actual N fixation rates been reported from the Mediterranean Sea. One study, from the Western Mediterranean, averaged $\sim$7 nmol N l^{-1} day^{-1} (Garcia *et al.*, 2006) while measurements from a warm-core eddy near Cyprus yielded rates of $\sim$129 nmol N $l^{-1}day^{-1}$ (Rees *et al.*, 2006). However, these studies lack any identification or characterization of the organisms responsible for this fixation.

We recently described a diverse diazotrophic community with differential patterns of *nifH* expression from an east–west transect off the Israeli coast (Man-Aharonovich *et al.*, 2007). One of the organisms identified was 99% identical to *R. intracellularis*. Previous observations indicated the presence of the diatoms *Rhizosolenia* spp. and *Hemiaulus hauckii* (B Kimor, personal communication), which harbor these cyanobionts although, to date, no published studies exist on their dynamics anywhere within the Mediterranean.

In this study, we focused on the diatom–diazotrophic associations in the eastern Mediterranean and have identified, characterized and followed the seasonal and spatial dynamics of the filamentous nitrogen-fixing cyanobacteria–*R. intracellularis* and its diatom hosts: *Rhizosolenia* spp. and *Hemiaulus* spp. at 2 monthly monitored stations off the Mediterranean coast of Israel.

Materials and methods

Sample collection and processing

Water samples were routinely collected during daytime in monthly cruises (June 2006–May 2007) on board the R/V Mediterranean Explorer. Two stations were sampled: one coastal station TB200 (32° 09.982′ N and 34° 34.509′ E) at ca. 200 m bottom depth and one pelagic station TB1000 (34°14.909′ E and 32°10.059′ N), at ca. 1000 m bottom depth. Water samples for molecular distribution, nitrogen fixation and nutrients concentration determination were collected using Niskin bottles (8 l) mounted on a Rosette equipped with a CTD (seabird 19 Plus), *in-situ* fluorometer (Turner designs, Cyclops7) that was calibrated against extracted Chl *a* in the laboratory.

Samples for cell counts were taken from the surface waters ($\sim$5 m depth). A Nytex plankton-net (55–300 μm equipped with a digital flow meter (Hydrobios No 438110) was towed horizontally behind the research vessels and used to compute the total volume of water passing through the net.

Sample collection and processing for cell identification and enumeration

Samples were collected from the net and preserved in 50 ml tubes with 0.5 % gluteraldehyde and stored at 4 °C in the dark until further examination in the lab. Samples were identified and counted using an epifluorescence microscope (Nikon Eclipse 80i) equipped with three specific filters—chlorophyll fluorescence filter (Ex: 450 nm, Em: 680 nm), phycoerythrin fluorescence filter (Ex: 490 nm, Em: 580 nm) and phycocyanin fluorescence filter (Ex: 620, Em: 660 nm).

Five to 20 replicate samples (70 μl) per station/depth were counted microscopically (with a total of 200 to $>$3000 heterocysts counted depending on abundance). The chloroplasts of the host cell (diatom) were observed using the chlorophyll *a* filter only while the endosymbiotic cyanobacterium *R. intracellularis* was observed under both phycoerythrin and phycocyanin filter sets. Both the host and the endosymbiont *R. intracellularis* were identified and counted.

Nitrogen fixation rates

N fixation was measured on preconcentrated samples using the acetylene reduction technique which measures total nitrogenase activity (Capone, 1993; Montoya *et al.*, 2004) and has also been applied in the field on concentrated samples (Falcon *et al.*, 2002, 2004). Ten to 15 liters were pre-concentrated

from surface waters (~5 m) and size fractionated to 1–10 μm, 10–100 μm and >100 μm under low vacuum. Biomass was incubated in 125 ml serum bottles with 80 ml of 0.2 μm filtered seawater. Acetylene (9 ml) was added to the bottles and 2 ml of gas from the headspace were immediately removed to Vacuettes (Greiner Bio-One, Kremsmünster, Austria) for T_0. Sample bottles (at neutral pressure), and appropriate blanks (0.2 μm filtered seawater and killed blanks) were incubated for 24 h in a flowing seawater bath at 25 °C and ~100 μmol quanta $m^2 s^{-1}$. Ethylene production was measured with an FID-GC (SRI 310C) using known ethylene standards. Ethylene production was converted to N-fixation rates according to Capone and Montoya (Capone and Montoya, 2001).

Sample collection and processing for molecular analysis

Twenty-liter samples were collected with Niskin bottles from each depth in the euphotic zone (~5, 60, deep chlorophyll maximum and 150 m). Each sample was placed into an acid-washed container and pumped using a peristaltic (Cole Parmer-Master Flex, Vernon Hills, IL, USA) pump onto 0.2 μm-pore-size Supor filters (Pall Gelman Inc., Ann Arbor, Michigan). The filter was then placed into a cryotube with 1 ml of lysis buffer (Massana *et al*., 1997) and stored at −80°C until further processing.

Nucleic-acid extraction and reverse transcription

Nucleic acids were extracted from the samples using a phenol–chloroform extraction according to Massana *et al*. (1997). Nucleic acids were treated with RNase-free DNase I (Ambion, Cambridgeshire, UK) for 30 min at 37 °C to remove DNA residue. DNase was inactivated by heat denaturation at 75 °C for 10 min and samples were stored at −80 °C until further use. Total RNA (100–300 ng) was then reverse transcribed using *nifH* degenerate reverse primer *nifH*3 (5′-ATRTTRTT NGCNGCRTA-3′) and *nifH*723R (5′-GATGTTCGCGCGG CACGAADTRNATSA-3′) using Bio-RT (Bio-Lab Ltd, Jerusalem, Israel) according to manufacturer's instructions.

