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Spectral diversity of phycoerythrins and diazotroph abundance in tropical waters

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Abstract

Phycoerythrin (PE) spectral diversity was investigated in eastern tropical Australian waters and around New Caledonian and Fijian archipelagos. Colony sorting of filamentous cyanobacteria revealed slight differences in the PE excitation spectrum of *Trichodesmium thiebautii* and *T. erythraeum*. Spectra of PE from *Katagnymene spiralis* and *Richelia intracellularis* were examined for the first time. PE spectra of filamentous cyanobacteria (*Trichodesmium, Katagnymene*, and *Richelia*) showed a broader phycoerythrobilin (PEB) band than those of *Synechococcus*. The influence of PE *Trichodesmium* on the global spectrum of PE in natural waters was clearly visible at various stations. The PEB band was large at the surface and narrower at increased depth, suggesting a shift of the cyanobacterial community from a dominance of diazotrophic filamentous cyanobacteria to small *Synechococcus*. Size fractionation of water samples confirmed this. A good linear relationship was observed between PE concentration in the >10-μm cellular-size fraction and the abundance of filamentous cyanobacteria expressed by either trichome numbers, total trichome surface area, or total trichome volume. PE in the >10-μm fraction is a useful tool for rapidly quantifying filamentous cyanobacteria. Neither diel variations nor photoacclimation significantly influenced the PE fluorescence excitation spectra in *T. thiebautii* and *T. erythraeum*. Using this method, we identified green colonies of filamentous cyanobacteria in deep waters (50–120 m) of the Coral Sea with a novel high-phycourobilin PE. While morphologically similar to *Trichodesmium*, it possesses distinctive photosynthetic responses and could be a new species.

Phycobiliproteins (PBPs) play an important role in light-harvesting pigment complexes of three groups of photosynthetic organisms: cyanobacteria, Rhodophyceae, and Cryptophyceae. They are also of significant ecological interest in providing information on biomass and taxonomic composition of these groups in the photosynthetic community (Glazer et al. 1982; Lantoine and Neveux 1997; Neveux et al. 1999; Wood et al. 1999). However, contrary to liposoluble algal pigments, which are extensively measured in the field to assess the community structure, PBPs are rarely used and data on them are still scarce. In subtropical and tropical oceanic waters, the major PBPs are phycoerythrins (PEs) associated with cyanobacteria. Two functional groups can be distinguished within these cyanobacteria according to their capability to fix dissolved N_2 (diazotrophy). The smallest unicellular species (*Prochlorococcus*, cluster A *Synechococcus*: Waterbury and Rippka 1989) are unable to fix N_2, while other species, unicellular or colonial, free-living or symbiotic, are diazotrophic (Capone et al. 1997; Zehr et al. 2001; Falcón et al. 2002). Diazotrophy represents a useful adaptation to oligotrophic conditions where other N sources are exhausted (Karl et al. 2002), provided that supplies of other macro- (PO_4^{2-}) and micronutrients (Fe) are sufficient to sustain diazotroph growth (Mills et al. 2004; Moutin et al. 2005). The importance of oceanic diazotrophs in N cycling and N global budget has resulted in new interest in measuring both rates and abundance of diazotrophs (La Roche and Breitbarth 2005). N_2 fixation may supply up to half of the new particulate N in the subtropical north Pacific (Karl et al. 1997) and increases the sequestration of CO_2 via particulate export to deeper waters (Gruber and Sarmiento 1997).

Trichodesmium spp. are one of the dominant diazotrophs in tropical and subtropical seas (Capone et al. 1997). Their abundance is generally assessed by time-consuming counting of filaments (or trichomes) using microscopy (Carpenter et al. 2004). Spectrofluorometric measurement of PE concentration has promised to be a more rapid way to assess their abundance; however, there are relatively few field data on the fluorescence characteristic of PE and its

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variability in filamentous diazotrophs. In this article, we investigated the spectral diversity of PE in isolated filamentous cyanobacteria and natural samples from North Australian, New Caledonian, and Fijian tropical waters. Moreover, we show that PE concentration can be used to quantify the biomass of filamentous diazotrophs in the photosynthetic communities.

Material and methods

Sampling—Water and net samples were collected in waters north of Australia (RV *Maurice Ewing* cruise, EW9912, October–November 1999) and around various archipelagos of the tropical Pacific (RV *Roger Revelle* cruise, April 1998; Diapazon program including nine *Diapalis* cruises on the RV *Alis* in the 2001–2003 period around New Caledonia). Several procedures were used to collect different size fractions or taxa. Unless otherwise noted, all water samples were collected from CTD rosettes equipped with Niskin bottles: (1) 0.5–3 liters of seawater was filtered onto GF/F or/and 0.4-μm polycarbonate membrane for the estimation of total PE. (2) To study the distribution of PE in three different size classes, serial filtration was performed on 10-μm, 2- or 3-μm, and 0.4-μm polycarbonate. (3) To examine the relationship between the PE concentration and the biomass of filamentous cyanobacteria during the *Diapalis* cruises, the entire contents of two 8-liter Niskin bottles were filtered onto 10-μm polycarbonate: the one 8-liter sample for filament counting and measurements, the other 8-liter sample for PE characterization. This was repeated at four or six depths between 0 and 80 m on a special cast occurring around noon (36 stations during *Diapalis* cruises). (4) To characterize the PE of various species of filamentous cyanobacteria, we hand sorted filaments caught with 35-μm mesh plankton net. *Rhizosolenia* sp. containing the symbiont *Richelia* were collected in the Sainte-Marie Bay (New Caledonian Lagoon) and *Katagnymene spiralis* in oceanic waters near Bourail (New Caledonia). *Trichodesmium* spp. came from various locations (near Fiji Islands, North of Australia, and New Caledonia). They were identified from morphological characteristics. Filamentous colonies were also collected using General Oceanics opening/closing (mesh size = 153 μm) net tows between 0 and 120 m in the Coral Sea, near the Australian coast. Sorted material for spectrofluorometric assays was concentrated on GF/F and frozen. All field materials collected on polycarbonate membranes were immediately processed (EW9912) or frozen before processing (*Diapalis* cruises).

