

Iron limits the cell division rate of *Prochlorococcus* in the eastern equatorial Pacific

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Abstract

Prochlorococcus, a small unicellular cyanobacterium, is an important member of the phytoplankton community in the eastern equatorial Pacific. When these waters were enriched with iron during IronEx II, the chlorophyll per cell and cell size of *Prochlorococcus* increased, implying that they were iron limited. The extent of this limitation was unclear, however, and the number of *Prochlorococcus* remained constant. To examine whether cell division rates were stimulated significantly by iron, we used a cell cycle analysis approach to measure them in and out of the Fe-enriched patch and in Fe-enriched bottles. The cell division rate increased from 0.6 to 1.1 d⁻¹ over 6 d of exposure to the elevated iron concentrations in the patch. Cells incubated in bottles with additional iron had rates of 1.4 d⁻¹ or two doublings per day. *Prochlorococcus* mortality rates, measured independently, nearly doubled after the addition of iron. This matched the increase in the cell division rate and maintained a relatively constant population size. Thus the cell division rates of even the smallest phytoplankton in the equatorial Pacific are significantly iron limited, but biomass is constrained by both iron limitation and microzooplankton grazing. The differential response of individual phytoplankton groups to the addition of iron during IronEx II was at least partially a result of differential mortality rates over the time course of the experiment. How the community would respond to sustained fertilization, however, is not obvious.

Nitrate and phosphate concentrations are persistently high in the euphotic zone of the equatorial Pacific, and phytoplankton biomass and productivity are lower than expected based on nutrient availability (Cullen 1991; Frost and Franzen 1992). The phytoplankton community is dominated by small picoplankton (Chavez 1989), which have a large surface area to volume ratio and are less likely to be diffusion limited by either nutrients or trace metals than larger cells (Morel et al. 1991a). Measured cell division rates are often 0.5 d⁻¹ or higher, both for the phytoplankton community as a whole (Barber and Chavez 1991; Chavez et al. 1991; Cullen 1991; Cullen et al. 1992; Landry et al. 1995; Verity et al. 1996; Latasa et al. 1997; Lindley and Barber 1998) and for the small cyanobacterium *Prochlorococcus* (DuRand 1995; Landry et al. 1995; Vaultot et al. 1995; Binder et al. 1996; Latasa et al. 1997; Liu et al. 1997; Vaultot and Dom-

inique 1999). These are relatively high values for a picoplankton-dominated community (Chisholm 1992; Cullen et al. 1992), which suggests that these cells are growing at near maximal growth rates. Estimated ratios of new production to total primary production are low (McCarthy et al. 1996), which indicates that most of this growth is fueled by regenerated nutrients, which are produced by the intensive grazing that keeps the picoplankton community in check (Wheeler and Kokkinakis 1990; Chavez et al. 1991; Cullen et al. 1992; Frost and Franzen 1992; Price et al. 1994; Landry et al. 1997; Loukos et al. 1997).

Why is the equatorial Pacific dominated by small cells growing on regenerated nutrients, when the persistently high concentrations of nitrate and phosphate could support new production and the growth of larger phytoplankton? The iron hypothesis explains these observations by maintaining that phytoplankton productivity and biomass are limited by iron (Martin et al. 1991; Coale et al. 1996b). Indeed, ambient iron levels are low in high-nutrient low-chlorophyll (HNLC) areas (Martin et al. 1991; Coale et al. 1996b), and the addition of iron to incubation bottles has been shown to increase net growth rates, chlorophyll yields, nitrate uptake, and nutrient consumption, particularly of the larger cells (Chavez et al. 1991; Martin et al. 1991; Price et al. 1991, 1994; Takeda and Obata 1995; Fitzwater et al. 1996). The ecumenical hypothesis (Morel et al. 1991b; Price et al. 1994; Cullen 1995) and the recent synthesis of data from the equatorial Pacific by Landry et al. (1997) extend this explanation by invoking both iron limitation and grazing to explain the HNLC regions. This hypothesis, in its broadest form, states that the cell division rates of the small phytoplankton that dominate the indigenous community are relatively fast and

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are "not strongly" limited by iron (not to the extent that μ is $<1/2 \mu_{\max}$; Cullen 1995). These cells are dependent on recycled nutrients (Price et al. 1994; Landry et al. 1997), and any increases in number following iron addition are rapidly cropped by quickly growing microzooplankton grazers (Banse 1982; Landry et al. 1997). In contrast, large cells with relatively small surface area to volume ratios are at a competitive disadvantage for NH_4^+ and Fe assimilation because of diffusion limitation (Morel et al. 1991a) and are iron limited. Therefore, transient iron enrichment significantly stimulates diatoms, which bloom because their mesozooplankton grazers cannot respond quickly enough to prevent an increase in cell numbers (Price et al. 1994; Coale et al. 1996b; Cavender-Bares et al. 1999).

Results from two in situ iron-fertilization experiments have confirmed the importance of iron limitation in the equatorial Pacific, at least at these sites. Addition of approximately 4 nM iron during IronEx I resulted in a threefold increase in chlorophyll *a* and productivity (Martin et al. 1994). The response during IronEx II, in which three pulses of iron were added, was even greater. Total chlorophyll increased at least 12-fold from 0.2 to 2.6 $\mu\text{g L}^{-1}$ 6 d after the iron addition (Cavender-Bares et al. 1999) and in some parts of the +Fe patch increased as much as 27-fold (Coale et al. 1996b). At the same time, the nitrate concentration decreased by approximately 5 μM (Coale et al. 1996b).

