environmental microbiology

Environmental Microbiology (2013) doi:10.1111/1462-2920.12079

Effects of phosphorus starvation versus limitation on the marine cyanobacterium *Prochlorococcus* MED4 I: uptake physiology

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Summary

Recent measurements of natural populations of the marine cyanobacterium *Prochlorococcus* indicate this numerically dominant phototroph assimilates phosphorus (P) at significant rates in P-limited oceanic regions. To better understand uptake capabilities of *Prochlorococcus* under different P stress conditions, uptake kinetic experiments were performed on *Prochlorococcus* MED4 grown in P-limited chemostats and batch cultures. Our results indicate that MED4 has a small cell-specific V_{max} but a high specific affinity (α_P) for P, making it competitive with other marine cyanobacteria at low P concentrations. Additionally, MED4 regulates its uptake kinetics in response to P stress by significantly increasing V_{max} and α_P for both inorganic and organic P (PO_4 and ATP). The Michaelis–Menten constant, K_M, for PO_4 remained constant under different P stress conditions, whereas the K_M for ATP was higher when cells were stressed for PO_4, pointing to additional processes involved in uptake of ATP. MED4 cleaves the PO_4 moieties from ATP, likely with a 5′-nucleotidase-like enzyme rather than alkaline phosphatase. MED4 exhibited distinct physiological differences between cells under steady-state P limitation versus those transitioning from P-replete to P-starved conditions. Thus, MED4 employs a variety of strategies to deal with changing P sources in the oceans and displays complexity in P stress acclimation and regulatory mechanisms.

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Introduction

The ubiquitous marine cyanobacterium, *Prochlorococcus*, numerically dominates the phytoplankton assemblages in the oligotrophic areas of the oceans (Partensky *et al.*, 1999). *Prochlorococcus*, widespread geographically from 48°N to 40°S and vertically from the surface to 200 m of depth, is most prevalent where macronutrients (N and P) are the lowest (Johnson and Howd, 2000) and has the potential to significantly influence oceanic biogeochemical cycles of carbon (C), nitrogen (N) and phosphorus (P) (Partensky *et al.*, 1999; Karl *et al.*, 2001a; Richardson and Jackson, 2007; Zwirglmaier *et al.*, 2008). Success of *Prochlorococcus* in these nutrient-limited regions has been attributed to a variety of characteristics, such as high intra-genus adaptability (Johnson *et al.*, 2006; Zwirglmaier *et al.*, 2008; Martiny *et al.*, 2009b), small cell size (Ting *et al.*, 2007), adaptable cell content of P and N (Bertilsson *et al.*, 2003; Heldal *et al.*, 2003; Bragg, 2011) and reduced genome size (Dufresne *et al.*, 2003; Rocap *et al.*, 2003; Kettler *et al.*, 2007; see review by Partensky & Garczarek, 2010 and references therein).

Within the *Prochlorococcus* genus there exist multiple genetically and physiologically distinct ecotypes, each adapted to different oceanic environmental conditions (Bouman *et al.*, 2006; Johnson *et al.*, 2006; Zwirglmaier *et al.*, 2008). The two major ecotypic divisions are the low-light (LL)-adapted ecotypes, inhabiting the nutrient-rich waters of the deep euphotic zone, and the high-light (HL)-adapted ecotypes, dominating the nutrient-depleted surface waters (Moore *et al.*, 1998; Moore and Chisholm, 1999; West *et al.*, 2001; Rocap *et al.*, 2003; Zinser *et al.*, 2006; Partensky and Garczarek, 2010). The HL-adapted group can be further subdivided into two groups: HLI (ecotype ‘eMED4’) and HLII (ecotype ‘eMIT9312’), which differ in their geographical distributions (West *et al.*, 2001; Johnson *et al.*, 2006; Zinser *et al.*, 2006; Zwirglmaier *et al.*, 2007; 2008; Malmstrom *et al.*, 2010), temperature tolerances (Johnson *et al.*, 2006) and aspects of their P physiology. MED4 is able to grow on a variety of organic

P sources and substantially increases alkaline phosphatase activity (APA) when reaching P starvation, whereas MIT9312 grows on only one kind of organic P source, ATP, out of six organic P sources tested and displays small amounts of APA upon P starvation (Moore *et al*., 2005).

Not only are *Prochlorococcus* one of the smallest known photosynthetic organisms, they also have low cellular P content. This is reflected in an N : P ratio that is above the Redfield ratio of 16:1 (Redfield, 1934) under P-replete (N : P = 16–33) and particularly P-limited conditions (N : P = 59–109; Bertilsson *et al*., 2003; Heldal *et al*., 2003). In accordance with a low cellular P content, *Prochlorococcus* has been found to use sulfolipids in place of phospholipids in their cell membranes (Van Mooy *et al*., 2006; 2009). In addition to these physiological characteristics *Prochlorococcus* has a streamlined genome that lacks the low-affinity phosphate (PO_4) uptake system (*pitAB*) found in other prokaryotes but contains a high-affinity phosphate uptake system encoded by *pstS* and *pstABC* (Rocap *et al*., 2003; Moore *et al*., 2005; Kettler *et al*., 2007). These genes, along with other P acquisition genes mostly found on genomic islands (Coleman *et al*., 2006), are upregulated under conditions of acute P starvation in *Prochlorococcus* (Martiny *et al*., 2006) and have been used as markers of P stress in natural populations (Fuller *et al*., 2005). Interestingly, recent metagenomic analyses indicate that *Prochlorococcus* is under selective pressure to acquire or maintain P acquisition genes in the North Atlantic gyre in response to P deficiency (Martiny *et al*., 2009a; Coleman and Chisholm, 2010). In the North Pacific subtropical gyre (NPSG), which is not as severely P-limited as the North Atlantic gyre (Karl *et al*., 2001b; Ammerman, 2003), *Prochlorococcus* genomes are not as enriched in P acquisition genes (Coleman and Chisholm, 2010). Conditions may be changing, however, as recent evidence suggests that the dissolved inorganic phosphorus inventory in NPSG is currently decreasing, and with further climatic warming and ocean stratification the NPSG could become more P-stressed (Karl, 2007; Morán *et al*., 2010). These conditions may favour further increases in *Prochlorococcus* populations (Karl, 2007) and increased selection for phosphorus acquisition genes within the *Prochlorococcus* genome (Martiny *et al*., 2009a; Coleman and Chisholm, 2010).

