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Growth kinetics of marine unicellular N_2-fixing cyanobacterial isolates in continuous culture in relation to phosphorus and temperature

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ABSTRACT: Unicellular N_2-fixing cyanobacteria from tropical marine oligotrophic environments have been proposed to be major contributors to the global N cycle but still remain poorly characterized. These organisms are likely to be limited by phosphorus availability *in situ*. The aim of this study was to identify growth kinetics of isolates from the tropical North Atlantic and subtropical North Pacific in relation to phosphorus and temperature in continuous cultures. Cells from the Atlantic measured 2.5 µm in diameter (A-2.5). Genetically identical isolates from the Pacific showed 2 diameters depending on P-media concentrations (small: 3 µm, 1 µM PO_4 [P-3] and large: 7 µm, 4 µM PO_4 [P-7]). All 3 isolates were highly stenothermal, and optimal growth temperatures ranged between 26 and 30°C. Small cells (A-2.5 and P-3) had lower half-saturation constants (K_s) for PO_4 than large cells (P-7) (0.06 to 0.21 µM vs. 0.20 to 0.25 µM). Maximum growth rates and N:P ratios increased with temperature for all isolates; N:P ratios were close to Redfield ratios (N:P = 16) when isolates approached maximum growth rates. N_2-fixation activity did not vary between growth rates, but did increase with temperature; rates were consistently lower than previously published rates for the same isolates under non-P-limiting conditions. From these studies, we conclude that both Atlantic and Pacific unicellular cyanobacteria that have the capacity to fix N_2 have a limited temperature range for growth and that smaller sized isolates could be better adapted for conditions of phosphorus limitation.

KEY WORDS: Growth kinetics · Phosphorus · Temperature · Unicellular cyanobacteria · N_2 fixation

INTRODUCTION

The availability of reduced forms of nitrogen (N) in nutrient-depleted areas on the ocean surface often limits primary productivity, and planktonic N_2-fixing microorganisms appear to be at an advantage in these environments (Karl et al. 2002). N_2-fixation events in marine surface waters represent the entry of 'new' N to the system, and are thought to be major controls on the rate of export of organic matter to deeper waters (Dugdale et al. 1961, Dugdale & Goering 1967, Mague et al. 1974, Carpenter & Romans 1991). Although it has been hypothesized that competition between N_2-fixing and non-N_2-fixing phytoplankton controls nitrate levels, phosphorus (P) is proposed to be the ultimate limiting nutrient regulating total oceanic productivity (Tyrrell 1999).

Large, colonial cyanobacteria of the genus *Trichodesmium* spp. are known to be the major N_2-fixing organisms in the tropical and subtropical oceans (Carpenter & Capone 1992, Capone et al. 1997, Capone & Carpenter 1999). Diverse studies of their population dynamics for limiting nutrient uptake have been carried out in the field and in the laboratory (Sanudo-Wilhelmy et al. 2001, Mulholland et al. 2002, Kustka et al. 2003), which suggest that their diazotrophic

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capacity can be limited by both P and Fe availability. Recently, unicellular cyanobacteria have been proposed to be major N_2-fixers in the tropical North Atlantic and subtropical North Pacific Oceans (Zehr et al. 2001, Falcón et al. 2002, 2004). Nevertheless, there are no reports that give information on the nutrient uptake kinetics of either P or Fe by unicellular cyanobacterial N_2-fixers. We carried out continuous culture (Herbert et al. 1956, Kubitschek 1970) experiments in order to understand the growth kinetics in relation to P and temperature on N_2-fixing unicellular cyanobacteria isolated from the tropical North Atlantic and subtropical North Pacific oceans.