PCR amplification of nifH *transcripts*

Nitrogenase Fe protein transcripts (*nifH*) were amplified from cDNA using the PCR primers of Zehr and McReynolds, (1989). A fragment of the *nifH* gene (approximately 360 bp) was amplified with a nested PCR strategy. The PCR mixtures contained 2.5 μl of 10× ExTaq buffer (Takara, Madison, WI, USA), 2 μl of a mix of deoxynucleoside triphosphates (2.5 mM each), 18.75 μl of DEPC water, 0.25 μl of 100 μM *nifH*3 (5′-ATRTTRT TNGCNGCRTA-3′), 0.25 μl of 100 μM *nifH*4 (5′-TTYT AYGGNAARGGNGG-3′) and 0.25 μl of ExTaq DNA polymerase (5 U μl^{-1}; Takara). The reaction mixtures were amplified for one denaturation step (5 min at 94 °C), followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, and one final 7-min extension cycle at 72 °C. To the nested PCR master mix, 0.5 μl of the first PCR product and 0.5 μl of DEPC water were then added. *nifH* primers previously designed (Zehr and McReynolds, 1989): 0.25 μl of 100 μM *nifH*1 (TGYGAYCCNAARGCNGA) and 0.25 μl of 100 μM *nifH*2 (ADNGCCATCA TYTCNCC). Second-round reactions were amplified with one denaturation step of 5 min at 94 °C, 30 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, and a final 7-min extension cycle at 72 °C. All of the tests for the presence of contaminating DNA in the RNA samples or in the reagent were carried out according to (Zani *et al*., 2000). PCR products were cloned using the QIAGEN–PCR cloning kit and selected clones were sequenced. The *nifH* sequences reported in this study are deposited with GenBank under accession numbers EU807710–EU807732.

Phylogenetic analyses of nifH *sequences*

nifH sequences were assembled and translated into amino-acid sequences using the *SEQUENCHER* program (version 4.5). All diversity and phylogenetic analyses were performed using deduced amino-acid sequences. *NifH* protein sequences were aligned using CLUSTALX version 1.81 (Thompson *et al*., 1997). Neighbor-joining and maximum-parsimony analyses were conducted on amino-acid data sets using Paup 4.0b10 (Swofford, 2002). Default parameters were used in all analyses. Bootstrap re-sampling of neighbor-joining (1000 replicates) and maximum-parsimony (1000 replicates) were performed.

Results and discussion

Study area

To examine spatial and temporal changes in *R. intracellularis,* one coastal (TB200) and one pelagic (TB1000) station, off the continental shelf, were sampled monthly during 2006–2007 (Figure 1). There were significant differences in the physical and chemical properties of the coastal and pelagic stations (Figure 2). The most apparent difference was the depth of the upper mixed layer during winter (January–March). At the coastal station, the winter deep mixing, which almost reached the bottom, could provide nutrients from the sediments while at the pelagic station, the deep mixing could not supply similar amount of nutrients from deeper layers.

The pelagic upper layer (100 m) had $NO_3 + NO_2$, PO_4 and $Si(OH)_4$ concentrations of <0.08−0.85 μM, <0.008–0.11 μM and 0.51–1.41 μM, respectively. Slightly higher concentrations of PO_4 (up to 0.13 μM) and $Si(OH)_4$ (up to 2.14 μM) were measured at the coastal station. These ranges of concentrations are consistent with previous studies from the Levantine basin (Berman *et al*., 1984; Azov, 1986;

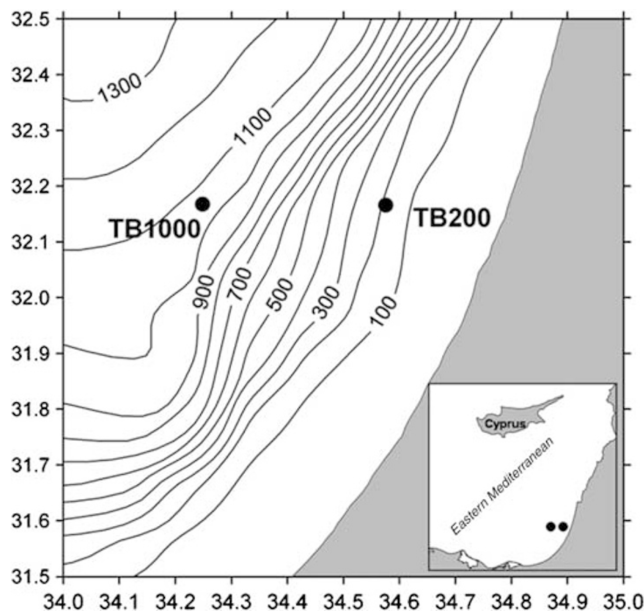

Figure 1 Map of the station locations: Station TB200, coastal station (on the continental shelf), bottom depth of 200 m (32° 09.982′ N and 34° 34.509′ E) and a pelagic station, TB1000 with a bottom depth of 900 m, off the continental shelf (34°14.909′ E and 32° 10.059′ N).

Bonin *et al*., 1989; Herut *et al*., 1999; Kress *et al*., 2005).

During stratified months (June–September) Chl *a* concentrations peaked to 0.45 μg l^{-1} at the DCM (~110–150 m) while during winter mixing, the DCM (~0.4 μg Chl *a* l^{-1}) was widely spread between 50–130 m at the pelagic station and was not developed at the coastal station showing uniform vertical Chl *a* distribution (~0.2–0.3 μg l^{-1}). The highest photosynthetic biomass at both stations, estimated by Chl *a* concentrations and by variable fluorescence, occurred during the winter mixing.

Identification and characterization of the diatom–diazotroph associations

Using epifluorescent microscopy we identified the presence of the two main known diatom hosts of *R. intracellularis* from our two sampling stations: *Rhizosolenia clevei* var. communis *and Hemiaulus hauckii*. The cyanobiont *R. intracellularis* was present in all samples containing *H. hauckii* whereas it was only sporadically found in *Rhizosolenia*.