Sample treatment and phycoerythrin determination—GF/F treatment:Material collected on GF/F, including hand-sorted filaments, was extracted by grinding in 5 mL 0.1 mol L^{-1} NaH_2PO_4 (pH = 6.5), maintained 3 h at 4°C in the dark and centrifuged for 20 min at 3,000 rpm before measurement. The PE fluorescence excitation spectra were recorded at 0.2-nm intervals between 450 and 580 nm (emission fixed at 605 nm) with an F4500 HITACHI spectrofluorometer equipped with a red-sensitive R928 Hamamatsu photomultiplier. Slit widths were set at 5 and

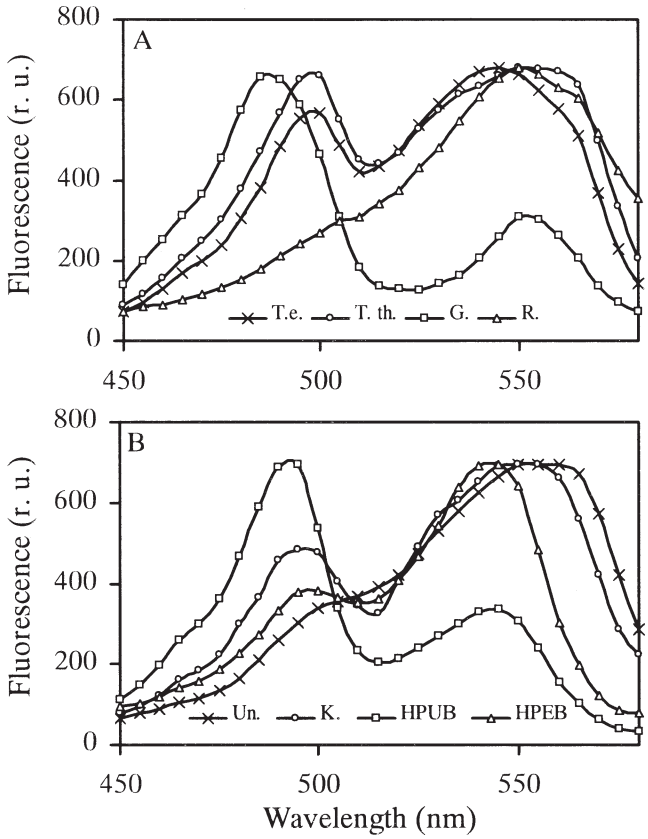

Fig. 1. Corrected fluorescence-excitation spectra of phycoerythrins (phosphate buffer: 0.1 mol L^{-1} NaH_2PO_4; pH = 6.5) in various filamentous cyanobacteria and *Synechococcus* (A), *Trichodesmium thiebautii* (T. th.), *Trichodesmium erythraeum* (T. e.), *Richelia intracellularis* (R.), and green colonies (G.); (B) high-PUB (HPUB) and high-PEB (HPEB) *Synechococcus, Katagnymene spiralis* (K.), and unidentified filament (Un.). Spectra are normalized at the fluorescence excitation maximum.

10 nm at the excitation and emission sides, respectively. Spectra were blank subtracted (new filter extracted in the phosphate solution). To obtain corrected spectra, correction factors for excitation wavelength-dependent energy were determined using Rhodamin B as quantum counter in the measuring compartment according to the manufacturer's protocol. Corrections are done to obtain spectra that theoretically match the absorption spectra, assuming that fluorescence quantum yield is independent of excitation wavelength. This procedure eliminates all disturbances related to the instrument characteristics and makes comparison between different studies easier. However, we observed that HITACHI-corrected spectra were different from spectra recorded with the same protocol on an MPF 66 PERKIN ELMER spectrofluorometer. In the latter instrument, a second Rhodamin B quantum counter is associated with the ratio energy compensation mode and leads to a better correction of the spectra. Then, it was possible to obtain additional correction factors (1-nm interval), assuming that fluorescence of a solution at 450 nm was the same whatever the instrument used and calculating the relative fluorescence changes at the other wavelengths. For taxonomic comparison (see Fig. 1), these

additional spectral corrections were applied. They did not change significantly the excitation maxima (maximum = 1 nm), but the PUB PEB (phycourobilin phycoerythrobilin) excitation ratio was increased around 19%. Other results represent direct measurement of the HITACHI-corrected spectra.

Polycarbonate membrane treatment: Polycarbonate filters were placed into a 4 or 5 mL 50/50 mixture of glycerol/phosphate buffer and shaken vigorously according to the procedure used in the in vivo method (Wyman 1992). This procedure is based on uncoupling between PE, phycocyanin, and chlorophyll, and is a more PE quantitative assay (Wyman 1992; Neveux et al. 1999).

Quantitative estimates of phycoerythrin were obtained from the area below the fluorescence excitation curve and a calibrating procedure described previously (Lantoine and Neveux 1997).

Filament counting and measurements—Filaments collected onto 10-μm pore-size polycarbonate membrane during the *Diapalis* cruises (47-mm-diameter Nuclepore) were preserved on board with 4% formalin solution. For counting at the laboratory, they were recovered by washing the filters with filtered seawater. Then, a convenient volume of an acidified formalin solution was added to obtain a 0.4% final concentration (Carpenter and Carmichael 1995). Acidification allows breaking the gas vesicles and facilitates sedimentation of the filaments. After a 24-h sedimentation, *Trichodesmium* trichomes were counted on an OLYMPUS IM inverted microscope using the Utermöhl (1958) technique. Biomass of filamentous cyanobacteria was expressed generally as trichome number and sometimes as total surface area or total volume of filaments.

Pulse amplitude modulation (PAM) measurements—Individual colonies were picked from net tow samples using plastic transfer loops and were immediately placed in filtered seawater. Colonies were examined using a Heinz Walz GmbH microscopy PAM system equipped with a far-red filter (Corion S10-720-F) to minimize nonphotochemical quenching of chlorophyll fluorescence induced by the microscope's transmitted light path. The actinic, saturation, and measuring light were provided by a single blue light-emitting diode (470 nm), and the photomultiplier detector was equipped with a >650-nm cut-off filter. PAM systems are multiple turnover systems, and the nomenclature used in this article follows van Kooten and Snel (1990). Prior to examination, cells were in dim (<20 μmol photons m^{-2} s^{-1}) ambient room light for 10–15 minutes since handling considerations did not permit full dark adaptation. The resulting measurement is intermediate between a true F/F_m and F/F_m' under actinic light (Kromkamp and Forster 2003). F_m and $F_{m'}$ represent the maximum fluorescence quantum yield with full dark adaptation or under actinic illumination, respectively.