MATERIALS AND METHODS

N_2-fixing unicellular cyanobacteria were isolated from the tropical North Atlantic (12° 18.70′ N, 56° 02.79′ W) and subtropical North Pacific (22° 45.31′ N, 158° 0.61′ W) following the protocol of Waterbury & Willey (1988), with modifications. Cells were first grown in batch cultures in artificial medium lacking reduced forms of nitrogen (Waterbury & Willey 1988) under low incident light intensity (100 µE m^{-2} s^{-1}), with diel periodicity on a 12 h dark:12 h light regime. Axenic unicyanobacterial isolates were obtained by separating colonies through plate streaking in Noble agar and SO medium plates. Also, in order to avoid contaminating algae, we used cyclohexamide (details in Falcón 2003). To check that cultures were axenic, we examined for the presence of contaminating bacteria using DAPI (Sherr et al. 2001) on a regular basis. One isolate (measuring 2.5 µm) was obtained from the Atlantic (A-2.5), and 2 genetically identical isolates (measuring 3 and 7 µm) were obtained from the Pacific (P-3 and P-7) (Falcón 2003). When grown under batch culture conditions at an initial P concentration of 112 µM, Pacific isolate cells measured 7 µm. We observed that if these isolates were not transferred within 3 wk after reaching a steady state of growth, they decreased their diameter to 3 µm, presumably due to nutrient limitation. We calculated the limiting amount of P required for growth by cells measuring 2.5, 3 and 7 µm in diameter following particulate organic nitrogen (PON) quantification of cells with the protocol for mass spectrometry of Dugdale & Wilkerson (1986), and by converting N units to P units, applying a Redfield ratio of 16:1 N:P. We then prepared solutions for media with different P (K_2PO_4) concentrations: 1 µM for 2.5- and 3-µm cells and 4 µM for 7-µm cells. We observed that cells grown under these P concentrations in batch cultures and transferred every 3 wk to fresh media retained their diameter. In order to avoid P contamination of the media (Button et al. 1973), we prepared artificial seawater following the recipe of Chen et al. (1996) and added all of the minerals in the exact concentrations described previously for SO media (Waterbury & Willey 1988), except for K_2PO_4. Cells were grown in batch culture under P limitation for at least 3 transfers, which added up to 9 wk of growth and approximately 30 generations before initiating continuous growth experiments at different temperatures.

Continuous culture growth chambers were designed following the descriptions of Brewer & Goldman (1976), Goldman & McCarthy (1978) and Goldman et al. (1981), with modifications. The chambers had a volume of 500 ml, with a surrounding water jacket with influent and effluent ports for circulating water, and a cell suspension overflow port. Media flow was regulated with a Cole Parmer, Master Flex L/S peristaltic pump, and temperature was controlled with a Thermo Haake DC10 circulating pump, connected to the influent and effluent ports of the water jacket. Silicon stoppers were used as lids and had openings for a thermometer, air and media tubing, and for Pasteur pipettes, which served for gas exchange. Mixture of the cultures was maintained with an air curtain at the bottom of the chambers and with small magnetic stirrers.

Experiments started with addition of cells from unicyanobacterial axenic batch cultures grown under P limitation. Cell abundance increased until it reached a point were no significant changes in cell density were observed, and, once this remained for over 3 d without oscillating, we turned the peristaltic pump on to the lowest possible dilution rate (0.1 d^{-1} = 50 ml d^{-1} media flow rate). Abundance was monitored daily using a hemocytometer with a Neubauer ruling and an epifluorescence microscope (Zeiss, Axioscope). We waited, for at least 5 d after experiments at a certain dilution had started and no significant change in cell abundance was observed, before we started the 24-h N_2-fixation experiment and measured dissolved inorganic phosphorus (DIP) concentrations. We then changed the dilution rate to 0.3, 0.7, 1 d^{-1} and so on until cell abundance collapsed and reached washout. In order to obtain information on the optimal temperature for growth of these isolates, we started experiments at 29°C, and, once cultures reached washout, we started a new experiment 1°C lower and continued until we reached a temperature at which no growth was observed under chemostat conditions. The same was done starting at 29°C and increasing the temperature by 1°C at a time.