H. hauckii found in our study period was characterized by chains composed of 3–15 cells. Each cell contained one or two *R. intracellularis* trichomes though occasionally *H. hauckii* cells contained up to four *R. intracellularis* trichomes (see Figures 3a–d). The *R. intracellularis* trichome consisted of 3–5 vegetative cells and one or two heterocysts at the end of each trichome depending on the size and stage of the *H. hauckii*–*R. intracellularis* life cycle. The diameter of the *H. hauckii* varied and averaged ~30 μm whereas the diatom chain could be as long as 200 μm. The average diameter of *R. intracellularis* inside *H. hauckii* was ~10 μm.

Under bright-field light microscopy only the *H. hauckii* cells were apparent (Figure 3b). The distinguishing spindles connecting the *H. hauckii* cells and their length (40 μm), enabled identification of the *Hemiaulus* as *H. hauckii* (Heinbokel, 1986; Kimor *et al*., 1987, 1992; Villareal, 1994). Under chlorophyll excitation only *H. hauckii* chloroplasts were visible (Figure 3a). The high chlorophyll autofluorescence of the diatom masked the chlorophyll contained in the cyanobiont vegetative cells. *R. intracellularis* was located in the diatom intercellular space (Villareal, 1991; Rai *et al*., 2000). The physical proximity between the cyanobiont and the diatom's chloroplasts may be due to the cyanobionts' high demand for carbohydrates (Wolk *et al*., 1994) required for the energetic costs of nitrogen fixation (Villareal, 1992; Janson *et al*., 1995a; Rai *et al*., 2000). Yet, we are not aware of any documented evidence for this carbon exchange.

The cyanobiont cells were visible only under phycocyanin or phycoerythrin (PE) excitation filters (Figures 3c,d,e,h, and i). These pigments are unique to the cyanobacterial endosymbionts (Villareal, 1992; Janson *et al*., 1995a; Rai *et al*., 2000) and are not found in the diatoms. Heterocysts appeared to contain high concentrations of PE (discernable by very high fluorescence under PE filters). This may be due to the pigment role in energy transfer to PSI or to the lack of protein degradation during heterocyst differentiation (Peterson, 1981; Fay, 1992; Janson *et al*., 1995a). Phycobiliproteins are also known to have a role in cyanobacterial nitrogen storage (Allen *et al*., 1984; Kromkamp, 1987). This increase in fluorescence might indicate that the heterocysts are using PE for an intracellular nitrogen storage facility (Figures 3d and i) (Wyman *et al*., 1985; Janson *et al*., 1995b; Wingard *et al*., 2002; Zhang *et al*., 2006). Nitrogen storage as PE, combined with the close physical proximity between cyanobiont and host, may imply that nitrogen is rapidly assimilated once fixed by the *R. intracellularis*.

In *Rhizosolenia spp*. the trichomes of *R. intracellularis* lie within the diatom's periplasmatic space (Villareal, 1992; Janson *et al*., 1995b) and not between the host chloroplasts. Thus, under bright-field light microscopy, the vegetative cells of *R. intracellularis* were visible inside the *Rhizosolenia* and contained one to two trichomes with the heterocyst located toward the end of the *Rhizosolenia* valve (Figure 3e). The diameter of the *Rhizosolenia* cells varied between 20–30 μm whereas the R. *intracellularis* diameter was ~10 μm. R. *intracellularis* contained ~10 vegetative cells with one enlarged heterocyst (Figures 3h and i). The heterocysts were visible only when the organisms were excited with the PE or PC filter