After measuring F/F_m' (saturation pulse of 0.6 s), the cell was exposed to 8 increasing actinic light levels (6, 36, 60, 90, 132, 192, 264, 444, 660 μmol photon m^{-2} s^{-1}). After 5 s at each step, the yield $(F_m'–F_s)/F_{m'}$ was measured using an 0.6-s saturation pulse (rapid light curve: White and Critchley 1999). This rapid light curve generated a plot of yield versus irradiance; electron transport rate (ETR = yield $\times$ PAR $\times$ 0.5 $\times$ 0.85), where PAR is photosynthetic available radiation, was calculated by the software. Because absorption was not measured, these ETR values are only relative and the ETR (μmol e^- m^{-2} s^1) will be termed the unitless relative electron transport rates (rETR: Morris and Kromkamp 2003).

Absorption spectra—Spectral optical densities of filamentous cyanobacteria recovered on GF/F filters were recorded at 1-nm intervals between 330 and 800 nm using a BECKMAN DU-26 dual-beam spectrophotometer according to Dupouy et al. (1997).

Results

Phycoerythrin and taxonomy of cyanobacteria—Fluorescence excitation spectrum of PE depends on (1) the relative proportion of two prosthetic chromophores, the PUB and the PEB and (2) the chromophore–chromophore and chromophore–apoprotein interactions (Sidler 1994). PUB has an absorption (fluorescence excitation) band with a peak at 495 $\pm$ 6 nm, and PEB has an absorption band with a peak between 536 and 565 nm, depending on the species. Figure 1 shows the corrected-fluorescence excitation spectrum of PE (C-FESPE) in the different filamentous species and compares them to two spectra of field *Synechococcus* sampled in Australian waters and characterized by extreme values of the PUB PEB excitation ratio. All filamentous cyanobacteria exhibited a large PEB excitation band. Slight differences could be observed between C-FESPE of *T. thiebautii* and *T. erythraeum* (Fig. 1A). The PEB band had a peak at 558 $\pm$ 2 nm for *T. thiebautii* or at 548 $\pm$ 1 nm with a shoulder at 562 nm for *T. erythraeum*. The two spectra presented the same PUB peak at 499 nm, but their PUB PEB ratio was 0.98–1.00 for *T. thiebautii* and 0.84–0.87 for *T. erythraeum*. During our studies, several analyses of PE were performed on the two species and we did not observe important variations in their C-FESPE. The C-FESPE of *K. spiralis* showed a PUB PEB ratio of 0.68 with PUB and PEB peaks located at 499 and 555 nm, respectively (Fig. 1B). The C-FESPE of *R. intracellularis* was distinctly different from those of *Trichodesmium* spp. and *K. spiralis*, showing only a shoulder related to PUB and a PEB maximum at 554 nm (Fig. 1A,B). Furthermore, a long (2.5 cm), unidentified filament floating at the surface presented a C-FESPE with only a shoulder at 500 nm and a PEB peak located at 558 nm (Fig. 1B). The PEB band in *Synechococcus* were clearly narrower than that of filamentous cyanobacteria, except that of green colonies, which were observed between 50 and 120 m in the Coral Sea (Fig. 1A). These green colonies had a C-FESPE similar to that of high-PUB *Synechococcus* with a PUB PEB ratio of 2.11, but the PUB maximum was 8 nm shifted toward lower wavelength (487 nm), whereas the PEB peak was 7 nm shifted toward higher wavelength (553 nm).

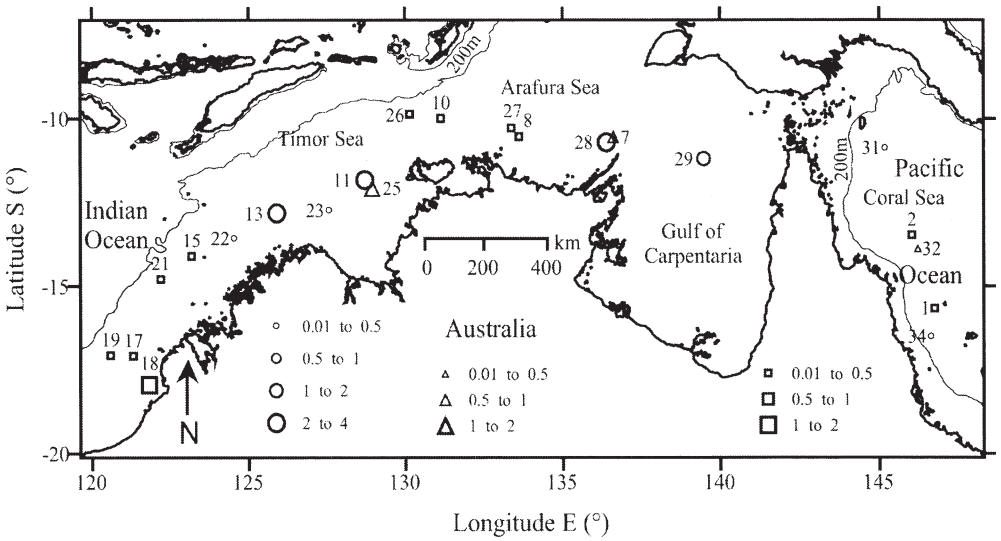

Fig. 2. Surface phycoerythrin (PE) concentrations (μg L^{-1}) at stations sampled during the EW9912 cruise north of Australia. Symbol shape indicates the % of the total PE pool that was due to *Trichodesmium*: open squares (not detected); open triangles (25–50%); open circles (50–80%). Numbers associated to symbols indicate the range of total PE found at that station. Highest symbol size represents highest PE concentration range.