DIP was measured using a Bran and Luebbe Autoanalyzer II at each dilution rate. Calculation of half-saturation constants (K_s) and maximum growth rates (μ_{max}) were carried out with Splus software using a non-linear, least-squares, curve-fitting method follow-

ing the equation of Monod (1942). Even though we did not keep the cultures growing for at least 5 generations at each dilution rate (Button et al. 1973), we started experiments with populations of cells that had previously been adapted to limiting-P conditions and, before taking any measurements, waited at each dilution rate until we observed that cell abundance was not significantly different than the abundance at lower dilution rates. Dilution rates (fractional rate of replacement of nutrient medium) were calculated as the rate of flow of media over the total volume of the growth vessels.

N_2 fixation was assayed following the protocol of Capone (1993) for acetylene reduction using a Shimadzu, GC8A gas chromatograph and a CR3A integrator. Measurements were carried out on cell populations at each dilution rate after we had observed for several days that cell abundance did not vary significantly from the abundance at a lower dilution rate. Experiments to quantify nitrogenase activity started at the beginning of the dark phase by injection of acetylene into samples in 7 ml serum vials; readings were taken every 3 h. Rates of N_2 fixation were measured during the dark phase, since these isolates have been shown to fix N_2 during the night time only due to the O_2 inhibition of nitrogenase caused by photosynthetic O_2 production (Postgate 1998). The dark:light cycle of the cells was inverted (dark phase: 09:00 to 21:00 h), and chemostats were kept in a dark room to avoid stray room light.

RESULTS

The isolate from the Atlantic (A-2.5) had maximum abundance between 12 and 31×10^6 cells ml^{-1} for dilution rates ranging from 0.1 to 0.7 d^{-1} at temperatures of 26 to 30°C.

The small Pacific isolate (P-2.5) had maximum abundance between 7 and 18×10^6 cells ml^{-1} for the dilution rates and temperatures described above. The Pacific large isolate (P-7) reached maximum abundance at the lower dilution rates of 0.1 and 0.3 d^{-1} ranging from 2 to 4×10^5 cells ml^{-1} at 28 and 29°C.

Maximum growth rates attained by each cell isolate are shown in Fig. 1, and the half-saturation constants for P (K_s) are given in Table 1. The A-2.5 and P-3 isolates showed the highest maximum growth rates between 28 and 30°C, which appeared to be their optimal range of temperature for growth according to Moisan et al. (2002). The P-7 isolate had lower maximum growth rates than the smaller isolates, and their optimal temperature was between 28 and 29°C.

Small unicellular cyanobacteria (A-2.5 and P-3) had lower K_s values for P than the larger strain (P-7) (Table 1). We observed an increase in K_s with temperature for all isolates. We noticed that the K_s for the P obtained were within the range of concentration of total dissolved phosphorus found in nature (Wu et al. 2000).

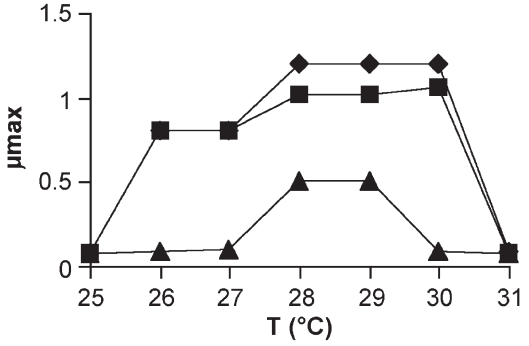

Fig. 1. Unicellular cyanobacterial isolates: maximum growth rates (μ_{max}) with temperature. ◆: A-2.5; ■: P-3; ▲: P-7

Further, we calculated the amount of P in the cells at the dilution rates before the populations reached their maximum growth rates (difference between the concentration of P in the media and the remaining concentration of P measured as DIP). We then calculated the amount of N fixed per cell from our integrated N_2-fixation rates at those same dilution rates and came up with an estimate of the cellular N:P ratio (Fig. 2). The general tendency was an increase in the N:P value with temperature; cells had lower N:P than Redfield (1958) ratios at the 0.1 and 0.3 d^{-1} dilution rates, but approached Redfield values at the 0.7 d^{-1} dilution rate. The A-2.5 isolate surpassed Redfield N:P ratios at 0.7 d^{-1} for temperatures above 27°C. Both the A-2.5 and P-3 isolates showed a decrease in N:P above 29°C for 0.3 and 0.7 d^{-1} dilution rates.

