

## N<sub>2</sub> Fixation by Unicellular Bacterioplankton from the Atlantic and Pacific Oceans: Phylogeny and In Situ Rates

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**N<sub>2</sub>-fixing proteobacteria ( $\alpha$  and  $\gamma$ ) and unicellular cyanobacteria are common in both the tropical North Atlantic and Pacific oceans. In near-surface waters proteobacterial *nifH* transcripts were present during both night and day while unicellular cyanobacterial *nifH* transcripts were present during the nighttime only, suggesting separation of N<sub>2</sub> fixation and photosynthesis by unicellular cyanobacteria. Phylogenetic relationships among unicellular cyanobacteria from both oceans were determined after sequencing of a conserved region of 16S ribosomal DNA (rDNA) of cyanobacteria, and results showed that they clustered together, regardless of the ocean of origin. However, sequencing of *nifH* transcripts of unicellular cyanobacteria from both oceans showed that they clustered separately. This suggests that unicellular cyanobacteria from the tropical North Atlantic and subtropical North Pacific share a common ancestry (16S rDNA) and that potential unicellular N<sub>2</sub> fixers have diverged (*nifH*). N<sub>2</sub> fixation rates for unicellular bacterioplankton (including small cyanobacteria) from both oceans were determined in situ according to the acetylene reduction and <sup>15</sup>N<sub>2</sub> protocols. The results showed that rates of fixation by bacterioplankton can be almost as high as those of fixation by the colonial N<sub>2</sub>-fixing marine cyanobacteria *Trichodesmium* spp. in the tropical North Atlantic but that rates are much lower in the subtropical North Pacific.**

Growth rates of plankton in open ocean surface waters are often limited by the availability of reduced forms of N. New combined N enters surface waters either by advection, diffusion of NO<sub>3</sub> from deep water, or biological N<sub>2</sub> fixation (15). The last pathway can be significant in tropical and subtropical seas, where large cyanobacteria, *Trichodesmium* spp., have been considered the major organisms responsible (8, 9, 11). Ongoing research to identify other sources of N<sub>2</sub> fixation and alternate pathways of reduced N to the trophic chain in the vast oceanic basins has pointed out the potential role of certain N<sub>2</sub>-fixing unicellular bacterioplankton, which could have a significant impact on global biogeochemistry of N and C (14, 16, 44).

Previous research has identified the presence of fragments of the unicellular cyanobacterial and proteobacterial *nifH* genes (which encode one of the peptide molecules that form the dinitrogenase reductase subunit of nitrogenase, the enzyme responsible for N<sub>2</sub> fixation [31]) in the subtropical North Pacific and tropical North Atlantic oceans (16, 43, 44). Preliminary data on N<sub>2</sub> fixation rates for the 10- to 0.2- $\mu$ m-size fraction of the bacterioplankton have suggested that they could make an important contribution to the global N cycle (14, 44). These studies used <sup>15</sup>N<sub>2</sub> to measure 24-h N<sub>2</sub> fixation rates in 10- $\mu$ m-pore-size-pretreated water from the subtropical North

Pacific. Also, N<sub>2</sub> fixation rates were estimated based on unicellular phytoplankton cell numbers that came from literature values (6). Cyanobacteria are autotrophs and generate ATP necessary for N<sub>2</sub> fixation through photosynthesis; unicellular cyanobacteria that fix N<sub>2</sub> need to do so during the night (2) since nitrogenase, the enzyme that catalyzes N<sub>2</sub> fixation, is inhibited by O<sub>2</sub> (15). Thus, it is important to obtain nitrogenase activity rates for the daytime and the nighttime.

In order to better define the in situ rates of N<sub>2</sub> fixation for the unicellular bacterioplankton community in the tropical North Atlantic and subtropical North Pacific oceans, we sampled at different depths throughout diel cycles for different seasons by the acetylene reduction and <sup>15</sup>N<sub>2</sub> protocols with slight variations (7, 10). Further, we coupled the N<sub>2</sub> fixation activity to the phylogenetic characterization of the populations responsible throughout the analysis of *nifH* transcripts. Here we demonstrate that potential N<sub>2</sub>-fixing unicellular bacterioplankton differ between the tropical North Atlantic and North Pacific; we also show that *nifH* transcripts of unicellular cyanobacteria occur during the nighttime only while those for proteobacteria are present during both the day and the night. Major differences are also found in N<sub>2</sub> fixation rates between the two oceans; in the tropical North Atlantic, the unicellular bacterioplankton activity is almost as high as that of the major oceanic N<sub>2</sub> fixers *Trichodesmium* spp. but in the tropical North Pacific is much lower. Our findings provide unique and new information essential to understanding N and C cycles in the Atlantic and Pacific oceans.