A major portion of the biological signal in the IronEx experiments was attributable to large phytoplankton, especially diatoms (Chavez et al. 1991; Price et al. 1994; Takeda and Obata 1995; Fitzwater et al. 1996; Zettler et al. 1996). During IronEx II, chlorophyll *a* in the $>10 \mu\text{m}$ fraction increased 60-fold, and the bulk diatom pigment concentration and pennate diatom abundance increased significantly (Coale et al. 1996b; Cavender-Bares et al. 1999). During the first 4 d after iron addition, pennate diatoms had a net growth rate (i.e., measured increases in cell number) of 1.0 d^{-1} (Cavender-Bares et al. 1999). Since the grazing rate of the mesozooplankton did not decrease, the cell division rate of the diatoms must have increased dramatically (Coale et al. 1996b). Finally, cells $>5 \mu\text{M}$ accounted for 85 to 98% of the total biomass specific nitrate uptake, which increased 5- to 7-fold as a result of iron fertilization (Coale et al. 1996b).

There are also some indications that the cell division rates of the picoplankton in the equatorial Pacific might be significantly iron limited in spite of their small size and relatively high growth rates. *Synechococcus*, a close relative of *Prochlorococcus*, has a relatively high demand for iron (Brand 1991). Moreover, the quantum yield of the total phytoplankton community, which is dominated by picoplankton, was low in this region because of iron limitation (Greene et al. 1994; Kolber et al. 1994; Lindley et al. 1995; Behrenfeld et al. 1996; Kudela and Chavez 1996), and models predict that an increase in iron supply would cause an increase in productivity (and by inference cell division rate; Lindley et al. 1995; Chai et al. 1996). In addition, Price et al. (1994) concluded that the indigenous (picoplankton-dominated) population in the equatorial Pacific is iron stressed because the short-term iron uptake rates, normalized to both chlorophyll *a* and particulate organic nitrogen, are rapid and comparable to rates seen in iron-limited cultures in the lab-

oratory. Finally, dilution series (Landry et al. 1995) carried out during IronEx II showed that the division rate of cells in the $<5 \mu\text{M}$ fraction doubled in the +Fe patch (Coale et al. 1996b).

In order to assess the degree of iron limitation of the indigenous picoplankton community during IronEx II, we focused our analysis on *Prochlorococcus*. As the smallest and most abundant component of the phytoplankton, it is likely to be the least iron-stressed member of the community. We observed that *Prochlorococcus* cell volume and chlorophyll *a* per cell increased after the addition of iron during IronEx II, but cell numbers remained constant over the course of the experiment (Cavender-Bares et al. 1999). There are two possible explanations for this; (1) either *Prochlorococcus* was slightly iron stressed, but already growing close to its maximal rate so the addition of iron did not have a significant effect on the cell division rate, or (2) cell division rate did increase significantly in response to the Fe additions, but the grazing rate increased commensurately, keeping the population levels constant. The latter is possible because the microheterotroph predators of small picoplankton can grow as quickly as, or more quickly than, their prey (Banse 1982). Without independent measures of cell division rate and grazing rate, it is impossible to distinguish between the two possibilities outlined above.

To this end, cell division rates of *Prochlorococcus* were calculated using DNA histogram analysis before and after the in situ addition of iron during the IronEx II experiment. Cells from the +Fe patch were also incubated on deck in bottles that contained additional iron. These cell division rates were compared with *Prochlorococcus* specific mortality rates measured using the dilution technique (M. Landry pers. comm.).

Methods

Sample collection and preservation—In the 1995 IronEx II experiment, three pulses of iron were added to the Equatorial Pacific (4°S , 105°W) on days 0, 3, and 7 in order to increase the iron concentration from 0.02 nM to 1.0 to 2.0 nM (Coale et al. 1996b; Rue and Bruland 1997). The cell cycle analysis we employed in this study requires high-frequency sampling over 24 h. This was done during a site survey to characterize the area before iron was added and on day 6 inside the iron-fertilized patch. For our purposes, the site survey data served as a control for the iron-fertilized patch because it was impossible to do 24-h sampling programs simultaneously in and out of the patch. During each diel sampling program, samples were collected approximately every 30 to 60 min from the ship's clean flow through seawater system (intake at 6 m). Given the multi-investigator nature of the IronEx II study, it was not possible to do high-frequency depth profile sampling.

In order to obtain more information on *Prochlorococcus* growth and diel patterns than was possible from in situ sampling, samples were also taken from bottle incubations. These were initiated on the morning of day 5 using water collected from the patch at 15-m depth with a trace metal clean Go-Flo bottle (Fitzwater et al. 1982). Two bottles were

Table 1. Glossary of important terms.

Parameter	Units	Definition
μ	d^{-1}	Cell division rate: the growth rate in the absence of cell losses
k	d^{-1}	Net growth rate: cell division rate minus cell mortality rate
m	d^{-1}	Mortality rate: cell losses
$f(S + G_2)_t$	unitless	Fraction of cells in the S and $G_2 + M$ phases of the cell cycle at time t
t_{S+G_2}	h	Duration of the S and $G_2 + M$ phases

placed in an on deck incubator that simulated the in situ light and temperature (bottles received approximately 40% of the irradiation at 0.5 m and were cooled with surface seawater) and sampled every hour for 48 h. In spite of trace metal clean precautions in setting up this experiment, the measured iron concentration in the bottles was 1.23 nM, at least 3.6 times the concentration inside the patch (R. M. Gordon pers. comm.). The concentrations of other metals were not measured, but it is likely that the behavior of *Prochlorococcus* in these bottles (see below) was primarily due to iron. Zinc contamination is a possibility, but the effect on *Prochlorococcus* is likely to be low. Although they do have a requirement for zinc, it cannot absolutely substitute for cobalt (M. Saito pers. comm.). The closely related cyanobacteria *Synechococcus* has a low Zn requirement compared to eukaryotic cells (Sunda and Huntsman 1995), and the addition of zinc to incubation bottles already containing high iron and silicate concentrations had no effect on the growth of these cells (Coale et al. 1996b). The results from this analysis, hereafter referred to as the “+Fe bottles,” were used to determine the growth rate of *Prochlorococcus* at iron concentrations higher than those in the patch, rather than as a proxy for additional in situ data as was originally intended. All samples were preserved in a final concentration of 0.1% glutaraldehyde for 10 min at room temperature in the dark and were then stored in liquid nitrogen (Vaulot et al. 1989).