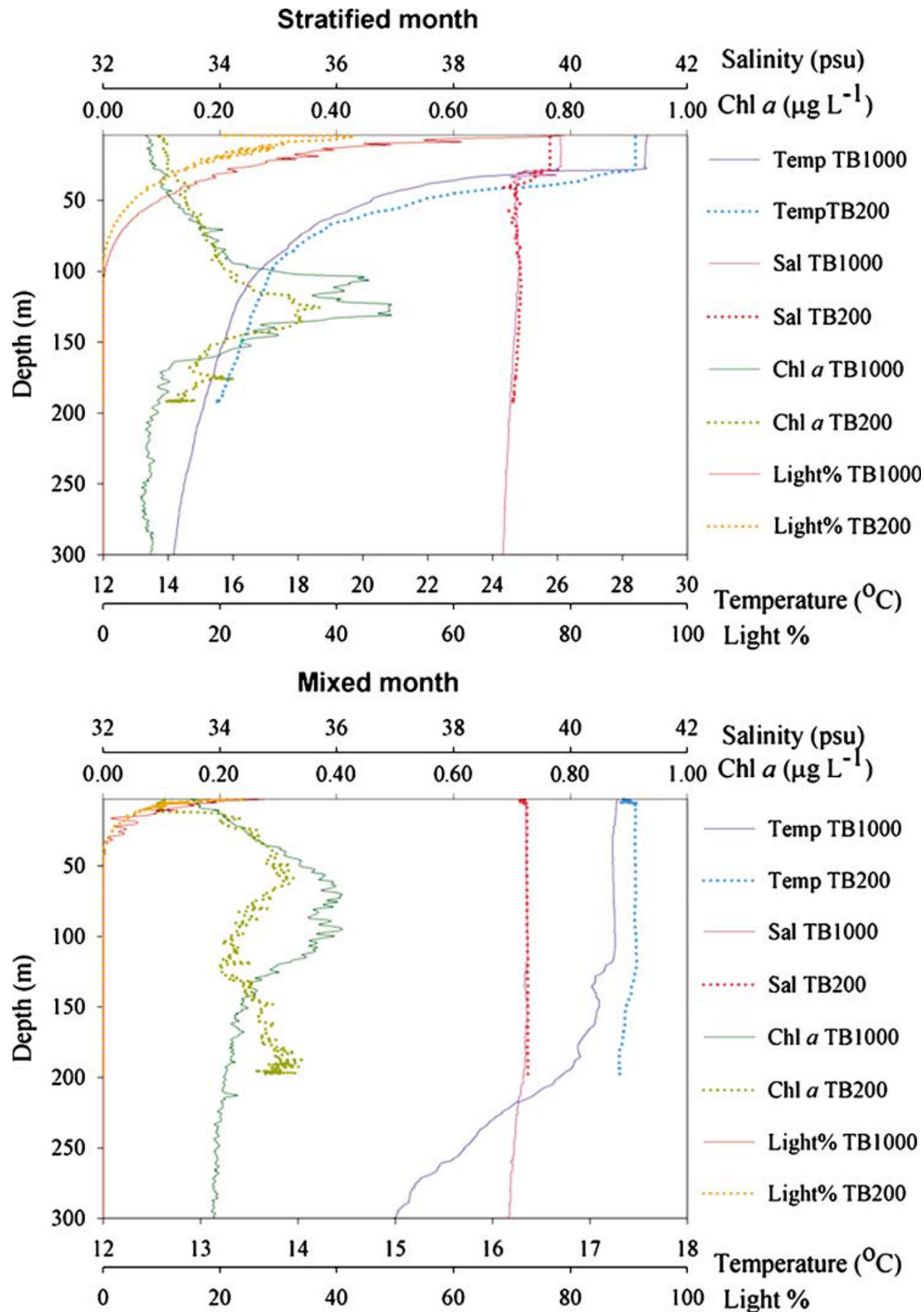

Figure 2 Representative depth profiles of the two sampling stations (TB200 and TB1000) during a stratified month (upper panel) and a mixed month (lower panel). In both panels parameter shown are: Temperature (blue and light blue), salinity, PSU (pink and red); Chl *a*, $\mu g\,l^{-1}$ (green and light green); and % light at depth (purple and orange).

sets. This might be due to low concentrations of Chl *a* in these specialized nitrogen-fixing cells where no PSII activity occurs (Zhang *et al.*, 2006). The diatom host can acquire their cyanobiont via two major pathways: either directly from the aquatic environment, or, through the parental cells following cell division (Villareal, 1989; Rai *et al.*, 2000). The initiation mechanism is not known at present and the published literature on transmission and division is scarce (Villareal, 1989). Moreover, during our study period we did not observe any free living *R. intracellularis* as have been observed in other studies (Gomez *et al.*, 2005; Foster and O'Mullen, in press). We observed the propagation of the symbiosis only via cellular division (Figure 4) and not by direct acquisition of symbionts from the seawater. Bright-field and epifluorescent microscopy revealed the division of the parent *H. hauckii* into two

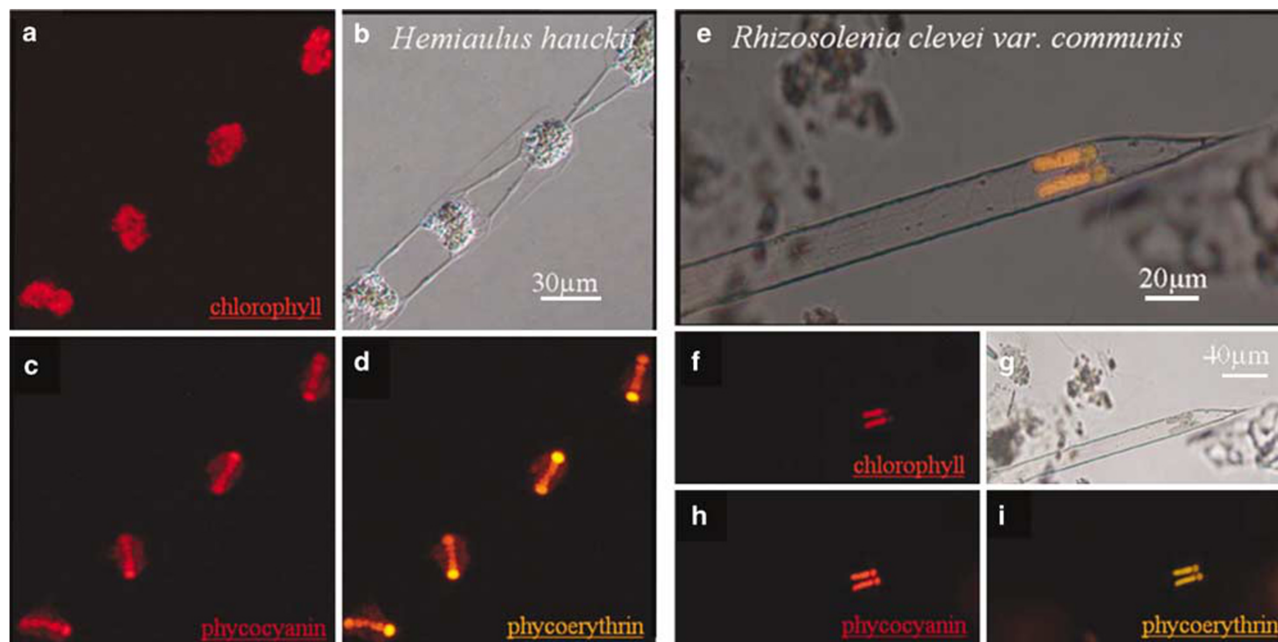

Figure 3 Diatom- Richelia symbioses. The diatom *Hemiaulus hauckii* and its endosymbiotic cyanobacterium *Richelia intracellularis* (**a–d**). The diatom *Rhizosolenia* spp and its endosymbiotic cyanobacterium *Richelia intracellularis* (**e–i**). (**b**, **g**) bright-field microscopy; (**a**, **f**) chlorophyll *a* fluorescence (ex: 450 nm, em: 680 nm); (**d**, **i**) phycoerythrin fluorescence (ex: 490 nm, em: 580 nm); (c.h) phycocyanin fluorescence (ex: 620, em: 660 nm).

daughter cells (Figures 4a and b). Intertwined and visible under PE fluorescence, we also observed concurrent division of the symbiont *R. intracellularis* (Figure 4c). These results contrast with the asynchronous division reported from the only known culture of *Rhizosolenia-Richelia* that had been isolated, maintained, and was documented up to seven generations (Villareal, 1989). What controls the timing of those processes is still unknown. We hypothesize that nutrient cycling may play a significant role in cellular division of the DDA's, yet further work is required to validate this.