P uptake studies on size-fractionated natural phytoplankton populations indicate that the picocyanobacterial size fraction (operationally defined as 0.6–2 μm), which includes *Prochlorococcus*, displayed the fastest uptake of PO_4 (Cañellas *et al*., 2000; Donald *et al*., 2001; Tanaka *et al*., 2004; Sohm and Capone, 2006). In more recent studies PO_4 and ATP uptake rates measured on flow-cytometrically sorted phytoplankton indicated that *Prochlorococcus* populations in the North Atlantic are more competitive for P sources than *Synechococcus* and eukaryotic algae (Zubkov *et al*., 2007; Casey *et al*., 2009). These studies, as well as reduced cell P content, substitution of phospholipids with sulfolipids and ability to utilize both inorganic and organic P sources, indicate that *Prochlorococcus* is especially adapted to P limitation. However, no studies have been carried out to examine the P uptake kinetics of cultured *Prochlorococcus*.

To better understand the ability of *Prochlorococcus* to thrive under P-deficient conditions and compete with other phytoplankton, we performed P uptake kinetic experiments on axenic cultures of *Prochlorococcus* MED4 (i.e. free of any contaminating bacteria) under various levels of P stress. We selected the MED4 strain of *Prochlorococcus* because it has been well studied, its genome has been sequenced and it was the only axenic strain available when these experiments began. We performed experiments on batch cultures under P-replete and short-term P-starved conditions and also established chemostat cultures to measure physiology under long-term, steady-state P-limited conditions that better reflect conditions in their natural environment. Both PO_4 and ATP uptake were measured, and basic kinetic parameters, such as the Michaelis–Menten constant (K_M) and maximum velocity of uptake (V_{max}), are presented along with measurements of cellular P quota and APA. Additionally, samples were collected for gene expression of selected P-related genes; these results are presented in Nahas Reistetter and colleagues (2013).

Results

Culture and cell conditions used for physiological measurements

Batch cultures of *Prochlorococcus* MED4 supplied cells in P-replete phase and P-starved phase, while 1 l chemostat cultures were maintained at various growth rates to provide cells at specific levels of P limitation. Once replicate chemostat cultures reached steady state they maintained a fairly constant culture concentration and relative chlorophyll fluorescence per cell (Fig. 1). Altering the dilution rate of the chemostat changed the growth rate but did not change the long-term culture concentration or relative chlorophyll fluorescence per cell. In all chemostat cultures, the residual PO_4 concentration in the medium was drawn down to below the SRP detection limit of 0.05 μM (data not shown). Replicate batch cultures of *Prochlorococcus* MED4 grew from P-replete exponential (growth rate $\mu = 0.68\ day^{-1}$) to P-starved stationary phase ($\mu = 0\ day^{-1}$) (Fig. 2a). P starvation was reached on day 9 as indicated by both a decrease in residual PO_4 concentration in the medium to the limit of detection and a decrease in P cell quota (Fig. 2b). Interestingly, the

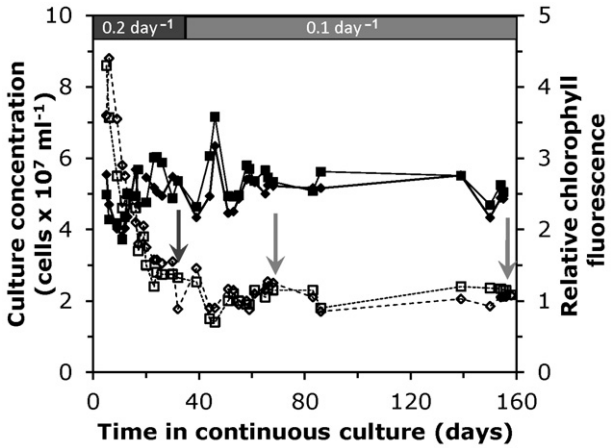

Fig. 1. Culture concentration (open symbols) and relative chlorophyll fluorescence per cell (filled symbols) versus time for replicate representative chemostat cultures of *Prochlorococcus* MED4. Growth rate determined by dilution rate is indicated along the top. Arrows indicate time points at which uptake experiments and ancillary measurements were taken.

relative chlorophyll fluorescence per cell did not drop until a day after the culture became P-starved.