DNA staining with Sybr Green 1—To eliminate interference from RNA, thawed samples were incubated with RNase A (Sigma-Aldrich R-4875) for 30 min at 37°C at a final concentration of 1 $\mu\text{g ml}^{-1}$. Potassium citrate (final concentration 30 μM) and a 1 : 10⁻⁴ final dilution of the DNA and RNA specific stain Sybr Green I (Molecular Probes) were then added, and the samples were maintained at room temperature in the dark for 15 min before analyzing by flow cytometry. All working stocks of Sybr Green I were diluted in dimethylsulfoxide (Marie et al. 1997).

The amount of DNA per cell, as well as *Prochlorococcus* number and forward angle light scatter (FALS, which is a measure of cell size), was quantified using a modified EPICS 753 flow cytometer as described in Binder et al. (1996), except the instrument was used in single beam mode and the optical filters were arranged to detect green fluorescence from Sybr Green I, which is excited at 488 nm. After passing through 630 and 560 nm short-pass dichroic filters, green signals passed through a 515 nm long-pass filter. Samples were delivered at a constant rate using a syringe pump (Har-

Table 2. The timing of cell cycle progression and values of critical parameters used in cell division rate calculations for *Prochlorococcus* populations in and out of the patch, and in Fe-enriched bottles.

Cell cycle parameters	Site survey	Inside patch	+Fe bottles
S phase initiation (h)	1640	1600	1630
S phase peak (h)	1900	1750	1730
$G_2 + M$ phase initiation (h)	1900	1730	1730
$G_2 + M$ phase peak (h)	2110	1900	1900
First increase of cells in G_1 (h)	2000	1810	1830
First increase in cell number (h)	2110	1810	ND
FALS max : min ratio	3.0	3.6	4.6
t_{S+G_2} (h)	4.3	2.3	3.0

vard Apparatus, Model 22). The lowest coefficients of variation (CVs) in terms of fluorescence were achieved by using low sample flow rates (5 $\mu\text{l min}^{-1}$) and sample lines pre-equilibrated with the stain for at least 1 h.

Calculating the cell division rate—Cell cycle analysis method: The cell cycle of *Prochlorococcus* is similar to the eukaryotic cell cycle in that it has a discrete DNA synthesis phase, even when the cells are growing more quickly than one division per day (Shalapyonok et al. 1998). As a result, the eukaryotic terms G_1 , S , and $G_2 + M$ are often applied to the *Prochlorococcus* cell cycle for convenience. Moreover, cell division in *Prochlorococcus* is tightly synchronized to the light:dark cycle (Vaulot et al. 1995; Liu et al. 1997; Shalapyonok et al. 1998), which allows one to estimate the duration of the S and $G_2 + M$ phases of the cell cycle and apply the DNA histogram method of estimating cell division rates (McDuff and Chisholm 1982; Carpenter and Chang 1988; Vaulot et al. 1995; Liu et al. 1997). In order to determine the duration of S and $G_2 + M$, DNA fluorescence histograms were modeled and divided into G_1 , S , and $G_2 + M$ using ModFit software (Verity Software House). Data from at least 4,000 cells were collected per sample in order to ensure accurate modeling of the DNA histograms. The average CV of the G_1 peak was 6.8%, with a standard deviation of 2.2%.

Following the method of Liu et al. (1997), the cell division rate (μd^{-1}) was obtained from the DNA histograms using

$$\mu = \frac{1}{t_{S+G_2}} \int_{0\text{hrs}}^{24\text{hrs}} \ln[1 + fS + G_{2,t}] dt \quad (1)$$

where $f(S + G_2)_t$ is the fraction of cells in the S and $G_2 + M$ phases of the cell cycle at time t , and t_{S+G_2} is the time (in h) required for cells to pass through S and $G_2 + M$ (see Table 1). The latter can be estimated as twice the distance between the peak of cells in the S phase and the peak of cells in the $G_2 + M$ phase (Carpenter and Chang 1988). With this approach, the t_{S+G_2} values for the site survey, inside the patch, and the +Fe bottles were 4.3, 2.3, and 3.0 h, respectively (Table 2). t_{S+G_2} can also be estimated as the time between the beginning of S phase and the first increase of cell

number or cells in G_1 (Shalapyonok et al. 1998); the results using this method agree well with those given above (Table 2). All of these values are significantly shorter than the 6 h used by Vaultot et al. (1995) and Liu et al. (1997) for *Prochlorococcus* in the equatorial Pacific and the subtropical north Pacific, respectively. We suspect that this is partially a result of the higher frequency sampling program used in our study, which allows greater resolution of cell cycle phase durations (McDuff and Chisholm 1982; Carpenter and Chang 1988). Cell cycle duration times shorter than 6 h have also been found for *Prochlorococcus* in the Arabian Sea (2 to 3 h; Shalapyonok et al. 1998).

Cell number method: The cell division rate can also be calculated if the net specific growth rate (k , d^{-1}) and mortality rate (m , d^{-1}) are known. The net growth rate was calculated as

$$k = \frac{\ln N_1 - \ln N_0}{t} \quad (2)$$

where N_0 and N_1 are the numbers of cells at times 0 and 1 d, respectively and t is 1 d. *Prochlorococcus* mortality rates, m , were calculated from the slope of the decrease in abundance over the time interval when the cells were not dividing (Vaultot et al. 1995; Liu et al. 1998). This method assumes that mortality remains constant throughout the day, and in some cases this is clearly not true. For instance, the decline in *Prochlorococcus* numbers during the site survey was faster from 2400 to 0600 h than from 0600 to 1530 h (Fig. 1). Despite such deviations from linearity, mortality rates calculated in this manner can be helpful in bounding the problem, thus we include them here. Once values of m and k are at hand, the cell division rate can then be determined as