Molecular identification and nifH *expression of* R. intracellularis

We confirmed the microscopical identification of the diatom–diazotroph associations using molecular tools. In both stations (TB200 and TB1000) water samples were collected during 2006 throughout the euphotic zone for molecular characterization of the symbiotic associations. Our recent study at an east–west transect in the Levantine basin identified expressed *nifH* with high identity to *R. intracellularis* from size-fractionated samples between 11 to 60 μm (Man-Aharonovich *et al*., 2007). Here, we expanded and sequenced a total of 52 randomly picked clones containing *nifH* inserts from samples >0.2 μm collected during daylight hours (as heterocystous cyanobacteria including *Richelia* are known to actively fix during the day). From the total clones that were sequenced, 54.5% had between 98–99% identities at the DNA level to *R. intracellularis*, while only a small number of clones from each of the other diazotrophs were found representing any of the other diazotroph groups. This suggests that *R. intracellularis* is the main daytime *nifH*-expressing organism throughout the water column during our study period. We could not decipher from our phylogenetic analyses which organism (*Rhizosolenia spp* or *H. hauckii)* was the host of *R. intracellularis* (Figures 3a–d). However, as >95% of the microscopical counts of the diatom containing the endosymbiont were of *H. hauckii,* we assume that *R. intracellularis* found in this study was predominantly from *H. hauckii*.

Surprisingly, all of the sequences that had high identity to *R. intracellularis* (~99%) fell into two *nifH* clusters, referred as Clusters A, B (Figure 5). Both subclades were statistically supported (bootstrap values >80) in the phylogenetic analysis based on amino-acid sequences. To examine the reasons for variation in the different clades from the two stations, we explored changes in the physical/chemical characteristics of the water column (see area of study and Figures 2 and 6). Cluster A was comprised of *R. intracellularis nifH* sequences recovered from the pelagic station (TB1000) during the mixed period (February, March 2006) and throughout the euphotic zone from surface samples to 120 m depth. Cluster B was comprised of *R. intracellularis nifH* sequences recovered from the coastal station (TB200) during the mixed-period (February, March 2006) and at the onset of stratification (May 2006). The only sequences

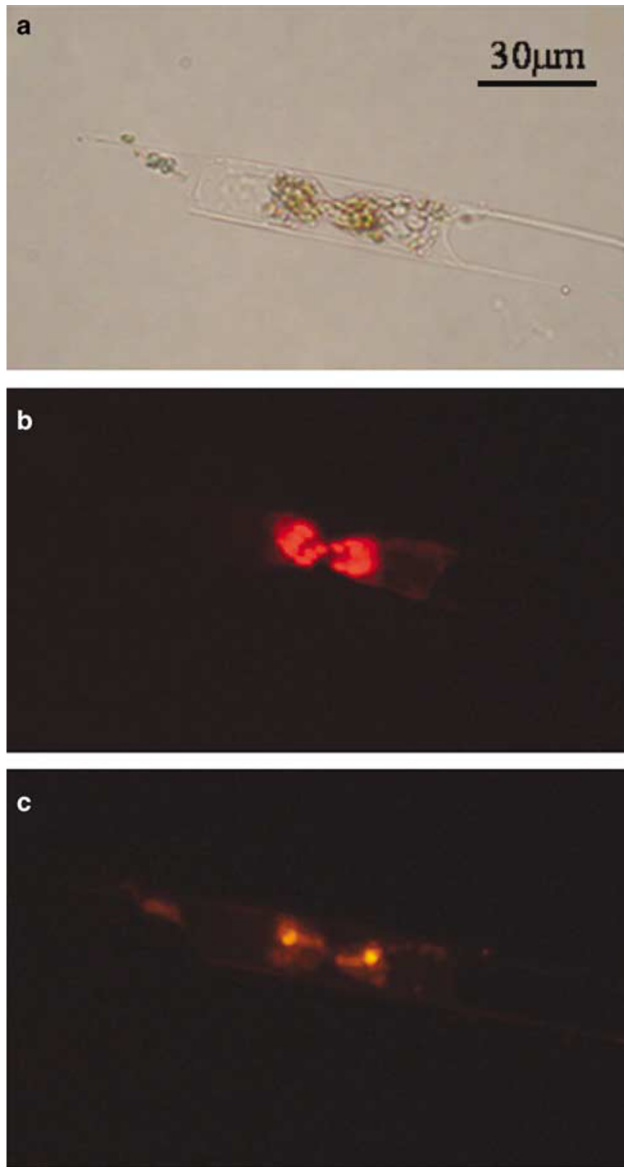

Figure 4 Cellular division of the diatom host and cyanobiont. Division of the diatom *Hemiaulus hauckii* under bright-field microscopy (**a**) with the diatom's chloroplast under Chlorophyll *a* fluorescence (**b**), and the cyanobiont *Richelia intracellularis* under phycoerythrin fluorescence (**c**).

resulting from these months were from surface-layer samples.