PO_4 *and ATP uptake experiments*

Short-term, cell-specific PO_4 uptake showed a Michaelis–Menten type, hyperbolic relationship between uptake rate and PO_4 concentration up to 10 μM (Fig. 3a). Furthermore, MED4 altered its P uptake rates according to P status of the cells, i.e. P-starved, P-limited or P-replete (Figs 3a and 4a). Cells replete in P, during exponential growth phase in batch cultures and in N-limited batch cultures, had similarly low PO_4 uptake (data not shown for N-limited cultures). Relative to P-replete conditions in batch cultures, maximum uptake rates (V_{max}) for PO_4 increased significantly under conditions of P stress in both P-starved batch cultures and P-limited chemostat cultures (Fig. 4a, Table 1). Interestingly, the P-starved batch cultures showed significantly lower V_{max} values for PO_4 than the P-limited chemostat cultures (Table 1). When comparing cell-specific V_{max} values for MED4 with values for marine cyanobacteria (Table 2) and eukaryotic phytoplankton (Perry, 1976; Burmaster and Chisholm, 1979; Riegman *et al*., 2000), we found values for MED4 are at least an order of magnitude lower, consistent with the smaller cell surface area of *Prochlorococcus* and limited space available for uptake sites (Stolte and Riegman, 1995). When V_{max} for *Prochlorococcus* MED4 is normalized to internal P concentration in the cell (Q), the $V_{max,P}$ is higher and not significantly different between P-starved batch and P-limited chemostat cultures (Tables 1 and 2). Compared with other, larger cyanobacteria, the values of $V_{max,P}$ for *Prochlorococcus* MED4 are surprisingly higher

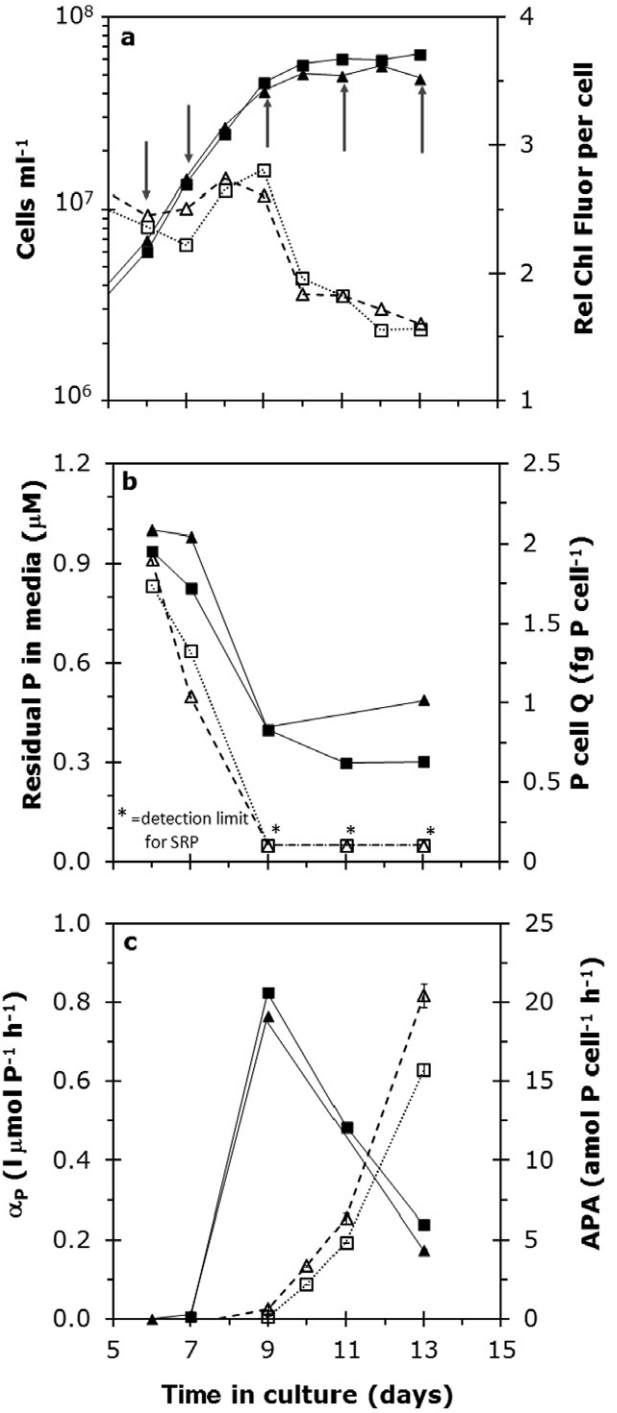

Fig. 2. Time course of replicate batch cultures of *Prochlorococcus* MED4.
a. Culture concentration (filled symbols) and relative chlorophyll fluorescence per cell (open symbols).
b. Residual PO_4 concentration (open symbols) and cellular P quota (filled symbols). There is no P quota data point for one replicate batch culture on day 11.
c. Specific affinity (α_P; closed symbols) and APA (open symbols).

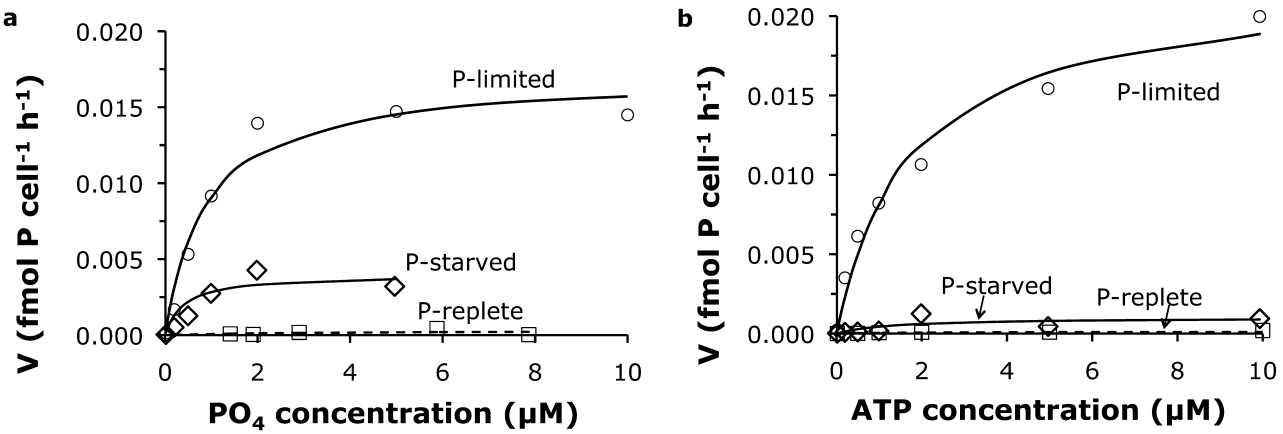

Fig. 3. Representative intracellular uptake curves by *Prochlorococcus* MED4 grown under P-replete, P-limited and P-starved growth conditions. P-replete and P-starved data are from cells grown in batch cultures and the P-limited data are from cells grown in a chemostat maintained at 0.2 day^{-1} growth rate. Solid lines represent a non-linear regression curve fit to the Michaelis–Menten equation.
a. Velocity of PO_4 uptake versus PO_4 concentration.
b. Velocity of ATP uptake versus ATP concentration.