$$\mu = k + m. \quad (3)$$

Results and Discussion

Cell cycle progression patterns—The progression of *Prochlorococcus* through the cell cycle and the degree of synchronization during the site survey (the “no iron added” control) were similar to previous data from the equatorial Pacific (Vaultot et al. 1995; Liu et al. 1997). Cell division was tightly synchronized to the light:dark cycle (Fig. 1A,B,C), with cells remaining in G_1 during most of the day, which indicates that there was no cell division during this period (Fig. 1C). As a result of grazing and other losses, cell numbers declined during the day (Fig. 1A). At the same time, the relative mean FALS per cell increased as the cells grew in size during G_1 , reaching a maximum at 1900 h (Fig. 1B). DNA synthesis started at approximately 1640 h and by 1900 h 75% of the cells were in the S phase and a few had progressed to $G_2 + M$ (Table 2). The peak of cells in $G_2 + M$ and the first increase in cell numbers occurred 2 h later, followed by a decrease in FALS per cell as the cells divided (Fig. 1B,C).

A qualitative comparison of *Prochlorococcus* cell cycle patterns in the site survey, +Fe patch, and +Fe bottles revealed significant differences that are consistent with the hypothesis that iron addition stimulated cell division rates. In

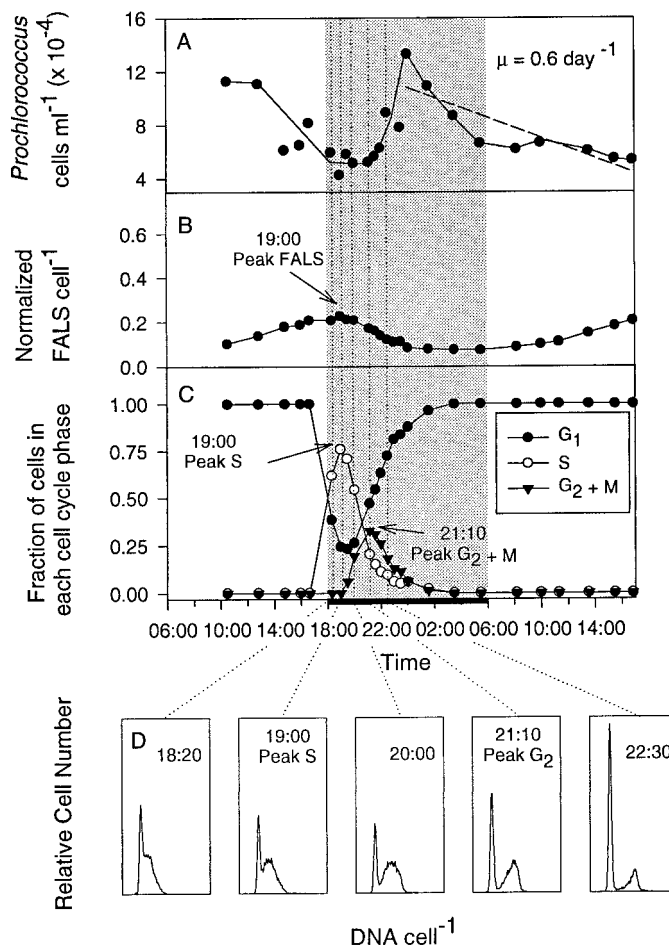


Fig. 1. Diel patterns seen in *Prochlorococcus* collected from 6 m during the site survey. The solid bar represents the dark period, vertical dotted lines point out times of interest: (A) Cell concentration, the dashed line represents the regression used to calculate the mortality rate (see text); (B) mean forward angle light scatter (FALS) normalized to standard beads; (C) the fraction of total *Prochlorococcus* in each cell cycle stage, DNA is synthesized during the S phase, G_1 is the gap before DNA synthesis takes place, and $G_2 + M$ includes the gap after the S phase as well as mitosis; (D) selected DNA histograms from the analysis, note the persistence of a substantial G_1 peak throughout the day.

all populations, we saw the classic diel patterns reflecting synchronized cell division in *Prochlorococcus* populations (Vaultot et al. 1995; Binder et al. 1996; Vaultot and Dominique 1999). These are characterized by cell abundance decreases (due to grazing in the absence of cell division) and FALS per cell increases (reflecting increases in cell volume and biomass) during the day, with a reversal of these trends at night when cell division takes place (Figs. 1A,B; 2A,B; 3A,B). We found, however, that the maximum FALS per cell ($FALS_{max}$) and the amplitude of the FALS change over the diel cycle ($FALS_{max} : FALS_{min}$) were significantly higher in populations exposed to elevated iron levels. The $FALS_{max}$ for the site survey population was 0.23, whereas it was 0.52 for the population inside the patch and 0.66 for the cells in the +Fe bottles (Figs. 1B, 2B, and 3B). The $FALS_{max} : FALS_{min}$