Phycoerythrins in global and size-fractionated oceanic samples—In the total autotrophic community of Australian samples, the PE spectra generally showed the dominance of either *Synechococcus* or filamentous cyanobacteria. For example, at station 29 (EW9912 cruise: Fig. 2), in the Gulf of Carpentaria, the PE exhibited a larger PEB band at the surface (0–20 m) than at 30–50-m depth (Fig. 3A). This suggested that the relative abundance of *Trichodesmium* PE in the cyanobacterial community decreased from the surface to 50 m and that *Synechococcus* PE dominated in deep samples. A more precise assessment of the two types of PE could be obtained by size fractionation (Fig. 3B,C). At 10 m, the >10-μm fraction showed a PE signal with a large PEB peak associated to filamentous cyanobacteria. It was higher (65% of total PE) than that observed in the <3 μm and related to *Synechococcus*. In the 3–10-μm fraction, it was insignificant. At 40 m, the *Synechococcus* signal was clearly dominating. It was characterized by a higher PUB PEB ratio than at 10 m. A similar but lower signal was observed in the 3–10 μm, suggesting some retention of *Synechococcus* cells by clogging of the filter rather than the presence of cells in the 3–10-μm size fraction. Generally, all *Synechococcus* PE was recovered in the <3-μm fraction and filamentous cyanobacteria on the >10-μm membrane. This pattern appeared in stations 5, 7, 11, 13, 23, 25, 26, 28, with a more or less great vertical extension of a recognizable influence of *Trichodesmium* PE in the total community. The highest concentration of *Trichodesmium* PE measured in the >10-μm fraction was observed near the surface at stations 11, 13, and 28 (2–4 μg L^{-1}, Fig. 2). Spectra suggested the dominance of *T. thiebautii* at most of the stations. At the stations located in the west coast (15–21) as well as at stations 8–10, *Trichodesmium* PE were not detected. At the shallowest station (18), *Synechococcus* PE showed a homogeneous vertical distribution (1.33 μg L^{-1}) with a low PUB PEB ratio (0.61). At the other stations, the PUB PEB ratio of *Synechococcus* PE generally increased with depth. The highest *Synechococcus* PE concentrations were observed at stations 7, 11, and 29 (2–3 μg L^{-1}) between 30 and 45 m.

Relationships between phycoerythrin concentration and biomass of diazotrophs in the >10-μ*m fraction*—A first attempt to correlate diazotrophs abundance and PE *Trichodesmium* concentration in the >10-μm fraction of Australian waters failed. This was attributed to a bad sampling strategy, which did not take into account the patchy distribution and the behavior of filamentous species in their environment. Samples for trichome counting were taken around noon and used all the water contained in 10-liter Niskin bottles, while PE concentrations were assessed on 1 liter taken at the same stations but on a different cast.

A second attempt in the New Caledonian archipelago (*Diapalis* cruises) used two Niskin bottles closed simultaneously on the same CTD-rosette cast. The entire contents of these bottles were filtered onto 10-μm filters. Microscopic examination of this fraction generally showed the presence of significant quantity of *T. tenue* (trichomes 4–5 μm wide and cell length 3–4-fold higher than width), a species for which the PE spectrum has not still been precisely characterized, but probably close to that of other *Trichodesmium*. Considering the samples taken at three stations near Lifou Island (New Caledonia: Fig. 4), *T. tenue* represented on average 57% ± 5% (Fig. 4A), 48% ± 12% (Fig. 4B), and 33% ± 10% (Fig. 4C) of the trichome total surface, respectively. At one station (Fig. 4B), *T. erythraeum* (trichomes of 8–13 μm width and cell length 0.5–1-fold higher than width) was in low abundance (4% ± 2%) and the PE spectra looked like that of *T. thiebautii* (trichomes 6–7 μm wide and cell length 1–2-fold higher than width), which represented 47% ± 14%. At the other stations, *T. erythraeum* reached 22–25% (Fig. 4A,C) and

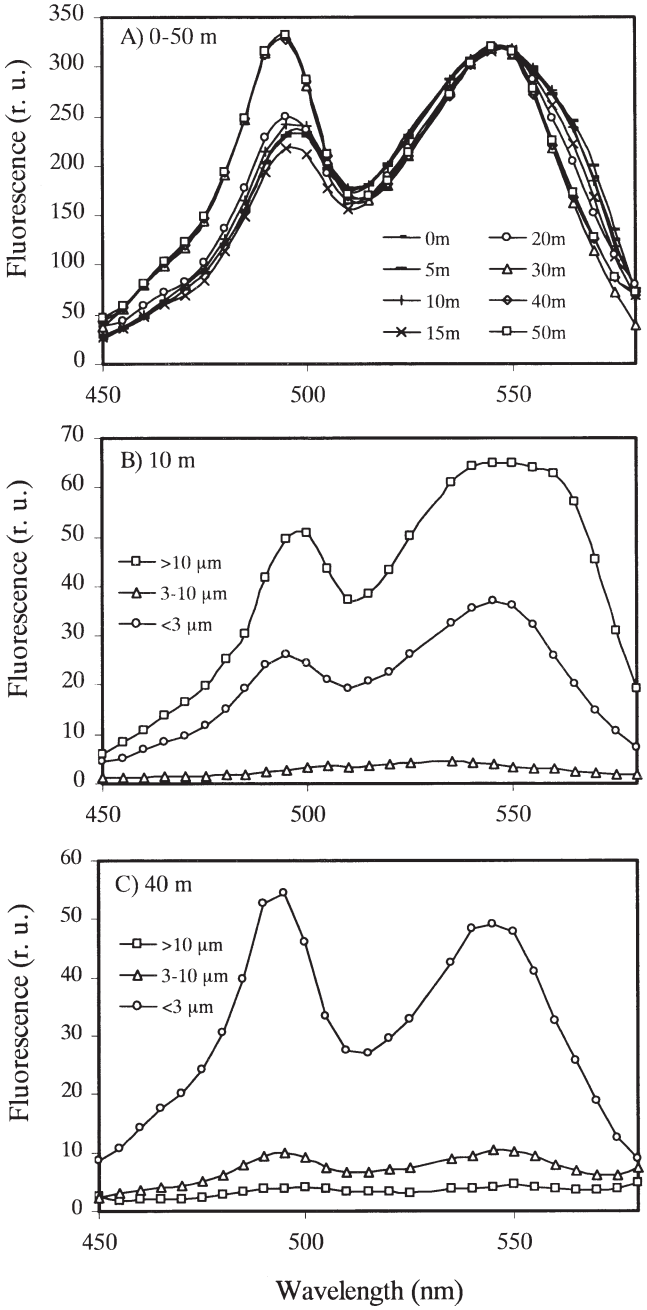

Fig. 3. HITACHI-corrected fluorescence excitation spectra of phycoerythrins (glycerol/phosphate buffer, 50/50) at station 29 (11.17°S; 139.55° W; bottom = 50 m) during the EW9912 cruise in North Australia: (A) vertical variations in the global communities (normalization at the second excitation peak; curves at 30, 40, and 50 m are superimposed; width of the second peak decreases from 0 to 30 m depth), (B) and (C) their characteristics in the >10-μm, 3–10-μm, and <3-μm size fractions at 10 and 40 m depth, respectively.