than those calculated for *Trichodesmium* species, although not compared with the one *Synechococcus* strain measured (Table 2; Fu *et al.*, 2005a,b).

Chemostat and batch MED4 cultures were also measured for ATP uptake, because P obtained from nucleotides may be an important source of DOP for microbes in oligotrophic oceans (Björkman and Karl, 2003; 2005). As observed for the PO_4 uptake, ATP uptake exhibited Michaelis–Menten kinetics (Fig. 3b), and V_{max} for ATP increased significantly under conditions of P limitation in chemostat cultures relative to P-replete conditions in batch cultures (Figs 3b and 4a). However, P-starved cells in batch cultures did not display an increased V_{max} of ATP uptake compared with P-replete cells in batch cultures. V_{max} values for ATP uptake were not significantly different from V_{max} values for PO_4 uptake under P-replete batch and P-limited chemostat culture conditions, while under conditions of P starvation in batch cultures, the V_{max} for ATP uptake were significantly lower (~ 3.5-fold) than for PO_4 uptake (Fig. 4a, Table 3). The $V_{max,P}$ for ATP is higher than V_{max}, and does not differ significantly from $V_{max,P}$ for PO_4 uptake except for the P-starved batch cultures (Tables 1 and 3).

The Michaelis–Menten constant, K_M, an indicator of enzyme affinity, was calculated for PO_4 uptake of MED4 and found to be essentially constant (0.9 ± 0.6 μM) between P-replete, P-limited and P-starved conditions (Fig. 4b, Table 1). The higher value and higher error on the K_M for PO_4 under P-replete batch culture conditions is likely a result of the difficulty in fitting the Michaelis–Menten equation to these low uptake rate data sets. The K_M for ATP uptake also was constant across all conditions (2.1 ± 0.9 μM; Fig. 4b), although they were significantly higher than the K_M for PO_4 when cells were P-stressed (Fig. 4b, Table 3). The K_M values found for *Prochlorococcus* MED4 are comparable with other cultured cyanobacteria, with the exception of *Synechococcus* WH7803 (Table 2; Donald *et al.*, 1997), although considerably higher than values determined for natural *Prochlorococcus* populations in the North Pacific subtropical gyre (Björkman *et al.*, 2012).

The specific affinity, α_P, for PO_4 uptake, i.e. the ability to accumulate substrate from limiting concentrations (Button, 1991), increased as much as 38- to 88-fold from P-replete conditions to P-limited and P-starved conditions (Figs 2c and 4c, Table 1). The α_P for ATP uptake did not exhibit this same trend, with α_P for ATP in the P-starved batch cultures not being significantly different from α_P for ATP under P-replete batch cultures (Fig. 4c, Table 1). Additionally, the higher K_M values for ATP uptake resulted in α_P for ATP that were significantly lower than the α_P for PO_4 (Table 3).

Utilization of organic phosphates

We detected minimal uptake of ^{14}C-ATP (expressed relative to uptake by no-cell blank = 4.5 ± 1.3) compared with uptake of ^{32}P-ATP (= 103 ± 1, $p = 0.012$) for duplicate batch cultures of MED4 in P-starved stationary phase. This was further confirmed with N-starved (P-replete) batch cultures. These results indicate that MED4 does not assimilate the entire ATP nucleotide; rather it cleaves and incorporates the terminal (γ) PO_4 molecule. We did not measure incorporation of the α or β PO_4 groups from ATP.

Alkaline phosphatase activity was measured concurrently with each uptake experiment to further describe the P physiology of *Prochlorococcus* MED4 under varying