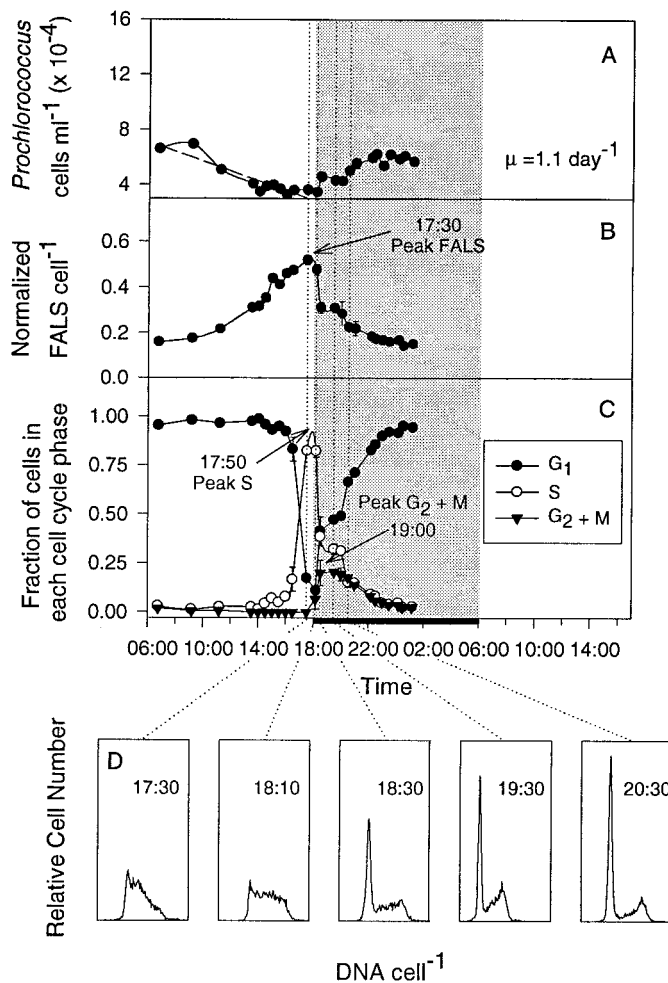


Fig. 2. Diel patterns seen in *Prochlorococcus* collected from 6 m inside the iron-fertilized patch. Samples from 1630 to 2100 h were run in duplicate, error bars represent 1 standard deviation and are occasionally smaller than the symbols. The solid bar represents the dark period, vertical dotted lines point out times of interest. Panels as in Fig. 1. Note the lack of a substantial G_1 peak at 1730 and 1810 h and the large changes that occurred between 1810 and 1830 h.

ratio also increased with increasing iron concentration, from 3.0 to 3.6 to 4.6 for the site survey, +Fe patch, and +Fe bottles, respectively (Table 2). If cell division were synchronized to the same extent in all of the groups, the cells with the greatest change in FALS ($FALS_{max} : FALS_{min}$) would have the highest cell division rate (DuRand 1995; Binder et al. 1996). The ratio of 3.6 inside the patch is comparable to that observed by Binder et al. (1996) for equatorial Pacific waters, whereas the value of 4.6 observed in the bottles—reflecting a decrease in mean FALS per cell over the diel cycle of 80% of the $FALS_{max}$ —is significantly higher than any previous data from the equatorial Pacific (DuRand 1995; Binder et al. 1996).

If we now look at the actual progression through the cell cycle stages in the three populations, the differences are even more apparent (Figs. 1C, 2C, and 3C; Table 2). Progression through the S and $G_2 + M$ phases was much faster in the

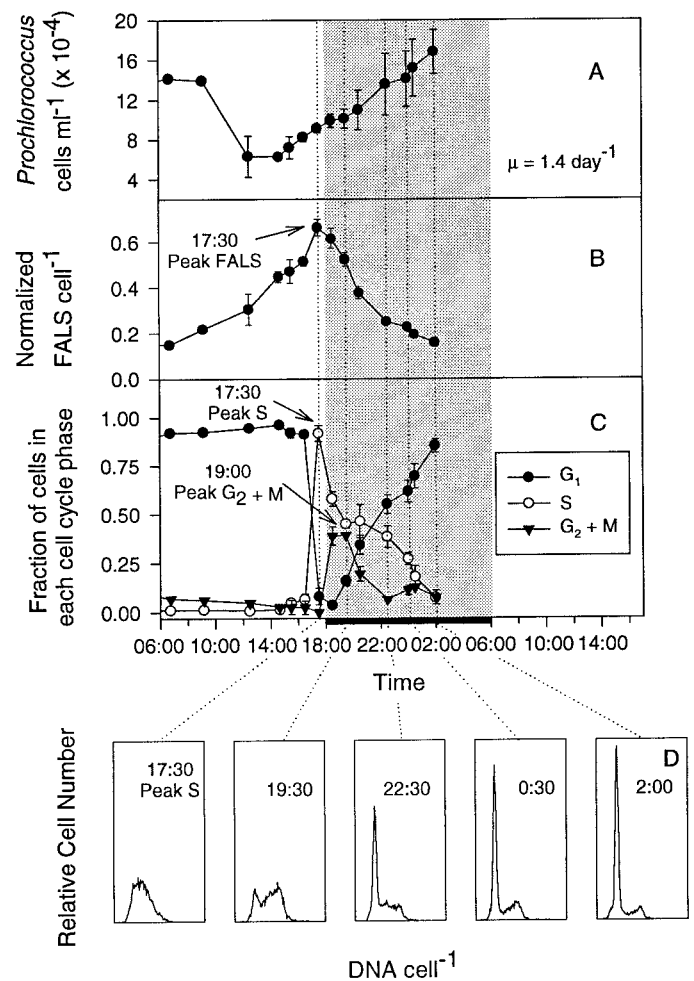


Fig. 3. Diel patterns seen in *Prochlorococcus* in the +Fe bottles. The concentration of iron in these bottles was 1.23 nM, approximately 3.6 times the concentration found inside the patch. The solid bar represents the dark period, vertical dotted lines point out times of interest. Panels as in Fig. 1. Note the lack of a substantial G_1 peak at 1730 h and the second peak of *Prochlorococcus* in both S and $G_2 + M$.

populations that had added Fe ($t_{S+G_2} = 2.3$ and 3.0 h for the +Fe patch and the +Fe bottles, respectively) compared to that of the site survey population ($t_{S+G_2} = 4.3$ h; Table 2). The timing of cell division was also shifted 1 to 2 h earlier in the day in the +Fe populations (Figs. 1C, 2C, and 3C; Table 2).

Finally, it can be seen clearly from the cell cycle progression data that the cells in the patch and in the +Fe bottles underwent a second round of division over a 24-h period, whereas those during the site survey did not (Figs. 1C,D; 2C,D; and 3C,D). For a population to have a division rate greater than 0.69 d^{-1} ($= 1 \text{ doubling d}^{-1}$) some cells must divide more than once per day. Because *Prochlorococcus* is tightly phased to the light:dark cycle, the second division occurs after the first division round is complete and manifests itself in the DNA histograms as a second peak in the S and $G_2 + M$ phases (Shalapyonok et al. 1998). This second round of division was observed only when Fe was added

Table 3. Summary of *Prochlorococcus* growth and mortality rates (d^{-1}) determined by a variety of methods. Standard deviations around the mean are included in those cases where replicates were present.