N_2-fixation rates for all isolates peaked during the third time point, which corresponds to activity between midnight and 03:00 h. The A-2.5 isolate had signifi-

Table 1. Half-saturation constant (K_s) under phosphorus limitation for different temperatures of unicellular cyanobacterial isolates A-2.5, P-3 and P-7. [a]Total dissolved phosphorus (TDP) in the tropical North Atlantic and subtropical North Pacific (Wu et al. 2000)

Temp. (°C)	K_s (nM PO_4)		
	Atlantic TDP (75 ± 42 nM)[a]	Pacific TDP (222 ± 14 nM)[a]	
	A-2.5	P-3	P-7
25	–	–	–
26	60 (0.021)	60 (0.009)	–
27	70 (0.017)	70 (0.005)	–
28	80 (0.005)	130 (0.040)	200 (0.035)
29	80 (0.005)	170 (0.057)	250 (0.037)
30	90 (0.029)	210 (0.045)	–
31	–	–	–

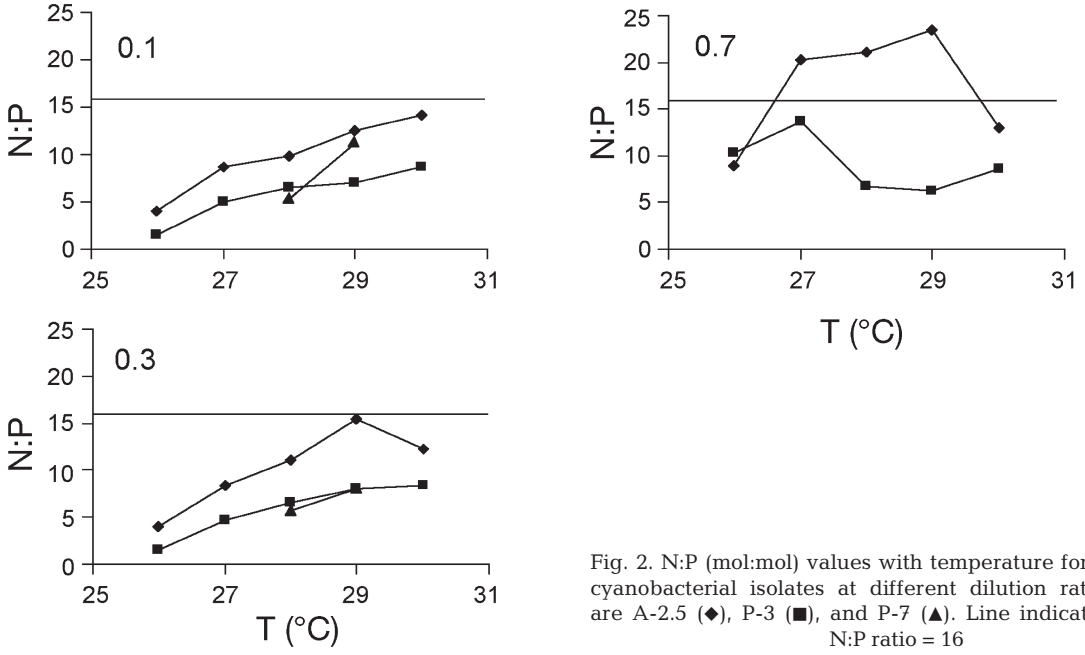

Fig. 2. N:P (mol:mol) values with temperature for unicellular cyanobacterial isolates at different dilution rates. Isolates are A-2.5 (◆), P-3 (■), and P-7 (▲). Line indicates Redfield N:P ratio = 16

cantly higher rates of N_2 fixation, which were maximal at the lower dilution rates for the higher temperatures (28 to 30°C). All of the isolates had the smallest N_2-fixation activity at the lowest temperatures. The P-7 isolate had the lowest N_2-fixation rates of all isolates (Figs. 3 to 5).