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## MATERIALS AND METHODS

**Seawater sampling.** Water samples were collected during two cruises to the tropical North Atlantic (summer 2001 and spring 2002) and during one cruise to the tropical North Pacific (fall 2002). Our research area in the Atlantic was concentrated between 7°10'23"N to 12°13'78"N and 45°29'60"W to 55°55'38"W, and in the Pacific it was in proximity to station ALOHA, 22°75'N to 158°00'W (19). Samples were obtained from discrete depths following a light level range of 100, 50, 25, and 1% which was determined previous to each conductivity-temperature-depth (CTD)-rosette system and which corresponded approximately to surface, 25, 50, and 100 m; also, samples were taken at the depth of increase in the nitracline, which was usually around 150 m. Seawater was gravity filtered from Niskin 10-liter bottles through 20- $\mu$ m-pore-size mesh (tropical North Pacific) and 10- $\mu$ m-pore-size polycarbonate (PCTE) membranes (Millipore Corporation, San Jose, Calif.) (tropical North Atlantic) to eliminate larger diazotrophs (e.g., *Trichodesmium* spp.) and collected in Nalgene bottles. In the Pacific, we observed by epifluorescence microscopy that large unicellular cyanobacteria (7  $\mu$ m) clustered together and clogged the 10- $\mu$ m-pore-size membranes.

**Nitrogenase activity.** The filtrate from each light depth (4 liters) was concentrated onto 0.2- $\mu$ m-pore-size membranes (47-mm diameter; polycarbonate PCTE; Millipore). Cell concentrates were diluted by washing the filters with 0.2- $\mu$ m-pore-size-filtered seawater, to the volume necessary to assay nitrogenase activity by the  $^{15}\text{N}_2$  and acetylene reduction protocols (7, 10). Each 0.2- $\mu$ m-pore-size membrane was screened under the epifluorescence microscope to verify that the cells had been washed off. Final enrichments were about 130- to 160-fold.  $^{15}\text{N}_2$  experiments were carried out during the summer 2001 cruise with 14 ml of cell concentrate (no headspace left in 14-ml vials) during 24 h and incubated with 9  $\mu$ l of  $^{15}\text{N}_2$  (data from three stations,  $n = 3$  per depth per station). Experiments were ended by filtering the contents of each vial onto a combusted glass fiber filter that was folded and left to dry in an oven at 60°C before being analyzed with a mass spectrometer. During the spring 2002 (Atlantic) and fall 2002 (Pacific) cruises, the diel and depth variations of  $\text{N}_2$  fixation were examined by the acetylene reduction procedure ( $n = 18$  per depth [Atlantic];  $n = 24$  per depth [Pacific]). Experiments were started with injection of acetylene into serum vials (14 ml) which contained 10 ml of cell concentrate; time zero values were registered immediately after. Samples were run for 24 h, and ethylene readings were carried out approximately every 4 to 6 h, preceded by ethylene standard quantifications. Twenty-four-hour  $\text{N}_2$  fixation rates were integrated over the water column depth for the times when nitrogenase activity was observed. Each depth experimental sample ( $n = 3$ ) was left inside a bag that mimicked its corresponding light level in a flowing seawater on-deck incubator. Control assays were carried out in parallel with 0.2- $\mu$ m-pore-size-filtered seawater.