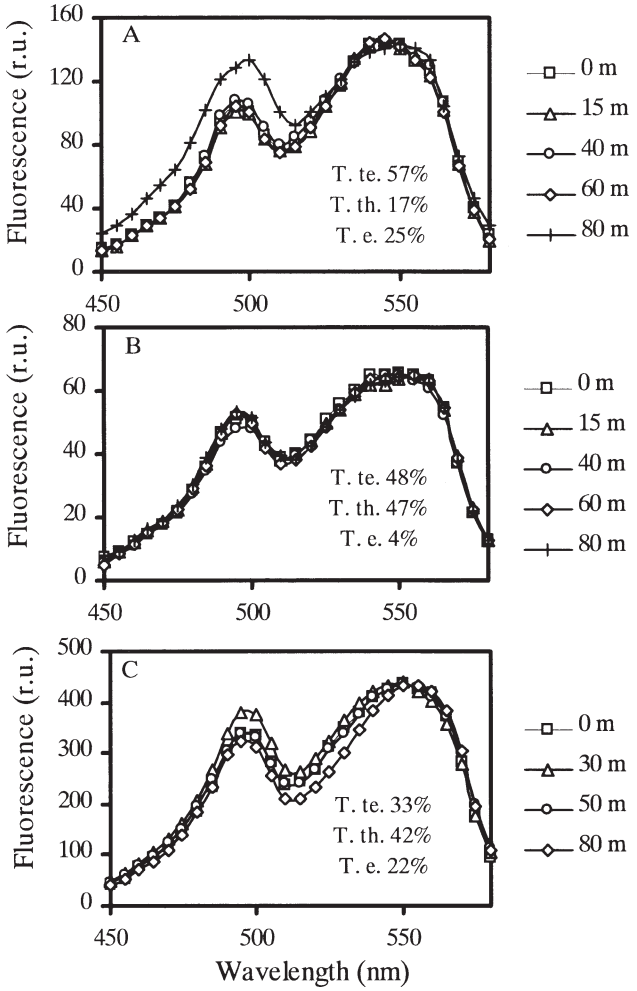

Fig. 4. HITACHI-corrected fluorescence excitation spectra of phycoerythrins (glycerol/phosphate buffer, 50/50) around Lifou island (New Caledonian waters) during *Diapalis* cruises in May 2002: (A) East Lifou, 20.62°S, 167.02°E; (B) Santal Bay, 20.83°S, 167.03°E; and in February 2003, (C) Santal Bay, 20.83°S, 167.03°E. Variations occur with depth in the >10-μm fraction (normalization at the second excitation peak). The mean percentage in the global trichome surface area (from microscopic counts) of the three main *Trichodesmium* species at each station is inserted on the graph: T. te. = *T. tenue*; T. th. = *T. thiebautii*; T. e. = *T. erythraeum*.

the spectra were rather intermediate between that of *T. erythraeum* and *T. thiebautii*. *Katagnymene* spp. and *R. intracellularis* were also frequent, but only in low abundance (<5%).

The clearest relationships between biomass of filamentous cyanobacteria and PE were obtained during the *Diapalis* 7 cruise (Austral summer, February 2003), where we recorded the highest trichome abundances (maximum around 4,500 trichomes L^{-1}) compared with those observed during the other *Diapalis* cruises. We discarded all data where *Trichodesmium* counts were lower than 50 trichomes per liter. Considering samples taken at all depths between 0 and 80 m, a significant Spearman correlation coefficient was calculated between PE concentration (>10-μm size fraction) and trichome number (Spearman = 0.91, p-value < 0.001, n = 30; Fig. 5). Although the data set was not normally distributed, it seemed relatively well described by a linear regression (R^2 = 0.95). Because the samples

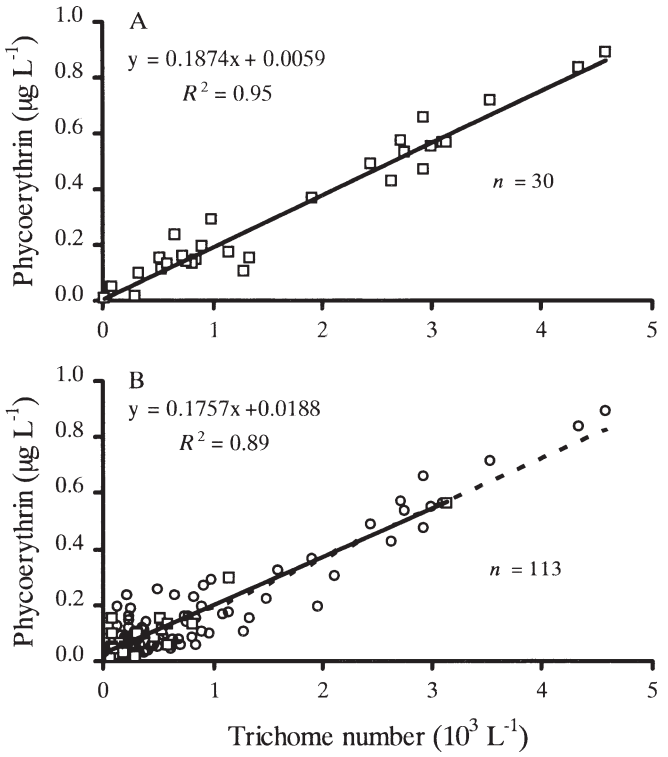

Fig. 5. Relationships between phycoerythrin concentrations and diazotrophs counts in the >10-μm fraction for samples taken during the *Diapalis* cruises: (A) *Diapalis* 7 cruise (February 2003), (B) all cruises; y, R^2, and n on the panel correspond to all data; open squares and dotted regression line ($y = 0.1763x + 0.0174$; $R^2 = 0.88$; $n = 93$) correspond to samples taken between 0 and 40 m depth; open circles, squares, and solid regression line ($y = 0.1772x + 0.0172$; $R^2 = 0.92$; $n = 19$) correspond to samples taken between 50 and 80 m depth. 1 × unit = 1,000 cells.

were composed of mixtures of different species with different dimensions, we expected a better correlation with total trichome surface or total trichome volume. The correlations were good but not better (surface: Spearman = 0.90, p-value < 0.001, $n = 24$, $R^2 = 0.89$; volume: Spearman = 0.90, p-value < 0.001, $n = 24$, $R^2 = 0.93$). Taking into account data of all the *Diapalis* cruises, the relationships were not significantly different (Fig. 5), but data points were more dispersed at low concentrations.