DISCUSSION

Our results show the importance of attempting to measure maximum growth rates of unicellular planktonic cyanobacteria in relation to temperature. Water temperature in the subtropical North Pacific in proximity to Stn ALOHA (data from 1988 to 2001, Dore et al. 2002) is above 25°C between June and November, when the deepest mixed-layer depths are found. The rest of the year, average surface water temperature is lower than 25°C. This implies that the growth of both small- and large-sized unicellular cyanobacteria is stressed by low temperature for at least half of the year. The question remains open as to whether these unicellular N_2-fixing cyanobacteria show shifts in distribution towards warmer waters throughout the year. Previous studies have mentioned the presence of genetically similar cells in this area during May and June (Zehr et al. 2001), but more data are needed to see if they are present around Stn ALOHA when temperatures are lower than 25°C. Li (1980) suggested that organisms in nature are commonly observed to grow at temperatures lower than those in their optimal range.

On the other hand, the temperature range for surface waters in the region of the Atlantic where the isolates were obtained is always above 25°C, and reaches a maximum of 29°C. It is striking that both Pacific and Atlantic isolates were unable to grow above 30°C, since even though the subtropical North Pacific reaches maximum temperatures of 27°C the tropical Atlantic, on the other hand, has maximum temperatures of 29°C or slightly higher. Global warming trends could easily affect populations of potentially major N_2-fixing unicellular cyanobacteria in the tropical North Atlantic (Gillet et al. 2003).

It is apparent that increases in water temperature lead to enhancement of unicellular N_2-fixing cyanobacterial growth rates and K_s. It appears as though unicellular N_2-fixing cyanobacteria are limited to waters with temperatures above 20°C, as is the major cyanobacterium group *Trichodesmium* spp. The upper and lower limits of temperature to sustain growth have to be taken into account when predicting population dynamics to understand their patterns of distribution (Goldman & Carpenter 1974), especially for the populations of bacterioplankton that are found in oceanic regions exposed to possible temperature changes by global warming trends (Trenberth et al. 2002, Gillet et al. 2003).

The concentration of dissolved inorganic phosphorus found in the subtropical North Pacific surface waters is higher than in the tropical North Atlantic (222 ± 14 nM vs. 75 ± 42 nM) (Wu et al. 2000). The subtropical North Pacific, however, is proposed to shift to P limitation after N_2-fixation events (Karl et al. 1997, Wu et al. 2000, Dhyrman et al. 2002). Wu et al. (2000) and Karl et al. (2002) have also proposed that N_2 fixation in the North Pacific is ultimately limited by iron (Fe). The central