**Cloning and sequencing.** After completion of the 24-h nitrogenase activity experiments, daytime and nighttime samples were filtered onto 0.2- $\mu$ m-pore-size Durapore membrane filters (25-mm diameter; Millipore) for RNA extraction (41) with an RNeasy kit; residual amounts of DNA were removed using an RNase-Free DNase set (Qiagen, Valencia, Calif.). An Access reverse transcriptase PCR kit (Promega, Madison, Wis.) was used to amplify cDNA for *nifH* genes (357-bp fragment) (42). The reverse transcription reaction was carried out for 30 min using 1  $\mu$ M primer *nifH* 3, 28  $\mu$ l of  $\text{H}_2\text{O}$ , 10  $\mu$ l of 5 $\times$  avian myeloblastosis virus buffer, 1  $\mu$ l deoxynucleoside triphosphates (10 mM each), 1  $\mu$ l of avian myeloblastosis virus reverse transcriptase, and 1  $\mu$ l of DNA-free RNA (41). Four liters of prefiltered water was collected onto 0.2- $\mu$ m-pore-size Durapore membrane filters (25-mm diameter; Millipore), and DNA was extracted according to standard protocols (26, 33). Primers (CYA 359F and 781b) that amplify a conserved 16S ribosomal DNA (rDNA) cyanobacterial region (approximately 422-bp fragment) (29) were used in a typical PCR. Purified *nifH* and 16S rDNA products were ligated with the pGEM-T Easy vector system (Promega) (33). Reaction mixtures (50- $\mu$ l final volume) contained the following: 33.5  $\mu$ l of RNA-DNA-free water, 5  $\mu$ l of PCR buffer, 5  $\mu$ l (16S rDNA) or 8  $\mu$ l (*nifH*) of  $\text{MgCl}_2$  (25 mM), 1  $\mu$ l of deoxynucleoside triphosphate mix, 2  $\mu$ l of each primer (12.5  $\mu$ M) (29, 42), 1  $\mu$ l of template (cDNA for *nifH*), and 0.5  $\mu$ l of *Taq* DNA polymerase (Promega). The thermal cycle for amplification of 16S rDNA was as follows: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 35 cycles. The thermal cycle for amplification of *nifH* was as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles. Purified amplified clone products were cycle sequenced with an ABI Prism BigDye kit (Perkin-Elmer, Foster City, Calif.).

Phylogenetic reconstruction analysis using PAUP\*4.0b8 (Sinauer Associates Inc., Sunderland, Mass.) included parsimony bootstrapping (250 replicates, random addition initial trees). Distance- and maximum-likelihood-based analysis (data not shown) gave topologies nearly identical to that of the parsimony

analysis. The resulting 72-taxon *nifH* data set included 217 parsimony-informative characters. The resulting 32-taxon 16S rDNA summarized data set (1) included 110 parsimony-informative characters.

**Cell counts.** Unicellular cyanobacteria cell counts were done on board within an hour of collection, using an epifluorescence compound microscope (Zeiss Axioscope) at 1,000 $\times$ . Seawater filtrates were collected onto 0.2- $\mu$ m-pore-size PCTE membranes (47-mm diameter; Millipore) and placed in microscope slides for observation. Blue and green excitations were used to observe phycoerythrin and chlorophyll *a* epifluorescence signals, respectively (25).

**Nucleotide sequence accession number.** The sequences generated in this study have been deposited in the GenBank database under the indicated accession numbers: tropical North Atlantic *nifH*, AF536983 to AF536986 and AY191972 to AY191976; tropical North Atlantic 16S rDNA, AY191920, AY191921, and AY191923 to AY191942; subtropical North Pacific *nifH*, AY191943 to AY191966, AY191969, and AY191971; subtropical North Pacific 16S rDNA, AY191919 and AY191904 to AY191922.