We hypothesize that the regional circulation pattern (Rosentraub and Brenner, 2007) may play a significant role in the creation of the two different clades. The sharp continental slope located between the continental and pelagic station could generate a hydrological barrier (Rosentraub personal communication) that separates the phytoplankton community and thus also the *R. intracellularis* endosymbionts. Thus, while heterocyst abundance was similar at the two stations, the phylogenetic analysis revealed two distinct clades of *R. intracellularis* (Figure 5). Bacterial diversity may not always exhibit a specific taxa-area relationship due to small size, horizontal gene transfer, physiological flexibility and resilience to the environments (Horner-Devine *et al.*, 2004a, b). Yet, taxa-area relationships have been found for bacterial populations driven by environmental heterogeneity (Horner-Devine *et al.*, 2004b). The two distinct clades we found for *Richelia* from the two stations concurs with the above findings by (Horner-Devine *et al.*, 2004b) or may present a case of allopatric speciation similar to isolated island populations that have emerged from one source population (Turelli *et al.*, 2001). The presence of *Richelia* within the diatom-hosts in a complex association must also be accounted when examining distribution and diversity. Further investigation into the genetic profiles of the host diatoms would be necessary.

Abundance and Nitrogen fixation

H. hauckii and *R. intracellularis* were present throughout the year at both stations (TB200 and TB1000) (Figure 6) and were also counted during several night-time samplings. The rare sightings of *Rhizosolenia*–*R. intracellularis* associations in our stations correspond to results from a study in the Caribbean and North Atlantic Ocean where 91–100% of *Hemiaulus* hosts contained the *R. intracellularis* symbionts while the *Rhizosolenia*–*Richelia* associations were rarer (5–254 times less abundant than *Hemiaulus*–*Richelia* associations) (Villareal, 1994). Recently, Foster *et al.* (2007) reported that *H. hauckii* and *R. intracellularis* phylotypes were more abundant compared with the other diatom–diazotrophic symbioses in most stations and depths sampled during a cruise to the Western tropical North Atlantic Ocean. Accordingly, they suggested that under similar conditions, the smaller and more rapidly growing *H. hauckii* and *R. intracellularis* have a competitive advantage over the other diatom–diazotroph assemblages (Foster *et al.*, 2007).

To date, we did not observe any substantial differences in heterocyst counts between the coastal station and the pelagic station except during October 2006 (11 heterocysts l^{-1} at the pelagic station and 52 heterocysts l^{-1} at the coastal station). In both stations heterocyst counts varied between 1–55 heterocysts l^{-1} and no significant bloom of *H. hauckii*–*R. intracellularis* was observed throughout the study period (Figure 6). Large blooms of the diatom–cyanobacterial association have been described in the tropical Atlantic, where heterocyst counts inside the bloom area ranged from hundreds to >1000 heterocysts l^{-1} compared with 39 heterocysts l^{-1} outside the blooms (Villareal, 1994; Carpenter *et al.*, 1999; Jahnke *et al.*, 1999). Recent determinations of the *H. hauckii*–*R. intracellularis* associations by quantitative (Q)PCR of *nifH* gene copies showed similar abundances (Foster *et al.*,