Effect of diel variations and photoacclimation processes on PE characteristics and biomass–PE relationships—We examined if photoacclimation processes could affect the shape of the PE excitation spectra and the relationships between PE concentration and filament counts. Considering all the data of the *Diapalis* cruises, the relationships were not significantly different if we separated the 0–40-m samples from deeper depth samples (50–80 m), suggesting there was no photoacclimation process in filamentous cyanobacteria related to an accumulation of PE in trichomes under low light (Fig. 5). We had relatively few significant values in the deeper layer because *Trichodesmium* spp. were generally more abundant in upper waters. Information on photoacclimation and photoadaptation in cyanobacteria can also be deduced from changes in the PUB PEB ratio. A high PUB PEB ratio represents an advantage for the cyanobacterial population (Alberte et al. 1984; Wood 1985) at depth because PUB absorbs mainly at wavelengths of the maximum light transmission of waters in oligotrophic systems. During our studies, we observed no important vertical changes in the PUB PEB ratio of the *Trichodesmium* populations. This could be illustrated with the three examples showing normalized spectra at the PEB maximum (Fig. 4). In May 2002, at the East Lifou station (Fig. 4A), a slight increase of the PUB PEB ratio was observed at 80 m (the signal at this depth was 10-fold lower than at other depths), while at the Santal Bay station, no important variation was noted (Fig. 4B). At the latter station, in February 2003, the highest PUB PEB ratio occurred at 30 and the lowest at 80 m, but the variations were relatively low; the spectrum at 0 m exactly matched that at 50 m. So, there was not a clear pattern suggesting the influence of photoacclimation processes in filamentous cyanobacteria. Two possibilities exist for this result: (1) a well-mixed thick surface layer or (2) regulation of trichome buoyancy by changes in the cellular carbohydrate–protein ratio (Villareal and Carpenter 2003), which will make vertical migration possible in the euphotic zone even in the presence of a physical barrier. Biological information related to the depth of vertical mixing can be deduced from cellular properties of *Synechococcus* cells as measured by flow cytometry. It is known indeed that red and orange fluorescence of cells will increase in proportion of their phycoerythrin and chlorophyll content following photoacclimation processes under low light. We noted a large increase between 40 and 80 m (4–7 factor) for the cellular orange and red fluorescence, suggesting that at least the 80-m samples were outside the mixing layer. As regular sampling was operated essentially at noon, we investigated also the possibility of diurnal changes in the PUB PEB ratio, which were suggested in previous work (Subramaniam et al. 1999). Colonies of *T. thiebautii* sampled from surface net tows before sunset until dusk did not show important modification in their fluorescence excitation spectra (Fig. 6). This was also true for *T. erythraeum* (not shown). All these results suggest that PE concentrations can constitute a good estimate of the *Trichodesmium* populations when the >10-μm fraction is considered.

However, the observation of green colonies in deep waters (50–120 m at stations 31, 32: see Fig. 2) of the Coral Sea (EWING 9912 cruise), which physically looked like *Trichodesmium* colonies, raised an evident question: Do these green colonies belong to a new species or represent an ecotype of known filamentous cyanobacteria photoacclimated to low light? As previously indicated, our PE data in the >10 μm did not exhibit a significant photoacclimation effect in *Trichodesmium* populations of the New Caledonian Archipelago. In the Coral Sea waters, there was no evidence of an important contribution of the green colonies to the PE in the global community. Moreover, size fractionation done at 0 and 100 m showed only PE *Synechococcus* signal in the <3-μm fraction and the presence of *T. thiebautii* only at the surface. This underlined

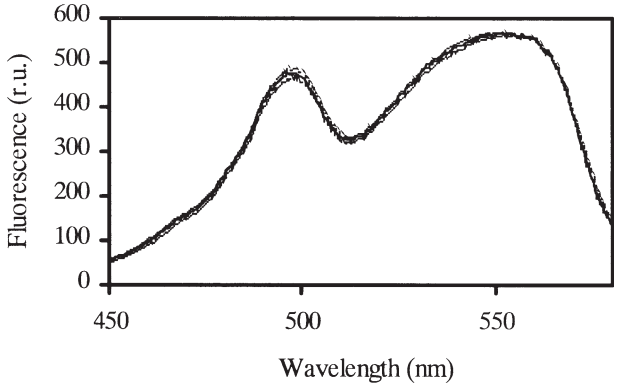

Fig. 6. Diurnal variations of the HITACHI-corrected fluorescence excitation of *T. thiebautii* phycoerythrin in surface waters (solution in phosphate buffer 0.1 mol L^{-1} pH = 6.5). Mean, mean–standard deviation and mean + standard deviation curves determined from five spectra recorded each 3 h between dawn and dusk (from 6:00 to 18:00 h). The coefficient of variation was lower than 3% beyond 479 nm and between 3% and 6% from 450 to 479 nm.

that green colonies were not concentrated enough in these waters to observe them during regular water sampling and that net sampling was necessary. In addition, PAM fluorometry on individual colonies showed distinct differences in the shape of the rETR curve between *T. thiebautii* and the green cyanobacterium with saturation at 300 and 100 μmol photons m^{-2} s^{-1} PAR, respectively (Fig. 7). The green cyanobacterium taken at different depths saturated at similar PAR value, but showed marked decreases in relative electron transfer rate at higher PAR (Fig. 7). These data suggest that the green filaments could belong to a new taxa. As indicated above, the high-PUB PE represents an adaptation to low light levels in oligotrophic waters. The