## RESULTS AND DISCUSSION

**Phylogenetic analysis.** Partial *nifH* sequences of both unicellular cyanobacteria and proteobacteria clustered separately depending on the ocean of origin (Fig. 1). Most of the cDNA *nifH* sequences obtained for the North Atlantic in April 2002 clustered mainly (32 clones out of a 37-clone library) with unicellular cyanobacterial sequences for this same region in August 2001 published previously (16) as well as with the unicellular cyanobacterial diazotrophs *Synechocystis* sp. strain WH8501, which was isolated in 1988 from the tropical North Atlantic (39), and *Cyanothece* strain ATCC 51142, isolated from the Gulf of Mexico (32). The remaining five Atlantic cDNA *nifH* clones clustered with  $\alpha$ -proteobacterium-like sequences. Five unique sequences from the subtropical North Pacific (10 clones out of a 34-clone library) clustered with unicellular cyanobacteria, which had been seen only in the North Pacific in May (44). Most of the Pacific *nifH* cDNA clone sequences (including all of those obtained from 100-m samples from incubations from 1200 to 1800 h for activity) clustered with  $\alpha$ -proteobacteria, and one unique sequence clustered with  $\gamma$ -proteobacteria. We cannot assign the observed nitrogenase activity with certainty to one group of bacteria; nevertheless, we obtained only  $\alpha$ -proteobacterium-type *nifH* transcripts from daytime incubations in the subtropical North Pacific;  $\alpha$ - and  $\gamma$ -proteobacteria and unicellular cyanobacterium-like *nifH* transcripts were obtained during the nighttime incubations (both oceans). The 16S rDNA cyanobacterial sequences from both oceans clustered together and with other proposed  $\text{N}_2$ -fixing unicellular cyanobacteria (Fig. 2) (*Synechocystis* sp. strain WH8501). This field observation is consistent with previous studies that have proposed that  $\text{N}_2$ -fixing unicellular cyanobacteria obtain ATP necessary for  $\text{N}_2$  fixation during the day through C fixation and temporally separate  $\text{O}_2$  production from nitrogenase activity (17, 23, 27, 28, 32, 35, 36, 37). Also, since proteobacteria are heterotrophic, their  $\text{N}_2$  fixation activity is not controlled by the day-night cycles of C fixation. Microanaerobic environments can occur in the surroundings of cells that are respiring, which could provide the environments adequate to carry out  $\text{N}_2$  fixation during the day (19, 30). In any event, the in situ  $\text{N}_2$  fixation rates measured were greater during the nighttime, and in fact, only the subtropical North Pacific showed a small daytime  $\text{N}_2$  fixation activity. Nevertheless, even though the obtained cyanobacterial *nifH* transcripts and 16S rDNA sequences clustered together with those from known  $\text{N}_2$ -fixing unicellular cyanobacteria