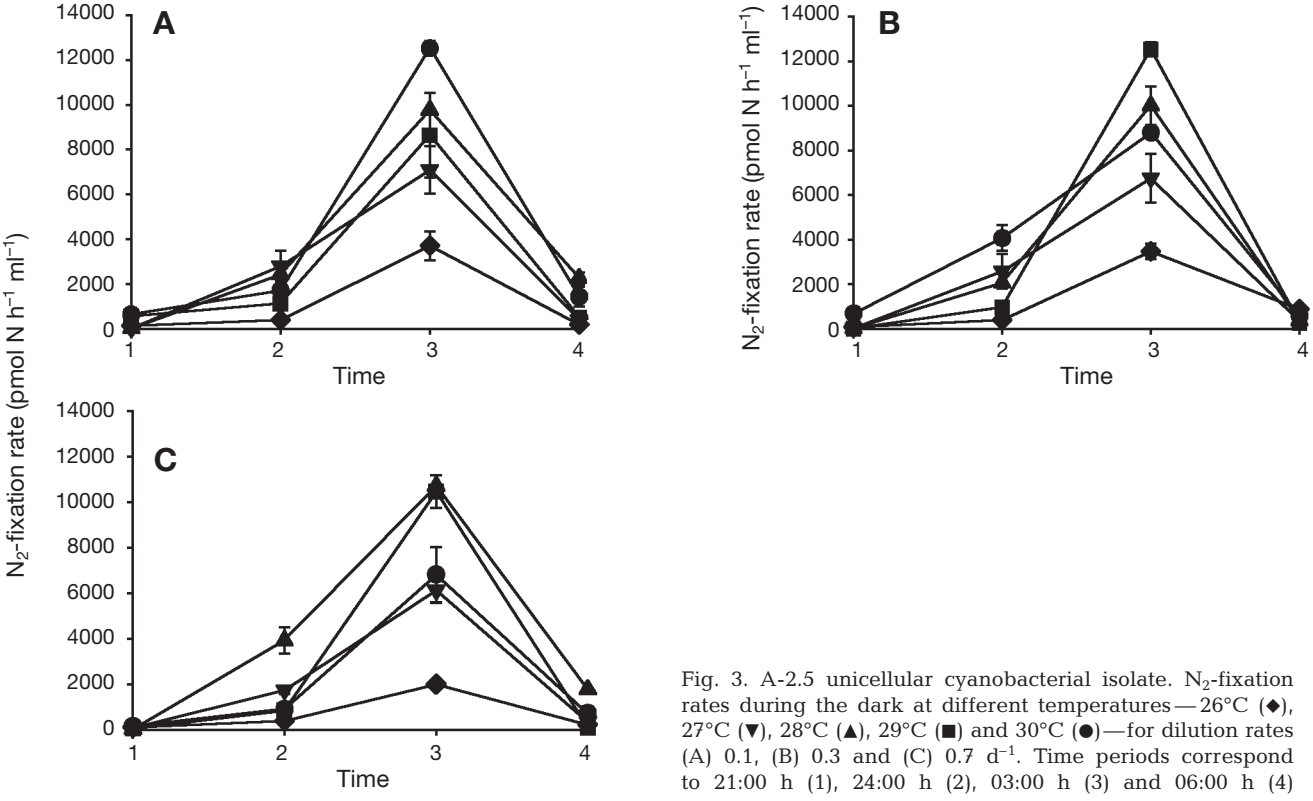

Fig. 3. A-2.5 unicellular cyanobacterial isolate. N_2-fixation rates during the dark at different temperatures — 26°C (◆), 27°C (▼), 28°C (▲), 29°C (■) and 30°C (●) — for dilution rates (A) 0.1, (B) 0.3 and (C) 0.7 d^{-1}. Time periods correspond to 21:00 h (1), 24:00 h (2), 03:00 h (3) and 06:00 h (4)