	Site survey	Inside patch	+Fe bottles
Iron concentration (nM Fe)	0.02*	0.02* to 0.34†	1.23‡
Cell division rate, μ			
Cell cycle analysis	0.6	1.1	1.4 \pm 0.1
Cell number data‡	0.7	1.3	ND
Mortality rate, m			
Cell number data	1.0§	1.7§	1.2 \pm 0.1
Dilution method¶:	0.5	1.2	ND
Mean	0.75	1.5	
Net growth rate, k			
Cell number data	-0.4 \pm 0.2	-0.2	0.2 \pm 0.1

* Rue and Bruland (1997)

† R. M. Gordon, pers. comm.

‡ Calculated using the mean mortality rate.

§ Mortality rate calculated from the decline in cell numbers when no cell division was taking place.

|| Mortality rate calculated from the net growth rate and the cell division rate based on cell cycle analysis.

¶ M. Landry, pers. comm.

(Compare Figs. 1C,D; 2C,D). The first division burst inside the patch, which can be seen clearly in all of the cell cycle parameters (Fig. 2A,B,C,D), occurred between 1810 and 1830 h. The second division event, which was smaller, occurred after 2000 h, when there was a second drop in the proportion of cells in *S* phase accompanied by an increase in the number of cells in *G*₁ and a continuing decrease in FALS (Fig. 2A,B,C). One division between 1810 and 1830 h followed by a second division after 2000 h would require a progression through the first cell cycle of approximately 2 h, which is close to the estimate of 2.3 h for t_{S+G_2} (Table 2). DNA histograms of *Prochlorococcus* from the +Fe bottles also indicate that these cells were growing much more quickly than one doubling per day and had a second round of division (Fig. 3). In these populations, the first peak in *S* phase occurred at 1730 h, and the second could be seen clearly at 2030 h (Fig. 3C). Two peaks in *G*₂ + *M* were also evident (Fig. 3C).

Cell division rates: cell cycle analysis—Using Eq. 1, the t_{S+G_2} values in Table 2 and the cell cycle data in Figs. 1C, 2C, and 3C, we were able to calculate cell division rates for the three *Prochlorococcus* populations growing at different levels of iron. In the +Fe populations it was assumed that the t_{S+G_2} values for the first and second rounds of cell division were identical. The cell division rate determined for the site survey using cell cycle analysis was 0.6 d^{-1} (Table 3), in agreement with previous estimates (0.5 to 0.7 d^{-1}) from similar depths for this region (DuRand 1995; Vaultot et al. 1995; Binder et al. 1996; Latasa et al. 1997; Liu et al. 1997). The division rate in the Fe-enriched patch was 1.1 d^{-1} , almost double that calculated for the site survey (Table 3). Populations in the +Fe bottles had cell division rates of 1.4

d^{-1} , which is equal to two doublings per day and significantly higher than any growth rate previously recorded for *Prochlorococcus* in the equatorial Pacific. Division rates greater than one division per day that are either supported by a second round of division seen in the DNA histograms or calculated by the dilution technique (Landry et al. 1995) have only been reported for the highly productive Arabian Sea (Reckermann and Veldhuis 1997; Liu et al. 1998; Shalapyonok et al. 1998). These results indicate that the maximum cell division rate of *Prochlorococcus* in the equatorial Pacific if iron is abundant is 1.4 d^{-1} , significantly higher than the cell division rates found in situ in this and other studies (DuRand 1995; Vaultot et al. 1995; Binder et al. 1996; Latasa et al. 1997; Liu et al. 1997). Thus, in some regions of the equatorial Pacific iron limitation of *Prochlorococcus* is more severe than originally envisioned in the ecumenical hypothesis, i.e., μ is less than half μ_{max} because of iron limitation (Morel et al. 1991b; Price et al. 1994; Cullen 1995).

One could argue that the second division burst observed in the Fe-enriched populations was due to a second *Prochlorococcus* population that was synchronized differently to the light : dark cycle. Although not seen in our samples from the equatorial Pacific, dual populations have been documented (Campbell and Vaultot 1993), and if this were the case here, our growth rates could be overestimated. We think this is unlikely, however, for the following reasons. First, similar behavior, i.e., a second round of division, has also been observed in *Prochlorococcus* cultures isolated from the Sargasso and Arabian Seas (Shalapyonok et al. 1998). Second, cell division rates calculated from cell number data (*see below*)—which would not be effected by the presence of a second *Prochlorococcus* ecotype—also indicate that two rounds of cell division took place. Finally, we note that the fraction of cells that remain in *G*₁ during the first round of replication is too small to account for the cells in the second *S* phase peak.

Cell division rates: Cell number, net growth rate, and mortality—Cell division rates calculated from changes in population numbers (Eqs. 2 and 3) are in good agreement with results based on cell cycle analysis (Table 3). Cell numbers, which are a function of both cell division rate and mortality, tend to be more variable than intrinsic cell properties such as size or DNA content. Cell number data are included here because they serve as a useful check on the previous results. Using the net change in population numbers to calculate the net growth rate, k (Eq. 2), we found the values were -0.4 ± 0.2 and -0.2 during the site survey and in the patch, respectively. Net growth rates were determined using the difference in cell numbers between days 6 and 7. In this time interval there were three pairs of time points approximately 24 h apart in the site survey (1530 to 1600 h, 1650 to 1640 h, and 1000 to 1030 h), these data were averaged to calculate the net growth rate. Data for inside the patch were less extensive; the net growth rate was estimated using the difference in cell numbers from 0645 h on day 6 to 0110 h on day 7, which should be long enough to eliminate diel effects (*see Fig. 1A*). Because this does not represent a full 24-h period, however, the net growth rate

Table 4. The maximum net carbon production by the community as a whole and carbon fixation by *Prochlorococcus* during the site survey and inside the iron fertilized patch.