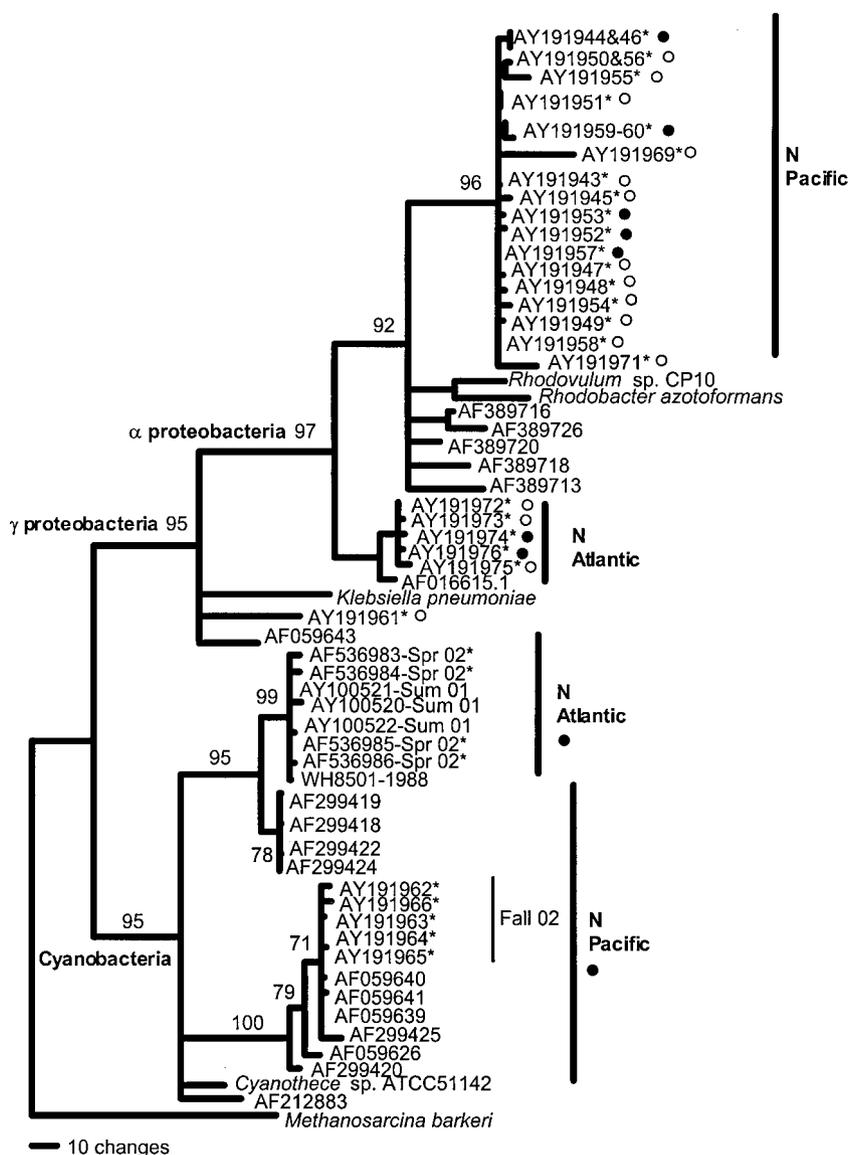


FIG. 1. Phylogenetic reconstruction (maximum parsimony) for *nifH* gene sequences from bacterioplankton in the tropical North Atlantic and subtropical North Pacific oceans. Asterisks indicate sequences obtained as part of this study. Daytime and nighttime transcripts are indicated by open and solid circles, respectively.

(*Synechocystis* sp. strain WH8501 and *Cyanothece* strain ATCC 51142), we cannot assume that unicellular cyanobacteria analyzed in this study were N<sub>2</sub> fixers. In fact, since the 16S rDNA region that we sequenced is only approximately 422 bp in length, it is not yet clear how closely related populations from the Atlantic and Pacific really are.

The differential clustering of unicellular cyanobacteria from the tropical North Atlantic and subtropical North Pacific based on 16S rDNA and *nifH* phylogenies suggests a common ancestry (16S rDNA) and long-term genetic divergence (*nifH*) between populations of diazotrophic unicellular cyanobacteria in the tropical North Atlantic and North Pacific. Further, it is interesting to relate unicellular cyanobacterial *nifH* sequences in the same region of the tropical North Atlantic in 1998 (43), in the summer of 2001 (16), and in the spring of 2002, which

could suggest that these unicellular cyanobacteria may be a permanent component of the spring-summer stratified water column in this area of the tropical oceans.

**In situ rates of N<sub>2</sub> fixation.** We measured unicellular bacterioplankton N<sub>2</sub> fixation in the mixed surface water layer of the tropical North Atlantic in the summer of 2001 (when we observed a strong thermocline and nutricline [http://biology.usc.edu/bc/]) and during spring 2002 (which again showed a stable surface water mixed layer [http://biology.usc.edu/bc/]). Nitrogenase activity was observed only during the night both in the summer of 2001 and in the spring of 2002 (Fig. 3). Integrated water column rates for 10 h of nighttime nitrogenase activity in the tropical North Atlantic averaged 47  $\mu\text{mol of N m}^{-2} \text{ day}^{-1}$  in summer 2001 and 37  $\mu\text{mol of N m}^{-2} \text{ day}^{-1}$  in spring 2002. Rates of N<sub>2</sub> fixation by *Trichodesmium* spp. were measured in

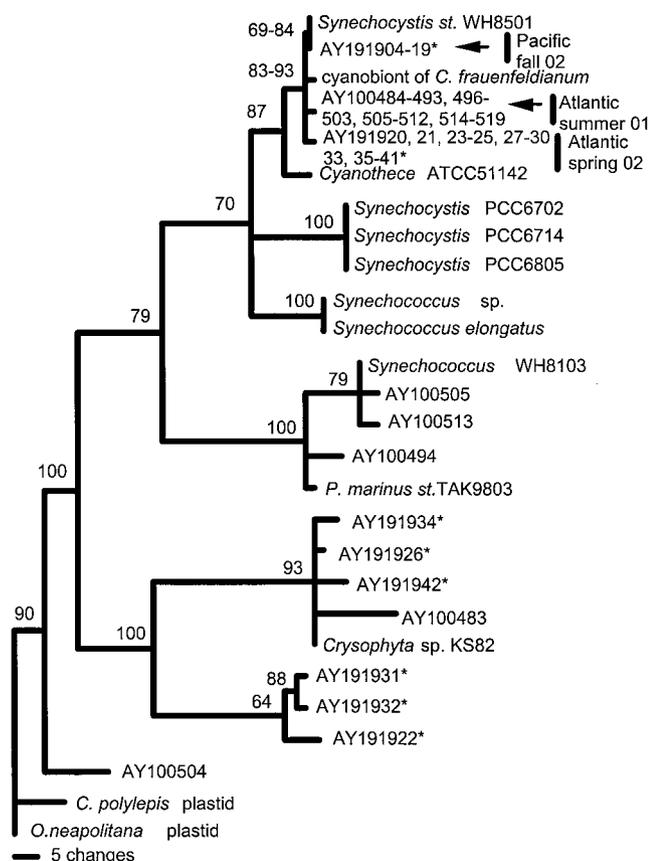


FIG. 2. Summary of phylogenetic reconstruction (maximum parsimony) for 16S rDNA sequences from unicellular cyanobacteria in the tropical North Atlantic and subtropical North Pacific oceans. Asterisks indicate sequences obtained as part of this study.