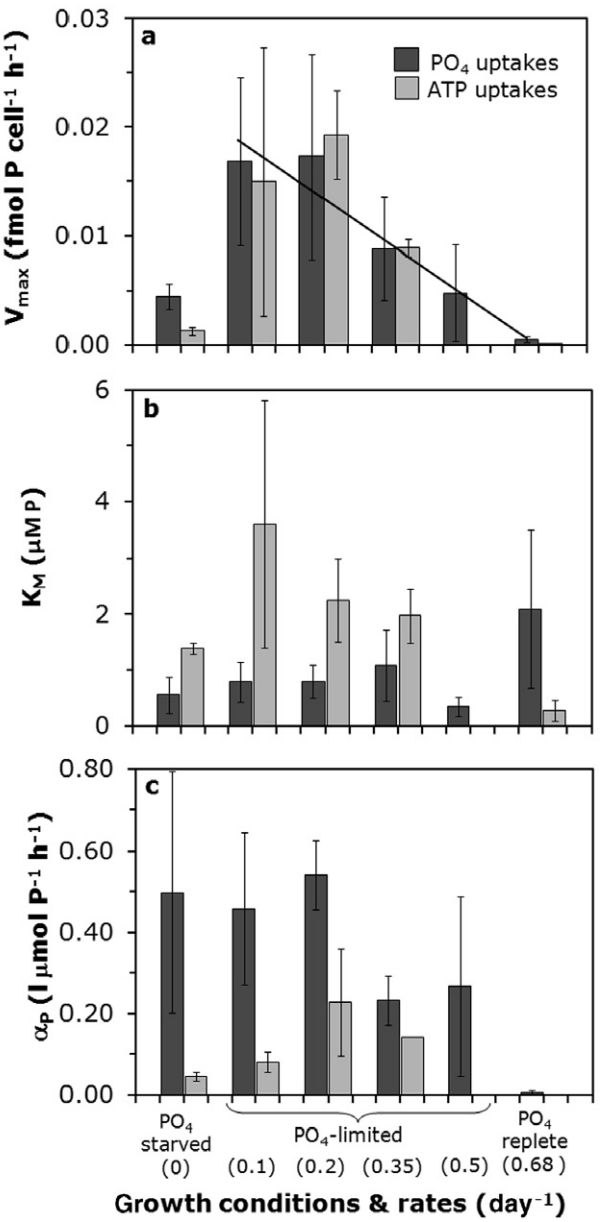

Fig. 4. Bar graphs of uptake parameters for PO_4 and ATP uptake by *Prochlorococcus* MED4 grown under varying degrees of P stress (= P-limited chemostat cultures and P-starved stationary-phase batch cultures); (a) V_{max}, with $R^2 = 0.9545$ for linear regression of V_{max} for PO_4 over growth rates of 0.1 day^{-1} P-limited to P-replete batch cultures, (b) K_M, (c) specific affinity (α_P). ATP uptake was not measured for 0.5 day^{-1} chemostats. Error bars represent standard deviation of two to six replicate cultures.

degrees of P stress. Consistent with previous studies of *Prochlorococcus* (Moore *et al*., 2005), APA increased for MED4 cells in stationary-phase batch cultures starved for PO_4 relative to cells in P-replete exponential phase of batch cultures (Fig. 2c). Although the APA during stationary phase continued to increase even as the cells were losing their relative chlorophyll fluorescence, we chose to combine the P-starved APA values for comparison to other P-stressed cells in the P-limited chemostats in Fig. 5a. A significantly greater, although variable APA (ranging from 10–76 amol P cell^{-1} h^{-1}) was observed for cells in the P-limited chemostats relative to both P exponential and starved conditions in batch cultures (Fig. 5a, Table 1).

To test whether APase or another enzyme, such as a 5′-nucleotidase-like enzyme, is involved in cleaving the PO_4 functional group from the ATP, competition experiments with other organic P substances were carried out on P-starved MED4 cells from batch cultures. Uptake (in pmol) of ^{32}P-ATP was observed in cells when no additional organic P source was present (Fig. 6). The addition of non-radioactive ATP lowered the ^{32}P-ATP uptake, as would be expected due to competition between non-radioactive and radioactive ATP molecules. The addition of another nucleotide, CTP, also lowered ^{32}P-ATP uptake. In contrast, the organic P compounds, GYP, G6P and cAMP, did not compete with ^{32}P-ATP uptake (Fig. 6).

Cellular P quota

The highest P quota was measured in P-replete batch cultures and the lowest in P-starved batch cultures (Figs 2b and 5b). The P-limited chemostat cultures yielded cells with intermediate P quotas that were significantly different from both P-replete cells and P-starved cells, but did not show a statistically significant difference between growth rates (Fig. 5b, Table 1). The overall values and trends in MED4 P quota are consistent with published values for *Prochlorococcus*, *Synechococcus* and other phytoplankton (Bertilsson *et al*., 2003; Heldal *et al*., 2003).

Discussion

P uptake characteristics

The results of this study indicate that *Prochlorococcus* MED4 alters its P uptake kinetics in response to conditions of P stress (corresponding both to steady-state P limitation as measured in chemostat cultures and to P-starved stationary phase as measured in batch cultures). Specifically, V_{max} of PO_4 and ATP uptake are highest for MED4 cells grown under steady-state P limitation and P-starved conditions relative to P-replete batch culture conditions, which we interpret as due to an increased capacity for uptake (Butler, 1998). Since uptake of PO_4 depends on the high-affinity P uptake system composed of a periplasmic binding protein, PstS, and associated membrane-bound ABC transporter, PstCAB (Scanlan *et al*., 2009), an increased V_{max} would likely be reflected in an increase in cell size and/or greater density

Table 1. A compilation of *P*-values obtained through a two-tailed Welch's *t*-test of uptake kinetic and other physiological parameters between P stress conditions: P-starved, P-limited (combined for all chemostat cultures) and P-replete cultures.

Physiological parameter	Uptake experiment	P-replete versus P-starved	P-replete versus P-limited	P-starved versus P-limited
V_{max}	PO_4 uptake	**0.0003**	**0.0002**	**0.0056**
	ATP uptake	0.1387	**0.0055**	**0.0080**
$V_{max,P}$	PO_4 uptake	**0.0011**	**0.0001**	0.1901
	ATP uptake	0.1491	**0.0038**	**0.0101**
K_M	PO_4 uptake	0.2030	0.2327	0.3622
	ATP uptake	0.5172	**0.0435***	0.0556
α_P	PO_4 uptake	**0.0205**	**0.0000**	0.5042
	ATP uptake	**0.0481****	**0.0091**	0.1175
APA	n/a	**0.0458****	**0.0012**	**0.0046**
Q	n/a	**0.0000**	**0.0015**	**0.0002**

Shaded rows correspond to values compared for ATP uptake parameters.
P-values in bold are significant ($P < 0.05$) except the two noted because they did not hold up to a sequential Bonferroni test to eliminate type I false positives: **P*-value > sequential Bonferroni correction factor of 0.01; ***P*-value > sequential Bonferroni correction factor of 0.025.
n/a, not applicable.

of these P uptake systems. Although we did not measure cell size, it is likely that the density of transport systems increased, consistent with observed increase in *pstS* gene expression for these cultures (Nahas Reistetter *et al*., 2013). An increase in *pstS* gene expression and PstS protein also have been observed for *Prochlorococcus* MED4 under acute (Martiny *et al*., 2006) and extended (Fuszard *et al*., 2010) P starvation.