Location	Maximum net carbon production (mgC m ⁻³ d ⁻¹)*	<i>Prochlorococcus</i> carbon fixation			Percentage of total net carbon production
		(mgC m ⁻³ d ⁻¹)	(fgC cell ⁻¹ d ⁻¹)	(fgC fgchl ⁻¹ d ⁻¹)	
Site survey	18	5	44	174	28%
Inside +Fe patch	162	13	197	295	8%

* Calculated from ¹⁴C incorporation data (D. Barber pers. comm.). Maximum productivity values were generally found at 6 to 8 m, which is comparable to the 6-m depth used for *Prochlorococcus*.

could be overestimated (which will also lead to an overestimate of the cell division rate).

Mortality increased in parallel with the cell division rate, and as a result *Prochlorococcus* abundance did not increase following iron fertilization. We assume that mortality was probably dominated by microzooplankton grazing (Landry et al. 1997), as direct losses due to sinking would be minimal for such small cells (Pedros-Alio et al. 1989), and the importance of viral lysis in mortality is unclear (Fuhrman and Suttle 1993; Waterbury and Valois 1993). Cell number data from midnight to 1650 and 0645 h to 1730 were used to calculate the mortality rates of 1.0 and 1.7 d⁻¹ in the site survey and in the iron patch, respectively. These agree well with mortality rates estimated from the dilution technique (Landry et al. 1995), which were at 0.5 and 1.2 d⁻¹ (M. Landry pers. comm.). Averaging the two estimates of mortality yields values of $m = 0.75$ and 1.5 d⁻¹ for the site survey and patch, respectively. It is not surprising that mortality rates increased in the +Fe patch, since the growth and grazing rate of heterotrophic protists that feed on picoplankton can be iron limited (Chase and Price 1997). Finally, applying Eq. 3 yields cell division rates of $\mu = 0.7$ and 1.3 d⁻¹ for these populations, which is in agreement with the rates calculated from cell cycle analyses (Table 3). For the bottle experiments we could only estimate mortality rates from measurements of μ (from the cell cycle analysis) and k (from limited cell number data) using Eq. 3 resulting in $m = 1.2$ d⁻¹. This is similar to the mortality rates seen in situ, an indication that confinement in bottles did not significantly affect *Prochlorococcus* grazers.

A tight coupling between cell division rate and mortality for the picoplankton is one of the basic tenants of the ecumenical hypothesis (Frost and Franzen 1992; Price et al. 1994) and the equatorial Pacific synthesis by Landry et al. (1997). In particular, *Prochlorococcus* cell division rate and mortality are tightly coupled in the equatorial Pacific (Chavez et al. 1991; Cullen 1991; Frost and Franzen 1992; Cullen 1995; DuRand 1995; Vaultot et al. 1995; Binder et al. 1996; Verity et al. 1996; Liu et al. 1997; Loukos et al. 1997; Strom et al. 2000). As pointed out in Landry et al. (1997) and seen in this study, balanced cell division and mortality are not incompatible with iron limitation, and biomass can be constrained by both iron limitation (in terms of cell size and pigment content) and microzooplankton grazing (in terms of cell numbers).

Prochlorococcus productivity—To compare changes in *Prochlorococcus* productivity with the relative change in community primary production, the amount of carbon fixed by *Prochlorococcus* was estimated using the cell division rates and then compared with the incorporation of ¹⁴C by the community as a whole. There are many ambiguities inherent in this comparison (Vaultot et al. 1995, but see also Andre et al. 1999), but it still gives some insights into the differential response of the community. The carbon fixed by *Prochlorococcus* in the absence of grazers, or net primary productivity, was calculated in and out of the patch using the following equation:

$$P = C_{\text{cell}}N(e^{\mu t} - 1), \quad (4)$$

where P is the amount of carbon assimilated by *Prochlorococcus* in mgC m⁻³ during the time interval t (1 d), C_{cell} is the carbon per cell, N the number of *Prochlorococcus* cells per m³ at dawn, and μ is the daily average cell division rate (Eppley 1968). We used a C_{cell} value of 53 fg cell⁻¹ for cells during the site survey (Campbell and Nolla 1994; Vaultot et al. 1995) and 85 fg cell⁻¹ for the cells inside the patch. The increase in C_{cell} was based on our observed 1.6-fold increase in *Prochlorococcus* cell volume inside the patch relative to the site survey (Cavender-Bares et al. 1999), assuming carbon increased proportionally with volume. The latter is a reasonable value for *Prochlorococcus* carbon per cell (Moore 1997) and has been used in a previous study (Andre et al. 1999). Values for the *Prochlorococcus* population number (N) were taken from Figs. 1 and 2 and are 1.1×10^5 and 6.8×10^4 cells ml⁻¹ for the site survey and inside the patch, respectively. Chlorophyll a_2 per cell was determined using HPLC and increased from approximately 0.25 to 0.67 fg cell⁻¹ after iron fertilization (Cavender-Bares et al. 1999). Net community particulate carbon productivity, or net carbon productivity (Barber et al. 1996; Bender et al. 1999), was also measured using ¹⁴C incorporation (D. Barber pers. comm.).

With this analysis, *Prochlorococcus* productivity during the site survey was calculated to be 5 mgC m⁻³ d⁻¹, whereas after 6 d in the iron-enriched patch it had increased by roughly 2.7-fold to 13 mgC m⁻³ d⁻¹ (Table 4). Productivity, measured at similar depths using ¹⁴C incorporation by the entire community, was 18 and 162 mgC m⁻³ d⁻¹ outside and inside the +Fe patch, a ninefold increase (Table 4, D. Barber pers. comm.). The relative contribution of *Prochlorococcus*

to net carbon production decreased from 28 to 8% as the phytoplankton community structure shifted to one dominated by larger cells (Table 4).