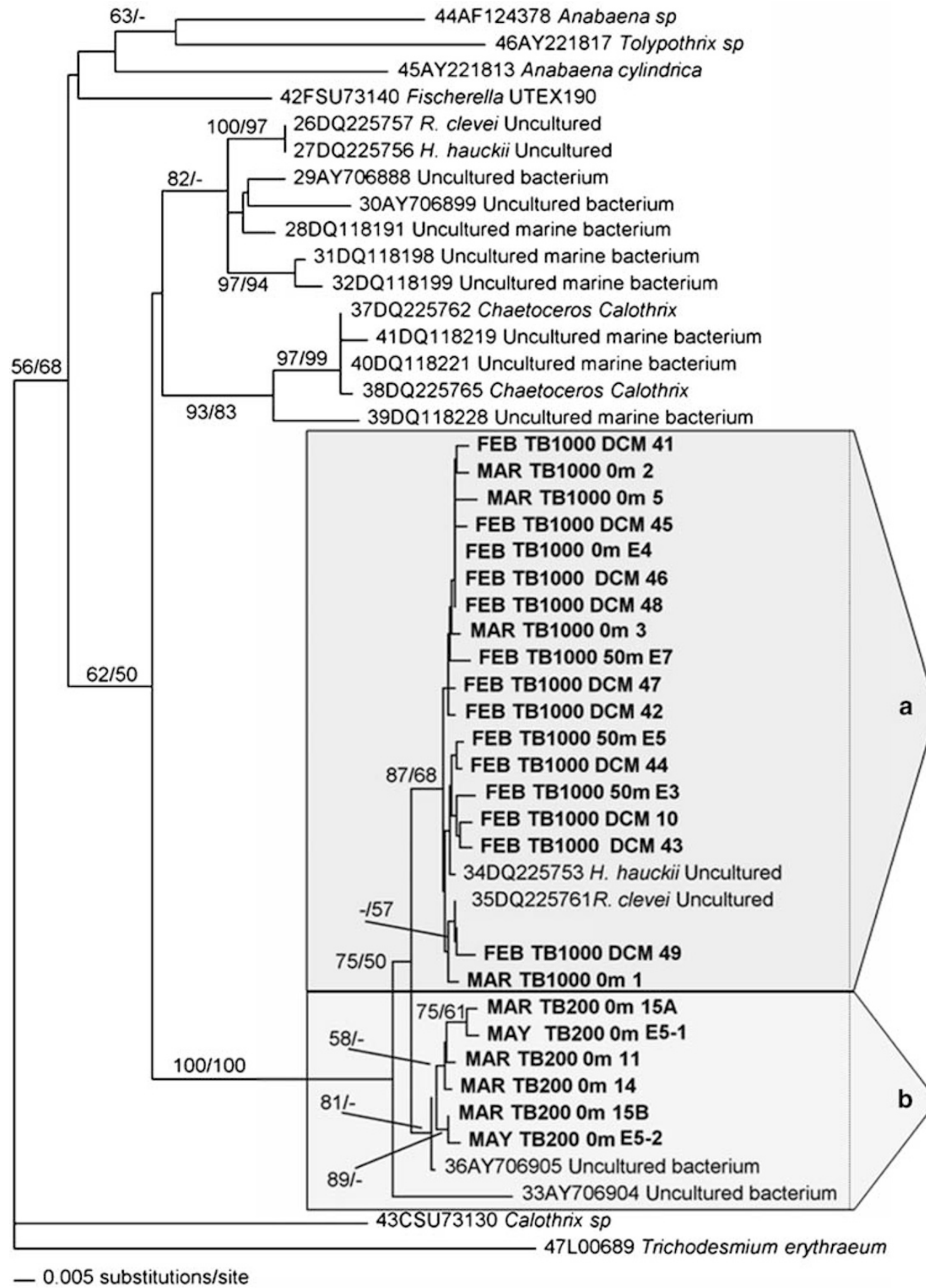

Figure 5 A Phylogenetic tree reconstructed from *nifH*-deduced amino-acid sequences. The sequences were obtained from the two sampling stations TB200 and TB1000 during 2006. The two groups obtained from the clone libraries sequences are marked in boldface letters. Bootstrap values (NJ/MP) exceeding 80% are indicated above the branches. The clusters (**a**) (open Ocean station—TB1000) and (**b**) (coastal station—TB200) represent the two groups.

2007). The highest numbers of heterocysts found at our coastal station (TB200) were counted in June and October 2006 ($\sim$50 heterocysts l^{-1}) and at the pelagic station (TB1000) in June and November 2006 ($\sim$50 heterocysts l^{-1}). Figure 6 clearly shows a seasonal peak of heterocyst concentrations during autumn (October–November), coinciding with the deepening of the mixed-layer depth at both stations. Dust events, delivering nutrients, in particular P, are also characteristic during these months (autumn and spring) (Herut *et al.*, 2002, 2005) and may enhance diatom–diazotroph occurrence.

Although total *Richelia* heterocyst counts were low, the actual presence of heterocysts and the expression of *nifH* detected by reverse transcribed-PCR (Figure 5), indicates active nitrogen fixation. According to our microscopical and molecular identification, *R. intracellularis* was the dominant

diazotroph in the 10–300 μm fractions. For this size fraction N-fixation rates averaged ~1 nmol N l^{-1} day^{-1} at both stations, and throughout the photic zone during the sampling period, and comprised 50%–70% of the total N-fixation rates measured (0.4–4.5 nmol N l^{-1} day^{-1} for >1 μm size-fraction). This is consistent with our findings that 54.5% of all sequenced clones were identified as *R. intracellularis* (see previous section Molecular identification and *nifH* expression).

A positive correlation was found between heterocyst numbers and the generally low nitrogen fixation rates only during stratified months (Figure 6, insert). We note that, at all times during the study period, to obtain any measurable rates of N fixation or statistically significant numbers of diazotrophs at our stations, we had to pre-concentrate large volumes of water. Species-specific rates of N-fixation by *R. intracellularis* as well as the total contribution of new N by DDA's and other diazotrophs to the N-budget of the Levantine basin remain uncharacterized and should be carefully examined at a wider scale. Moreover, our sampling and analyses for *nifH* expression performed predominantly during daytime does not take into account possible contributions of unknown active diazotrophs during night time. Yet, the low rates of N-fixation measured during this study, combined with the low *Richelia* abundances and lack of DDA blooms (Figure 6), may reflect a scenario controlled by limited availability of essential nutrients as has been recorded for bacterial and primary productivity in the eastern Mediterranean (Herut *et al.*, 2002; Flaten *et al.*, 2005; Zohary *et al.*, 2005; Tanaka *et al.*, 2007).

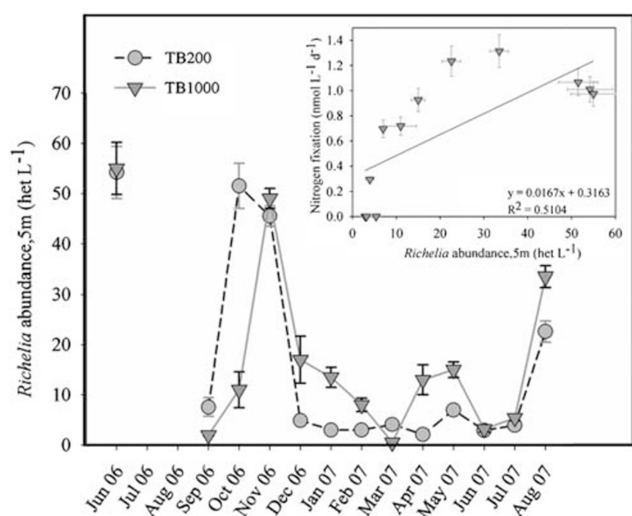

Figure 6 Spatial and temporal *R.intracellularis* heterocyst counts from surface waters (~5 m) at our two representative stations TB1000-open Ocean (triangles) and TB200—coastal (circles) from May 2006 through August 2007. Insert shows correlation between above heterocyst counts and N-fixation rates during stratified months only (May–September).

In present day oceans, diatoms are almost the sole photoautotrophs with an absolute requirement for silicon in the form of silicic acid that is synthesized to form their frustules. Previous studies show that there is no significant difference in the seasonal silicic acid depth integrated values between the 120 (shelf) and 400 m (continental slope) stations (Herut *et al.*, 2000). Moreover, the surface silicic acid concentrations in a westward section off the Mediterranean Israeli coast show no consistent gradient of lower concentrations towards the open sea, though seasonal changes are depicted in the range of 0.5–1.7 μmol l^{-1} (Kress and Herut, unpublished data).