The relationship of V_{max} with growth rate was not a straightforward inverse relationship for *Prochlorococcus* MED4 as has been observed with other phytoplankton (Rivkin and Swift, 1982; Riegman *et al*., 2000), because of the significant drop in V_{max} under conditions of P starvation relative to P limitation. However, there is a strong negative correlation (slope = –0.031, $R^2 = 0.9545$) for PO_4 uptake and a nearly identical, negative trend (slope = –0.030, $R^2 = 0.8557$) for ATP uptake when the P-starved data are not included (Fig. 4a). As growth becomes limited by lower P fluxes (flow rate into the chemostats), the $V_{max,P}$ data for both PO_4 and ATP uptakes also show a negative correlation with substrate concentration (slope = –0.675, $R^2 = 0.867$ for PO_4 and slope = –0.638, $R^2 = 0.829$ for ATP). Based on theoretical descriptions of uptake kinetics (Morel, 1987) and the flexible uptake description (Bonachela *et al*., 2011), these trends would indicate that the low V_{max} under P-replete conditions likely represents transporter limitation, while the higher V_{max} under P-limited growth conditions suggests that *Prochlorococcus* MED4 approaches diffusion limitation when the number of transporters (PstCAB) is highest. We estimated the concentration of P at which diffusion limitation of growth would occur for *Prochlorococcus* MED4 when growing at 0.1 day^{-1} to be 0.2 amol P l^{-1}, using the equation for molecular diffusion under steady-state conditions and solving for *S*:

$$S = J/4\pi rD,$$

where *J* is the measured V_{max} at 0.1 day^{-1}, *r* is the radius for MED4 (0.2 μm, Ting *et al*., 2007) and *D* is the molecular diffusion coefficient for P (10^{-5} cm^2 s^{-1}) as outlined in Chisholm (1992). This concentration is below the low values of P that have been reported in the euphotic zone of the P-limited regions of the North Atlantic Ocean (Wu *et al*., 2000; Cavender-Bares *et al*., 2001), implying that *Prochlorococcus* is unlikely to reach diffusion limitation, especially since inorganic P flux provides more than this every hour (SRP flux in the Sargasso Sea was estimated to be 0.005 μmol P l^{-1} h^{-1} based on annual flux of 43 mmol m^{-2} $year^{-1}$; Lomas *et al*., 2010) and since organic P sources are bioavailable (Björkman and Karl, 2003).

Since the uptake data displayed hyperbolic relationships with substrate, normalizing V_{max} to P cell quota ($V_{max,P}$) should give an estimate of maximum growth rate, μ_{max}. However, $V_{max,P}$ (when calculated on a per day basis) underestimated the P-replete growth rate of 0.68 day^{-1} ($V_{max,P}$ is 0.008 h^{-1} * 24 h day^{-1} = 0.19 day^{-1}; Table 2) and substantially overestimated the growth rates when *Prochlorococcus* MED4 is P-stressed. Uptake rates larger than required to maintain growth may be due to luxury uptake; however, this is not likely in these P-limited *Prochlorococcus*, as there is no evidence of differential expression of polyphosphate kinase gene or protein under P starvation (Martiny *et al*., 2006; Fuszard *et al*., 2010). Additionally no signs of polyphosphate bodies have been observed under P-replete conditions (Heldal *et al*., 2003). Another possible explanation is that the V_{max} measured in this study are actually reflecting gross uptake rather than net uptake. Unfortunately, we did not measure cellular P pool turnover in the samples to determine this. However, we can make a rough estimate of the cellular P pool turnover by assuming a total adenine nucleotide (TAN) turnover rate of 2.2% of the generation time (Karl and Björkman, 2001). For the P-limited chemostats with

Table 2. Comparison of PO_4-specific K_M (μM), V_{max} (fmol P cell^{-1} h^{-1}), $V_{max,P}$ (h^{-1}), α_P (l μmol P^{-1} h^{-1}) and Q (fg cell^{-1}) average values with standard errors in parentheses, except where noted, of marine cyanobacteria (*Trichodesmium* sp., *Synechococcus* sp. and *Prochlorococcus* MED4) under different growth conditions.