The increase in *Prochlorococcus* productivity resulted from changes in cellular performance, not changes in population size (which actually decreased inside the iron-fertilized patch). Carbon fixation per cell and per unit chlorophyll increased by 4.5 and 1.7-fold, respectively (Table 4). Chlorophyll-specific productivity did not increase to a greater extent because the carbon to chlorophyll ratio decreased as the cells synthesized more chlorophyll in response to the addition of iron (Cavender-Bares et al. 1999). This preferential synthesis of chlorophyll when iron limitation is relieved indicates that cell division rates estimated using productivity measurements and a fixed carbon to chlorophyll ratio may be incorrect (Cullen 1995; Landry et al. 1997). Although these estimates of *Prochlorococcus* productivity are rough, they parallel changes in productivity of the phytoplankton community as a whole during IronEx I, where normalizing carbon uptake to chlorophyll rather than to volume reduced the response seen upon the addition of iron (Martin et al. 1994; Cullen 1995; Lindley and Barber 1998).

Photoinhibition, nitrogen limitation, and iron—It is clear that the cell division rate of *Prochlorococcus* increased in the iron-fertilized patch, but it is possible that this was not a direct result of increased iron availability but rather an indirect response to changes in the ecosystem brought about by the Fe-induced bloom of the larger cells. One could argue, for example, that increased grazing rates in the patch caused elevated NH_4^+ levels, thus stimulating *Prochlorococcus* growth rates. However, NH_4^+ concentrations are high in the equatorial Pacific (0.1 to 0.5 μM). At these concentrations, small cyanobacteria, even if they are growing at high cell division rates, can satisfy their nitrogen quota using NH_4^+ without becoming diffusion limited (Morel et al. 1991a; Price et al. 1991, 1994). The estimated half-saturation constant for NH_4^+ is 2 to 10 times lower than the concentrations present (Chai et al. 1996), and in some cases NH_4^+ uptake by the phytoplankton community is saturated (Price et al. 1991; McCarthy et al. 1996). In contrast, half-saturation constants for iron have been estimated as 34 to 120 pM, which is close to four times higher than the ambient dissolved iron concentration (Price et al. 1994; Coale et al. 1996a). Finally, during both the IronEx I and II experiments, NH_4^+ concentrations were consistently lower inside the +Fe patch than outside, whereas chlorophyll and productivity increased (Martin et al. 1994; W. Cochlan pers. comm.).

A second caveat in interpreting our results involves the role of light in regulating cell cycle progression in *Prochlorococcus*. The increase in biomass inside the iron-fertilized patch significantly reduced the light intensity available to the cells at 6 m as the experiment progressed. The vertical attenuation of light (K_{PAR}) measured by Kudela and Chavez (1996) doubled abruptly between days 5 and 6, which reduced the irradiance at 6 m from approximately 70 to 45% of the incident irradiation (Kudela and Chavez 1996). Since *Prochlorococcus* cell division rates at the surface are often lower than those at depth, probably because of photoinhibition, (Cullen et al. 1992; Lindley et al. 1995; Vaultot et al.

1995; Liu et al. 1997), decreased irradiance could have contributed to the increased cell division rates that we observed in the patch. We are convinced, however, that although this could be a contributing factor, it is not the dominant explanation for the increase in cell division rates in the iron-enriched populations. First, independent measures of physiological stimulation in the patch reveal that the initial physiological response, i.e., increased *Prochlorococcus* chlorophyll and FALS per cell, as well as increased photochemical efficiency of the indigenous phytoplankton community, was detectable within 1 or 2 d of the iron addition, well before light intensity was significantly reduced on day 6 (Kolber et al. 1994; Behrenfeld et al. 1996; Kudela and Chavez 1996; Cavender-Bares et al. 1999). Second, depth profiles of *Prochlorococcus* cell division rates in the equatorial Pacific reveal that the maximum cell division rate at 30–45 m is 30 to 40% more than the cell division rate at the surface (Vaultot et al. 1995; Binder et al. 1996; Liu et al. 1997). This difference is not enough to account for the 80 and 130% stimulation in cell division rate we saw in the patch and +Fe bottles, respectively.

Conclusion

During IronExII, the addition of iron resulted in a shift in the structure of the phytoplankton community in favor of large cells (Coale et al. 1996b; Cavender-Bares et al. 1999). This shift could have its origins in different degrees of iron limitation in large and small cells and/or the extent to which different sized grazers can control increased growth rates of their prey (Morel et al. 1991b; Price et al. 1994; Cullen 1995). Our results show that the cell division rates of even the smallest cells in the phytoplankton community were significantly limited by iron, which suggests that the degree of limitation was severe across the entire community. Despite their small size, which minimizes diffusion limitation (Morel et al. 1991a), the cell division rate of *Prochlorococcus* roughly doubled with the addition of iron. Furthermore, this increased growth rate was matched by increased grazing rates, resulting in fairly constant population numbers in the patch. Thus, *Prochlorococcus* cell division rate and biomass (in terms of cell size and chlorophyll per cell) were limited by iron availability (Cavender-Bares et al. 1999), but any increase in cell numbers upon the addition of iron was kept in check by microzooplankton grazing. Although the actual increase in cell division rate of the large cells following iron fertilization is not known (Coale et al. 1996b; Cavender-Bares et al. 1999), these results imply that at least some of the differential response of the phytoplankton community in favor of these cells (Cavender-Bares et al. 1999) is due not to dramatic differences in the degree of iron limitation between small and large cells, but to the ability of microzooplankton to respond to increases in prey abundance more rapidly than the mesozooplankton grazers of the larger phytoplankton. How the community would respond to sustained fertilization, however, is not obvious. A comparison of the phytoplankton community in the equatorial Pacific during El Niño and normal upwelling, when the input of iron is higher, indicates that the structure of the community will probably

shift in favor of the larger cells—even if the iron input is smaller and on a longer timescale than in IronEx II (Landry et al. 1997).

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