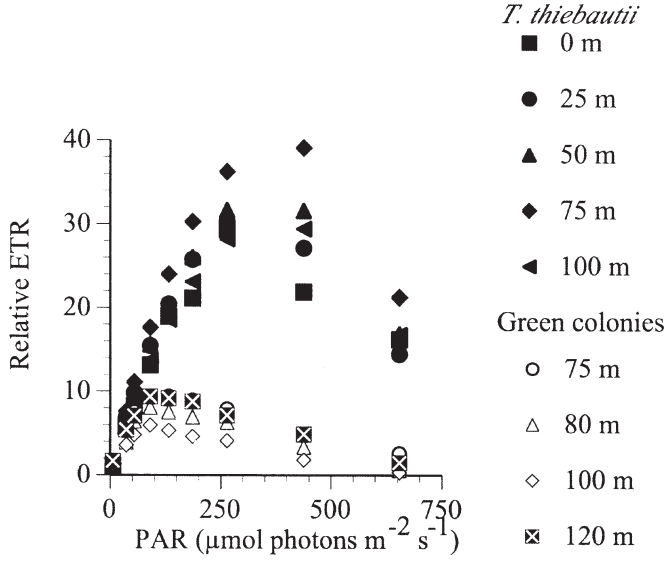

Fig. 7. Rapid light curves from *T. thiebautii* and the unidentified green cyanobacterium. Individual points are the mean of 3–9 colonies. Error bars (95% confidence interval) are omitted for clarity, but were between 5% and 20% of the mean. *T. thiebautii*—filled symbols; green colonies—open symbols.

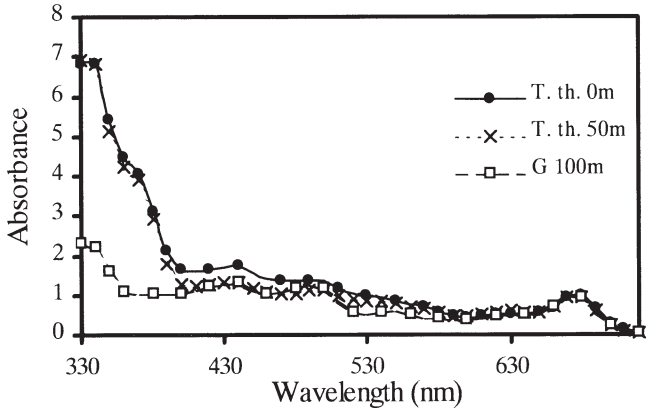

Fig. 8. Absorption spectra, normalized at the red peak (674–676 nm), for *T. thiebautii* taken at 0 (T. th. 0 m) and 50 m (T. th. 50 m) and green colonies taken at 100 m (G 100 m).

green population could be limited to relatively deep waters if for any reason it did not tolerate ambient high light intensities. Its in vivo absorption spectra indeed showed a relatively low absorption in the ultraviolet (UV) range compared with that recorded on *T. thiebautii* taken at 0 and 50 m depth (Fig. 8). Absorption in the UV by microsporine-like amino acids (absorption maximum around 330 nm) represents a photoprotective way for *Trichodesmium* to avoid cell damage (Subramaniam et al. 1999). The second main difference in the absorption spectra was mainly related to the difference in the absorption properties of phycoerythrin.

Discussion

Knowledge of the functioning of the ecosystems and their impact on the various compartments of the biosphere need information on structure, role, and behavior of the major component of the living communities in each compartment. The essential role of cyanobacteria in the oceans is now well recognized since the discovery of number of small *Synechococcus* and *Prochlorococus* (Johnson and Sieburth 1979; Chisholm et al. 1988; Neveux et al. 1989) and the awareness that N_2-fixing species, both filamentous and unicellular, also have to be taken into account in C and N cycling and the N global budget (Capone et al. 1997; Montoya et al. 2004). To assess N_2-fixation rates at a global scale, algorithms for remote-sensing estimates of the abundance of *Trichodesmiums* are being developed from their bio-optical properties (Subramaniam et al. 1999; Westberry et al. 2005). However, unequivocal identification and abundance estimation of *Trichodesmium* (or other filamentous diazotrophs) from remote satellite with relatively low pixel and spectral resolutions seems difficult in the absence of sea-truth measurements. *Trichodesmium* abundance is generally assessed by relatively tedious filament counting. Optical properties of PBPs in cyanobacteria could provide alternative methods to rapidly determine their abundance. PE spectra in oceanic *Synechoccocus* are relatively well documented (Lantoine and Neveux 1997; Wood et al. 1999). PE fluorescence excitation spectra in filamentous

cyanobacteria data are scarce. Moreth and Yentsch (1970) reported a maximum excitation peak at 490 nm and showed an uncorrected emission spectrum (max at 565 nm) for native and partially purified PE in *T. erythraeum*. Fujita and Shimura (1974) reported corrected excitation spectrum for purified PE from *T. thiebautii*, and Wood et al. (1999) documented native PE in an unidentified *Trichodesmium* (spectrum somewhat truncated because the scan stops at 550 nm) collected in the Arabian Sea. *T. thiebautii* exhibited three excitation peaks, at 500, 547, and 565 nm (Fujita and Shimura 1974). However, some absorption spectra of purified PE in some isolates (Qingdao, China) of *Trichodesmium aff. thiebautii* showed the absence of a PUB band and either a single broad peak at 550 nm or two equivalent peaks at 546 and 557 nm (Haxo et al. 1987). Our study represents a new and relatively large data set on fluorescence excitation spectrum of isolated species or mixed filamentous assemblages sampled in the tropical Pacific and north Australian waters. It concerned only fluorescence of native PE extract in phosphate buffer or PE uncoupled from photosynthetic electron transfer by glycerol (Wyman 1992). If we exclude a spectrum of one large unidentified filament and that of *R. intracellularis* (Fig. 1A,B), all spectra showed a clear PUB band and a large PEB band. Surprisingly, the complete C-FESPE of *T. erythraeum* had apparently not been published, and no comparison with our results could be done. For *T. thiebautii*, we observed a PUB and an asymmetric PEB band with one peak at 558 ± 2 nm, which differed from the previous observations of two peaks in the PEB band (Fujita and Shimura 1974). In the latter case, however, PE was partially purified by Sephadex-gel filtration. Furthermore, differences between absorption and corrected excitation spectra of PE extract were noted (Fujita and Shimura 1974), suggesting either the use of imperfect correction factors or wavelength-dependent quantum yield of PE fluorescence. We noted also that correction factors could be dependent on the spectrofluorometer type, even if we used the manufacturer's protocol and the same quantum counter. Nevertheless, differences were never represented by the emergence of a new peak or the disappearing of an existing peak. Good reliability of the spectrum was generally obtained from sorted *T. thiebautii* or *T. erythraeum* in the tropical Pacific. Nevertheless, unusual spectra could sometimes be obtained, which were considered as the result of a partial denaturation of the native protein. For example, one excitation spectrum of a sample of *T. erythraeum* extracted in phosphate buffer exhibited two emission peaks, at 502 and 555 nm, instead of a single peak around 566 ± 1 nm. Considering that in native PE, energy absorbed by PUB is transferred to PEB with very high efficiency (Ong and Glazer 1991), the existence of two peaks suggested an uncoupling of PUB and PEB, which emitted their own fluorescence. This uncoupling was not observed on the in vivo spectrum of the same sample. No diurnal variations of the PUB PEB ratio were noted. This contrasts with previously published work, which suggested a rapid and reversible interconversion of PEB to PUB in responses to change in ambient light regime during the day (Subramaniam et al. 1999). There was no clear photoacclimation effect on PE excitation spectra. Similarly, no absorption changes with depth related to chromatic adaptation were reported in *T. thiebautii* from the Central North Atlantic Ocean (McCarthy and Carpenter 1979). If we hypothesize that green colonies represented a photoacclimated ecotype of *T. thiebautii* (or other already known *Trichodesmium*), it seems evident that it would be the result of a relatively long stay of the colonies under low light. This long stay would be necessary to synthesize a new type of PE inside new phycobilisomes. Some *Synechococcus* strains indeed can respond to changes in light-quality conditions (white or green light to blue light transitions) by synthesizing high-PUB PE instead of high-PEB PE, but complete replacement of the one by the other needs 100–140 h under 10–20 μmoles photons m^{-2} s^{-1} (Palenik 2001; Six 2005). If an interconversion in a same protein of PEB to PUB by a lyase isomerase was involved, this would take certainly a shorter time, but whatever the processes that would lead to the high-PUB PE in green colonies, we would expect a more significant variation of the *Trichodesmium* PUB PEB ratio along the water column than we observed. The green colonies may indeed belong to a new species, but confirmation from gene-sequencing analysis is required (Lundgren et al. 2005).