Marine cyanobacteria	Culture conditions	K_M	V_{max}	$V_{max,P}$	α_P	Q	Reference
Trichodesmium							
IMS101	P-lim, batch (0.2 mM PO_4)	0.42 (0.05)	3.43 (0.1)	0.13 (0.004)	0.32 (0.03)	26.4	Fu and colleagues (2005a)
	P-rep, batch (20 mM PO_4)	0.34 (0.04)	0.59 (0.01)	0.012 (0.0004)	0.033 (0.003)	17.9	Fu and colleagues (2005a)
GBRTRLI101	P-lim, batch (0.2 mM PO_4)	0.64 (0.07)	7.14 (0.2)	0.14 (0.004)	0.21 (0.02)	51.0	Fu and colleagues (2005a)
	P-rep, batch (20 mM PO_4)	0.68 (0.08)	1.2 (0.03)	0.014 (0.0005)	0.021 (0.002)	85.7	Fu and colleagues (2005a)
IMS101	P-lim, batch (0.2 mM PO_4)	0.20 (0.01)	0.54 (0.05)	0.043 (0.003)	0.091	12.6	Fu and colleagues (2005b)
	P-rep, batch (20 mM PO_4)	0.28 (0.03)	0.16 (0.01)	0.0089 (0.0004)	0.028	18.0	Fu and colleagues (2005b)
Synechococcus							
WH7803	P-starv (PO_4 undetectable)	3.1 (0.96)	nc	nr	nc	n.d.	Donald and colleagues (1997)
	P-repl (172 mM PO_4)	67 (25)	nc	nr	nc	n.d.	Donald and colleagues (1997)
CCMP1334	P-lim, semi-cont (0.2 mM PO_4)	0.4 (0.08)[a]	0.21 (0.01)[a]	3.4 (0.1)	7.2	0.06	Fu and colleagues (2006)
	P-repl, semi-cont (20 mM PO_4)	1.25 (0.1)[a]	0.003 (0.002)[a]	0.021 (0.0007)	0.016	0.14	Fu and colleagues (2006)
NIBB 1071	P-lim ($\mu = 0.08$ day^{-1})	0.07	0.616	nr	nr	2.1–3.2	Ikeya and colleagues (1997)
	P-repl ($\mu = 0.58$ day^{-1})	1.5	0.0018	nr	nr	6.5–6.9	Ikeya and colleagues (1997)
Prochlorococcus							
MED4	P-starv	0.53 (0.37)	0.005 (0.001)	0.21 (0.05)	0.50 (0.30)	0.74	This study
	P-lim ($\mu = 0.1$ day^{-1})	0.79 (0.36)	0.017 (0.008)	0.33 (0.15)	0.46 (0.19)	1.38	This study
	P-lim ($\mu = 0.2$ day^{-1})	0.79 (0.30)	0.017 (0.009)	0.43 (0.19)	0.54 (0.08)	1.15	This study
	P-lim ($\mu = 0.35$ day^{-1})	1.1 (0.6)	0.009 (0.005)	0.23 (0.08)	0.23 (0.06)	1.24	This study
	P-lim ($\mu = 0.5$ day^{-1})	0.35 (0.16)	0.005 (0.005)	0.11 (0.10)	0.27 (0.22)	1.59	This study
	P-repl (1 mM PO_4)	2.1 (1.4)	0.0005 (0.0003)	0.008 (0.006)	0.006 (0.005)	1.95	This study

a. Standard deviation reported.
nr, not reported; nc, reported V_{max} in units (pmol μg protein^{-1} min^{-1}) not convertible to comparable units.

Table 3. A compilation of *P*-values obtained through a two-tailed Welch's *t*-test of uptake parameters V_{max}, K_M and α_P for PO_4 and ATP uptake on cultures under various P stress conditions: P-starved, P-limited (combined data from all growth rates of chemostat cultures) and P-replete cultures.

	P-starved ATP to P-starved PO_4	P-lim (combined) ATP to P-lim (combined) PO_4	P-replete ATP to P-replete PO_4
V_{max}	**0.0019**	0.5005	0.1459
$V_{max,P}$	**0.0064**	0.5166	0.3497
K_M	**0.0008**	**0.0151**	0.3800
α_P	**0.0269**	**0.0005**	0.4331

P-values less than 0.05 are in bold.

growth rates ranging from 0.5 to 0.1 day^{-1}, the TAN turnover rates range from 44 to 220 min. Since our incubation times for the P-limited and P-starved cultures were 45 min, it is possible that the P uptake rates we measured were closer to gross uptake rather than net uptake. Such high uptake rates would enable *Prochlorococcus* to rapidly take advantage of any short term P pulses that might occur in the oceans. This might also indicate that there may be a different time scale for regulation of gross, short-term uptake compared with net uptake (Morel, 1987).

Estimates of K_M have been used by ecologists as a measure of the relative competitive abilities between phytoplankton for a substrate at low concentrations: lower values of K_M represent higher affinities for the substrate (Dugdale and Goering, 1967). For *Prochlorococcus* MED4, K_M for PO_4 remained constant and independent of P stress condition, indicative of a single uptake system. This is consistent with the fact that the MED4 genome lacks a low-affinity P transporter system (*pitAB*) and contains only a single copy of a high-affinity P uptake system (*pstS* and *pstCAB*) (Rocap *et al*., 2003; Moore *et al*., 2005). This is in contrast to what has been observed for other marine cyanobacteria that exhibit a marked decrease in K_M values from P-replete conditions to P-limited conditions (Donald *et al*., 1997; Ikeya *et al*., 1997; Fu *et al*., 2005a,b; 2006). Multiple genes encoding the periplasmic phosphate binding protein, *pstS*, are found in *Synechococcus* genomes (Palenik *et al*., 2003; Scanlan *et al*., 2009), which likely contribute to different K_M values. Interestingly, the K_M for PO_4 measured for MED4 in this study is 50–100 times higher than that measured for natural populations of *Prochlorococcus* in the North Pacific subtropical gyre (8–18 nM; Björkman *et al*., 2012). These differences may be due to differences in experimental procedures, such as the artificial seawater and chemostat system used in this study compared with the native environment, or the much higher range of non-radioactive PO_4 and ATP used for the cultures in this study (0.02–20 μM P) compared with the additions in the