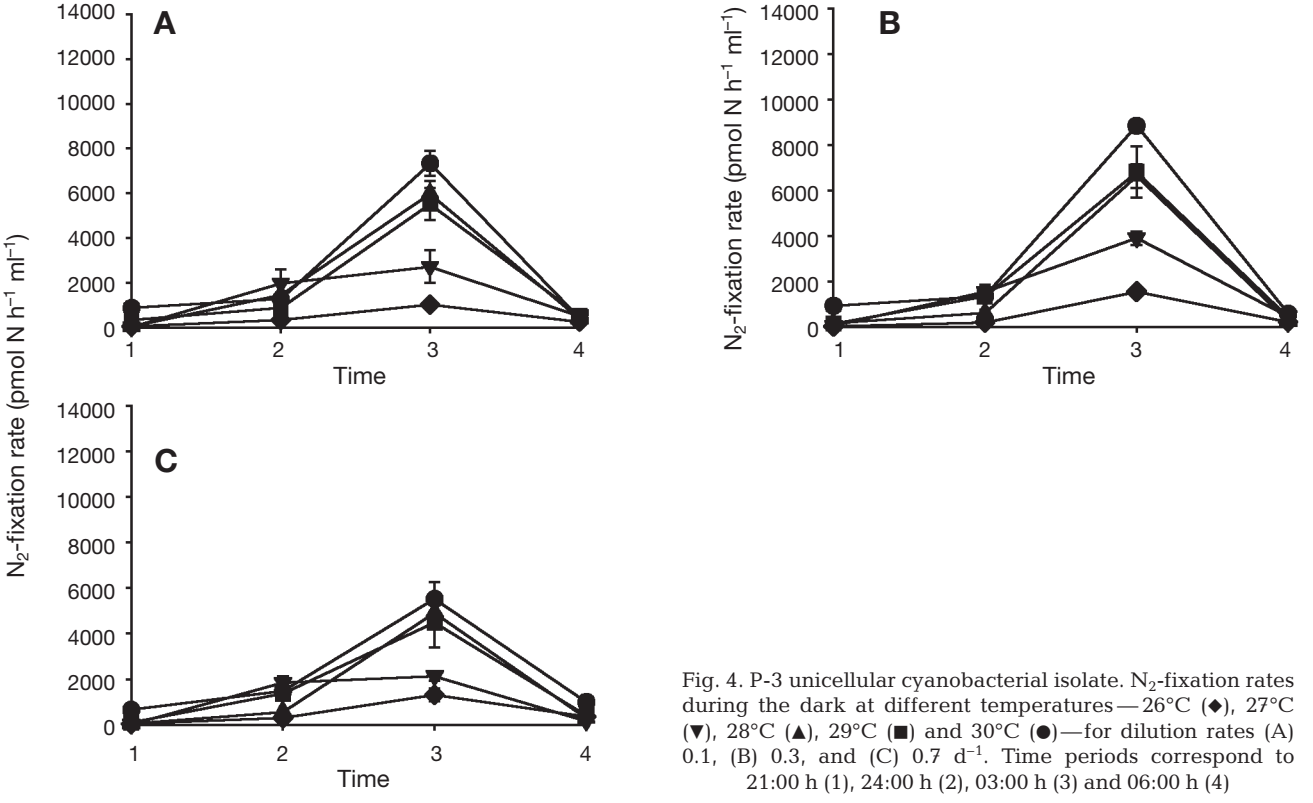

Fig. 4. P-3 unicellular cyanobacterial isolate. N_2-fixation rates during the dark at different temperatures — 26°C (◆), 27°C (▼), 28°C (▲), 29°C (■) and 30°C (●) — for dilution rates (A) 0.1, (B) 0.3, and (C) 0.7 d^{-1}. Time periods correspond to 21:00 h (1), 24:00 h (2), 03:00 h (3) and 06:00 h (4)