We examined whether the seasonal and depth-related changes in $Si(OH)_4$ and $NO_3 + NO_2$ influenced diatom–diazotroph abundance (Figure 7). The ratio between $Si(OH)_4/NO_3 + NO_2$ was used to indicate the relative availability of these nutrients. For non-symbiotic diatoms, blooms can occur when Si, P and bioavailable nitrogen sources (other than atmospheric nitrogen) are high (leading to a low $Si(OH)_4/NO_3 + NO_2$ ratio). A high ratio of Si to bioavailable N could provide a competitive advantage to the diatom–diazotroph associations where N is supplied via biological N-fixation.

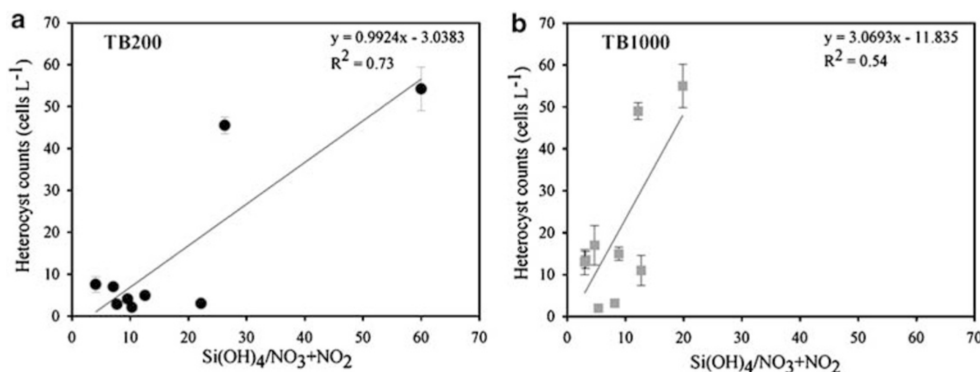

Figure 7 Correlations between $Si(OH)_4/(NO_3 + NO_2)$ and counts of *Richelia* heterocysts (het l^{-1}) from the coastal station TB200 (**a**) and the pelagic station TB1000 (**b**). Heterocysts were always counted within the diatom hosts.

We observed a positive correlation between the heterocyst counts (all heterocysts counted were within diatom hosts) and the $Si(OH)_4/NO_3+NO_2$ (Figure 7). Although during the observations of the *H. hauckii–R. intracellularis* association the diatom/heterocyst ratio was not necessarily 1:1, we found a positive relationship between heterocyst and diatom counts. As $Si(OH)_4$ concentrations did not increase or decline significantly at the two sampling stations to concentrations that would limit diatom growth, we deduce that the reduction in bioavailable N sources, such as nitrite and nitrate, stimulated increased N-fixation and enhanced the growth of symbiotic diatom–diazotroph associations.

For large-scale blooms of diatom–diazotroph symbioses such as *H. hauckii, Rhizosolenia* and *R. intracellularis* to form and persist requires two other essential nutrients: Fe and P. In the Eastern Mediterranean Fe availability may actually be quite high (Guerzoni *et al*., 1999). In contrast, P concentrations are at the nanomolar scale in the upper layers (Krom *et al*., 2005) causing intense competition between autotrophic and heterotrophic plankton for this limited resource (Psarra *et al*., 2005; Thingstad *et al*., 2005; Zohary *et al*., 2005).

Diatoms, with relatively small surface area/volume (SA/V), are competitively disadvantaged compared with the picoplankton's large SA/V ratio and high nutrient uptake rates that are suited for this ultraoligotrophic system. Thus, in the Eastern Mediterranean open Ocean where P is extremely limited (Flaten *et al*., 2005), we would not expect to find large-scale blooms of diatoms (Psarra *et al*., 2005) or the associations of *H. hauckii, Rhizosolenia* and *R. intracellularis*. This is consistent with our findings of limited abundances of both the diatom hosts and the diazotrophic cyanobacterial symbiont requiring high P.

Data from the late Pleistocene Mediterranean sapropels, including samples from the Levantine basin, show an annually-repeating seasonal flux dominated by *Rhizosolenid* diatoms including *H. hauckii* (Kemp *et al*., 1999) *Richelia* endosymbionts were not actually preserved in the sapropels. Yet the isotopic signatures of $^{15}N/^{14}N$ from these sapropels, suspended particles and extracted chlorophyll, have prompted claims that large diazotrophic blooms occurred and contributed 70–90% of new N to the Eastern Mediterranean (Sachs and Repeta, 1999; Pantoja *et al*., 2002). Those blooms could have formed in the past due to a shallower pycnocline (resulting from larger freshwater fluxes into the basin with high Si and P) and thus nutrient-enrichment of the euphotic layer (Sachs and Repeta, 1999; Pantoja *et al*., 2002). Moreover, such enhancement of carbon export may have triggered or amplified denitrification rates generating nitrogen deficient-water that would fuel subsequent nitrogen fixation (Deutsch *et al*., 2007).

Will large blooms of DDA's occur again in the Mediterranean? Future scenarios predict the enhancement of the scale and frequency of anthropogenic and/or atmospheric (for example, via Saharan dust) input of nutrients having combined N:P $\geqslant 16$ and high Fe. These conditions could result in one of two scenarios. If P is still limiting, and limited denitrification continues, nitrogen-fixation in this area would remain low. Alternatively, the geophysical characteristics of the semi-enclosed basin of the eastern Mediterranean combined with higher inputs of nutrients could increase the inventories of limiting resources such as *P* as well as those of dissolved Fe and shift the system towards mesotrophy. Moreover, the global shift in temperatures may cause reduced circulation and the formation of anoxic conditions as well as an increase in shelf areas due to enhanced coastal flooding. These conditions could promote increased denitrification rates and subsequent loss of dissolved nitrate source, thereby enhancing nitrogen-fixation and primary productivity (Deutsch *et al*., 2007) and enabling diatom–diazotroph blooms.

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