parallel with our experiments in the tropical North Atlantic during the summer 2001 cruise (D. G. Capone, personal communication). For that cruise, the average rate for 30 stations was  $62 \pm 21$  (standard error)  $\mu\text{mol of N m}^{-2} \text{ day}^{-1}$ . A cruise in the same vicinity in February 2001 found average rates of

$167 \pm 49$  (standard error)  $\mu\text{mol of N m}^{-2} \text{ day}^{-1}$  ( $n = 23$ ) (D. G. Capone, personal communication). Thus, unicellular bacterioplankton  $\text{N}_2$  fixation activity at several stations in the tropical North Atlantic was about equivalent to that of *Trichodesmium* spp. in the summer of 2001 and about 20% of *Trichodesmium* spp.  $\text{N}_2$  fixation activity in the spring of 2002.

During the fall of 2002, in the subtropical North Pacific, we observed  $\text{N}_2$  fixation by unicellular bacterioplankton primarily during the night; measurable daytime nitrogenase activity was also observed (Fig. 3). Integrated water column  $\text{N}_2$  fixation rates for 12 h of nitrogenase activity averaged  $2.2 \mu\text{mol of N m}^{-2} \text{ day}^{-1}$ . The  $\text{N}_2$  fixation rates for the fall in the subtropical North Pacific are only 2% of those previously estimated in the summer of 2000 based on cell abundances of the 3- to 20- $\mu\text{m}$  phytoplankton size fraction (44) and approximately 11% of those estimated from 24-h incubations with  $^{15}\text{N}_2$ -enriched water in November 2000 (14). In the subtropical North Pacific, reported rates of  $\text{N}_2$  fixation by *Trichodesmium* spp. (8) are  $84 \pm 50 \mu\text{mol of N m}^{-2} \text{ day}^{-1}$ ; thus, unicellular bacterioplankton would increase these rates by only 2.6%.

**Unicellular cyanobacterial cell abundances.** Unicellular cyanobacterial abundances were an order of magnitude higher in the Atlantic than in the Pacific, peaking from surface to 25 m in both oceans (Fig. 4); cells were larger in the Pacific (3 to 7  $\mu\text{m}$ ) than in the Atlantic (2.5  $\mu\text{m}$ ). The cell abundances reported here included all unicellular cyanobacteria (2.5  $\mu\text{m}$ , Atlantic; 3 to 7  $\mu\text{m}$ , Pacific), both the potential  $\text{N}_2$  fixers and the non- $\text{N}_2$  fixers. The fluorochrome system used (24) does not work on unicellular cyanobacteria since these have a similar fluorescent signal with or without a positive in situ immunolocalization of cells expressing nitrogenase.

Previous studies have shown that total bacterial cell numbers in marine oligotrophic environments tend to be balanced over time by bacterivores and viruses and are comparable between the tropical North Atlantic and the subtropical North Pacific (12).

Our data suggest a trend of increased unicellular cyanobacterial abundance and  $\text{N}_2$  fixation rates in the tropical North Atlantic compared to those in the subtropical North Pacific (Fig. 3 and 4); this is an interesting observation, since it has