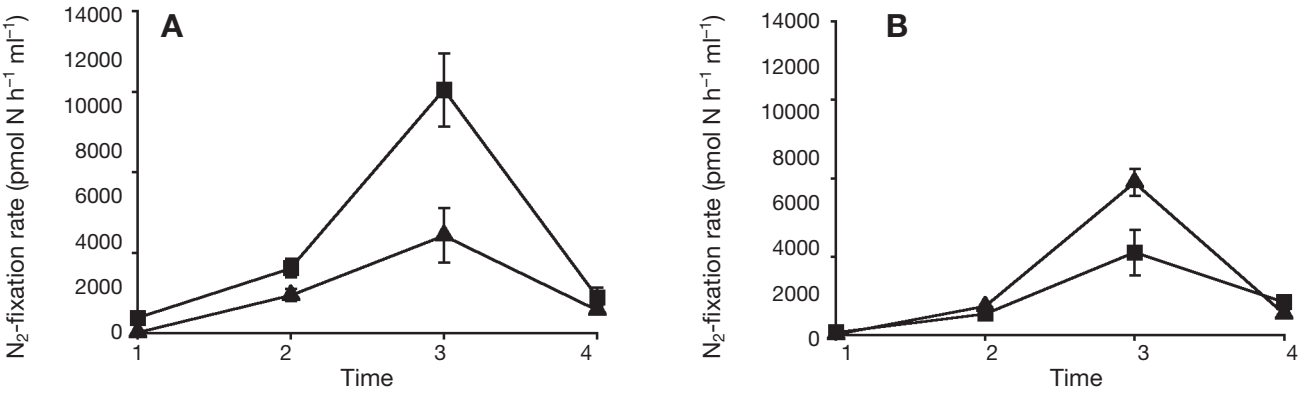

Fig. 5. P-7 unicellular cyanobacterial isolate. N_2-fixation rates during the dark at different temperatures—28°C (▲) and 29°C (■)—for dilution rates (A) 0.1 and (B) 0.3 d^{-1}. Time periods correspond to 21:00 h (1), 24:00 h (2), 03:00 h (3) and 06:00 h (4)

Atlantic Ocean has relatively high rates of planktonic N_2 fixation and is proposed to be limited by P availability once the cellular quotas of Fe for N_2-fixers have been met (Wu et al. 2000, Sanudo-Wilhelmy et al. 2001).

K_s values for P in the form of orthophosphate, which has been shown to be the most bioavailable form of P in the subtropical North Pacific (Björkman & Karl 1994, Björkman et al. 2000), were lower for the Atlantic isolate as compared with both of the Pacific isolates. This is consistent with results that show that the Atlantic is more severely limited in P than the Pacific (Wu et al. 2000, Sanudo-Wilhelmy et al. 2001, Dhyrman et al. 2002). It appears that unicellular N_2-fixing cyanobacteria isolated from the tropical North Atlantic and subtropical North Pacific Oceans have adapted to different P concentrations. P-3 cells had similar K_s values to those from A-2.5 at the lower temperatures; thus, it appears as if smaller cells have adapted to P-limiting conditions (O'Brien 1974, Smith & Kalff 1982).

Our results indicate that N:P assimilation values approach Redfield (1958) ratios when cells are at their maximum growth rate. Nevertheless, the N:P ratios reported here could have resulted from an increase in the cellular P storage of the isolates growing in continuous culture. More experiments are needed in order to confirm the N:P ratios obtained here. Interestingly, Goldman et al. (1979) also only observed Redfield ratios in chemostat-grown phytoplankton at maximal growth rates.

N_2-fixation rates of the isolates studied here were obtained previously from batch cultures, which were grown under non-limiting P conditions at 29°C and were at least 1 order of magnitude higher for all unicellular cyanobacterial isolates (Falcón et al. 2002) when compared to the rates measured under chemostat P-limited conditions. The low N_2-fixation rates in chemostats could be due to P limitation.

Analysis of field samples has to be carried out in order to better comprehend the nutrient uptake strategies of these potentially important N_2-fixers. More information is needed to understand the patterns of size change, which we propose to be an adaptation to P-limiting conditions for the cells in the subtropical North Pacific. To our knowledge, this is the first report on P nutrient kinetics on marine unicellular N_2-fixing cyanobacteria, as well as on temperature control on growth. These organisms can be major N_2-fixers in the oligotrophic oceans (Zehr et al. 2001, Falcón et al. 2002), thus influencing marine biogeochemical cycles.

The experiments reported here were carried out under non-limiting Fe conditions; Fe will limit N_2 fixation since it is an essential component of the nitrogenase enzymatic complex. The studies that propose P to be the limiting element in the Atlantic state that this is the case once N_2-fixers have met their cell quotas for Fe (Sanudo-Wilhelmy et al. 2001). Thus, it is a combination of a series of factors, including P and Fe availability, which will eventually control N_2-fixation rates in oligotrophic marine ecosystems. Further studies are needed that analyze Fe K_s and maximum growth rates as a function of temperature under Fe and P limitation for marine N_2-fixing cyanobacteria.

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