For ecological studies, fluorescence spectra of phycobiliproteins, and more particularly PE, constitute useful taxonomic tools for characterizing the structure of marine cyanobacterial communities and for the assessment of the relative abundance of cyanobacteria. It can supplement information obtained by other techniques, such as flow cytometry, microscopic observations, and quantitative polymerase chain reaction amplification of group-specific dinitrogenase genes (Church et al. 2005). Part of the diversity of marine cyanobacteria remains to be identified, as evidenced by literature reports of new types of unicellular cyanobacterial cells in the 2–10-μm size range (Neveux et al. 1999; Zehr et al. 2001; Falcón et al. 2002). The >10-μm fraction in New Caledonian waters also showed the presence of different forms of colonial coccoid cells, which most probably were cyanobacteria. Their abundance was not sufficient to measure phycobiliproteins with our method. The information on the relative abundance of all these cyanobacteria is limited, and we do not know their importance in the functioning of the oligotrophic ecosystems.

We showed that PE concentration in the >10-μm fraction provides a good estimate of the diazotrophic filamentous cyanobacteria. However, pelagic diazotrophic cyanobacteria were also reported in the 2–10-μm size range (Zehr et al. 2001; Falcón et al 2002; Church et al. 2005). The incorporation of PE related to diazotrophic unicellular cyanobacteria of <10-μm size in this estimate needs to complement our knowledge about the PE spectral diversity of these organisms and their recognition as N_2-fixers. Important concentrations of PE in this size fraction were not observed in North Australian waters. However, a dominant specific PE with PUB and PEB peaks, respectively, at 494 and 564 nm was observed around the Fijian Archipelago in April 1998 (R/V Revelle cruise) and was similar to that previously reported North of Tahiti in

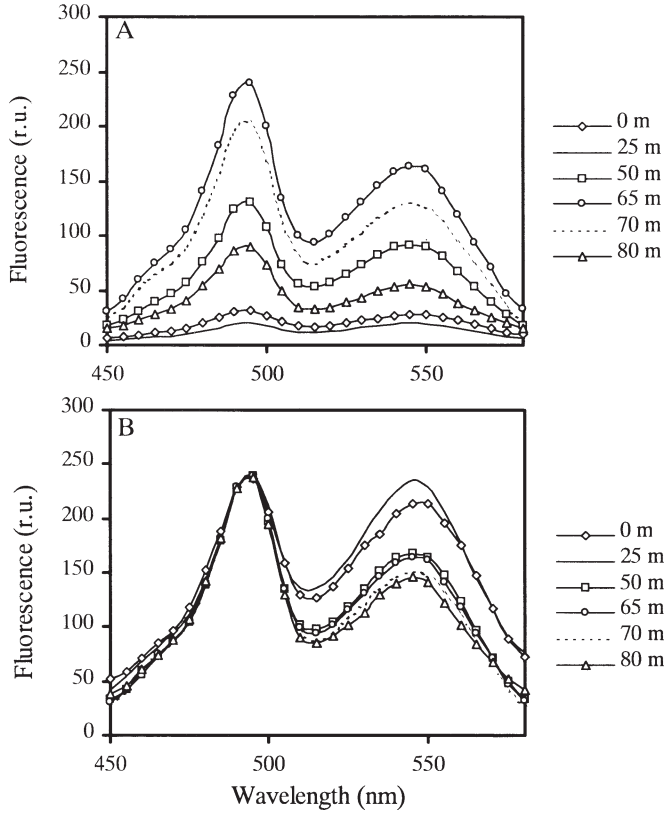

Fig. 9. HITACHI-corrected fluorescence excitation spectra of phycoerythrin at sta. 26 of the EW9912 cruise. Variations with depth (A) before and (B) after normalization at the first excitation peak.

3-μm unicellular cyanobacteria (Neveux et al. 1999). Furthermore, high rates of N_2 fixation by the $<$10-μm fraction was recently reported at 50–65 m (Sta. 26 and 27; see Fig. 2) in the deep chlorophyll maximum of the Arafura Sea (Montoya et al. 2004). In this fraction, PE excitation spectra was typical of high-PUB *Synechococcus* (Fig. 9). Cells of this *Synechococcus* had a similar size range to other *Synechococcus* observed in oceanic waters ($<$3 μm). This suggests the presence of either *Synechococcus* species that do not belong to the A cluster or N_2 fixers in the A cluster. Alternatively, fixation could be related at least partly to nitrogen-fixing heterotrophic bacteria.

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