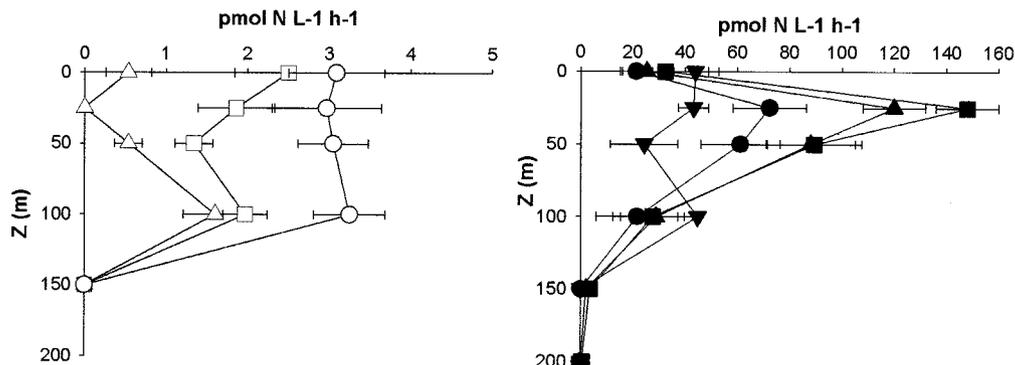


FIG. 3. Distribution of average rates of in situ  $\text{N}_2$  fixation (picomoles of N liter $^{-1}$  hour $^{-1}$ ) by bacterioplankton by depth for the tropical North Atlantic (solid symbols) and subtropical North Pacific (open symbols).  $^{15}\text{N}_2$  24-h incubations in summer 2001 are shown by  $\blacktriangledown$ . Symbols indicate periods of incubations and reading time in gas chromatography:  $\blacktriangle$ , 1800 to 2200 h;  $\blacksquare$ , 2200 to 0200 h;  $\bullet$ , 0200 to 0600 h for spring 2002, tropical North Atlantic;  $\triangle$ , 1200 to 1800 h;  $\square$ , 1800 to 2400 h; and  $\circ$ , 2400 to 0600 h for fall 2002, subtropical North Pacific. Error bars represent standard errors. Only readings where nitrogenase activity was detected are shown.

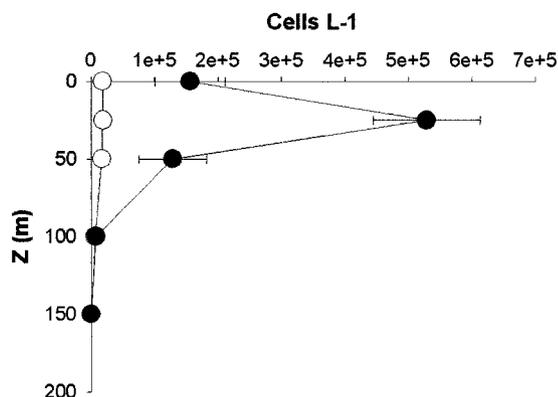


FIG. 4. Distribution of average cell counts of unicellular cyanobacteria by depth, in the tropical North Atlantic (solid symbols;  $\sim 2.5 \mu\text{m}$ ) and subtropical North Pacific (open symbols; 3 and 7  $\mu\text{m}$ ) oceans. Error bars represent standard errors.

been suggested that unicellular diazotrophic cyanobacteria might be major diazotrophs in oligotrophic marine ecosystems (44).

We hypothesize that differences in N<sub>2</sub> fixation rates of unicellular bacterioplankton and cell abundances of coccoid cyanobacteria ( $>2.0 \mu\text{m}$ ) observed between oceanic basins are in part due to the larger eolian Fe flux to the North Atlantic ( $0.2$  to  $0.8 \mu\text{mol of Fe m}^{-2} \text{ day}^{-1}$ ) (40) and to the high Fe/P ratio present in this area, which have been suggested (4, 5, 38) to favor N<sub>2</sub> fixation in comparison to the North Pacific Ocean (20) ( $0.08$  to  $0.16 \mu\text{mol of Fe m}^{-2} \text{ day}^{-1}$ ) (40). Nevertheless, to date, Fe cell quotas for proteobacterial and unicellular cyanobacterial diazotrophs are needed, as are field measurements of the Fe/C ratio of proteobacterial and unicellular cyanobacterial diazotrophs (22). The tropical North Pacific (20, 21) has low light levels and deeper mixed layers that show a pattern of diminished N<sub>2</sub> fixation rates (14). In the summer, the thermocline reaches its maximum in tropical oceans, bringing stability and increasing surface water temperatures, which could have a direct effect on the diazotrophic activity of unicellular bacterioplankton.

The magnitude of N<sub>2</sub> fixation by small unicellular bacterioplankton, the stability of their populations over time, and the differences found between the tropical North Atlantic and the subtropical North Pacific (3, 5, 8, 13, 18, 20, 21, 34, 38, 40) suggest that the marine N cycle of these spatially extensive ecosystems has more components, and is more complex, than previously thought.

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