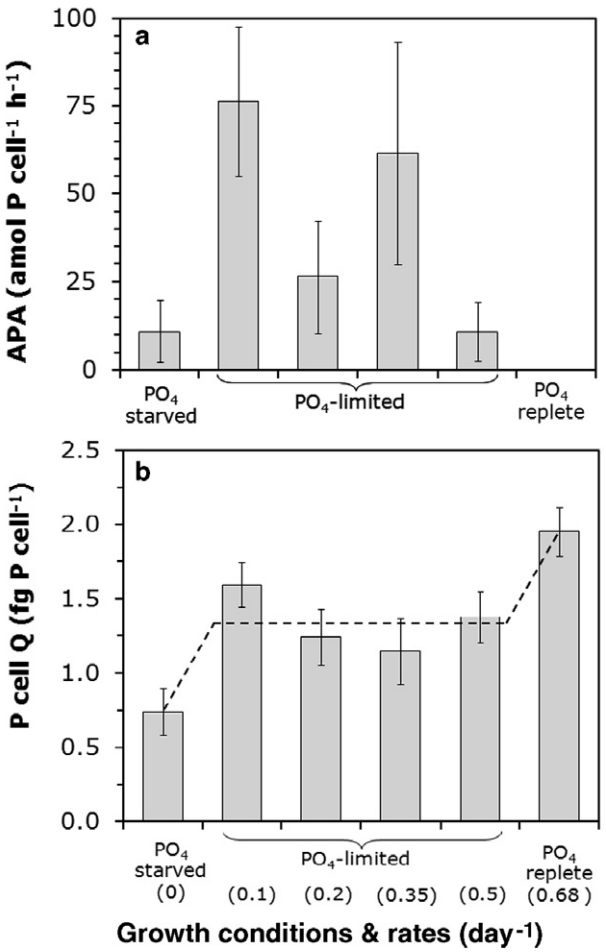

Fig. 5. Alkaline phosphatase activity (a) and P quota (b) for *Prochlorococcus* MED4 grown at varying degrees of P stress on cells harvested the same time as uptake experiments were carried out. Error bars represent standard error of two to six replicate cultures.

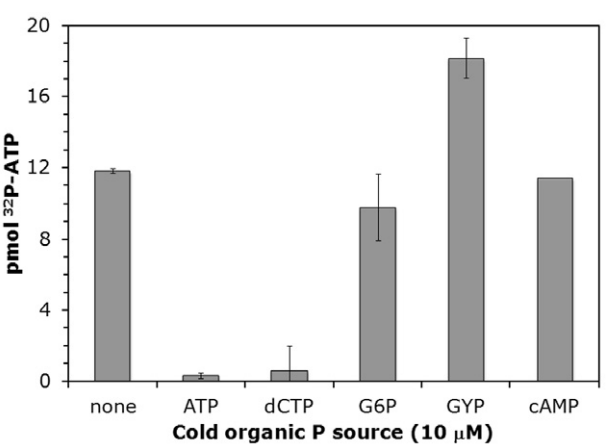

Fig. 6. Effect of various organic P compounds (10 μM concentration) on pmol ^{32}P-ATP taken up by the cells for P-starved *Prochlorococcus* MED4. Error bars represent standard error of two replicate cultures.

field study (10–150 nM P). It is also possible that the *Prochlorococcus* assemblages in the North Pacific subtropical gyre differ in their P physiological response to P stress, since many P-related genes in *Prochlorococcus* populations in the Pacific Ocean are lacking compared with MED4 (Coleman and Chisholm, 2010), which has one of the highest complements of P-related genes (Martiny *et al*., 2006).

We did observe a significantly higher K_M for ATP uptake relative to the K_M for PO_4 uptake, except under P-replete conditions in batch cultures (Table 3), implying a different uptake system than that measured for PO_4. Our ^{14}C-ATP experiments demonstrated that MED4 assimilates the terminal PO_4 group from ATP rather than the entire ATP molecule. Therefore it is likely that the observed shift in K_M can be attributed to an enzyme involved in cleavage of PO_4 from organic molecules. MED4 has an APase gene, *phoA* (Rocap *et al*., 2003; Moore *et al*., 2005), that is upregulated and expressed under P-limited and P-starved conditions (Martiny *et al*., 2006; Fuszard *et al*., 2010; Nahas Reistetter *et al*., 2013), and APA also increases under P-limited and P-starved conditions. However, our results from the ATP uptake competition studies point to the likelihood that ATP is being cleaved by a 5′-nucleotidase-like exoenzyme (Fig. 6; Ammerman and Azam, 1985). This hydrolytic enzyme is commonly produced by aquatic bacteria (Ammerman and Azam, 1985), including genes observed in some cyanobacteria such as *Synechococcus* WH8102 (Moore *et al*., 2005). Although all *Prochlorococcus* genomes contain a gene that encodes for 5′-nucleotidase surE (PMM1271 in MED4, EC 3.1.3.5), neither this gene nor its protein product in MED4 was observed to be upregulated in response to P starvation (Martiny *et al*., 2006; Fuszard *et al*., 2010). Thus, there may be another, as yet unidentified, gene for a 5′-nucleotidase-like enzyme in MED4.

The significantly higher APA in the P-limited chemostat cultures, as much as an order of magnitude higher than in the batch cultures, is likely due to the fact that the MED4 cells are in a long-term (steady-state), P-limited physiological state as opposed to the continually changing conditions of a batch culture transitioning into P-starved stationary phase. Additionally, the time course of the batch experiment (Fig. 2) indicates that stationary-phase cells approach the high APA levels observed in the P-limited chemostats (Fig. 5a) only when they become 'overstressed' several days into stationary phase. Increases in APA levels as the batch cultures transition into P-starved phase were surprising since there were decreases in other physiological responses, such as P cell quota, relative chlorophyll fluorescence and specific affinities (Fig. 2), as well as decreases in gene expression of key P acquisition and regulatory genes, including *phoA* (Nahas Reistetter *et al*., 2013). We can only speculate that the differences in the timing of different physiological changes during stationary phase and/or differences in physiological responses for batch cultures relative to P-limited chemostats may reflect different regulatory mechanisms for cells in steady-state P-limited conditions compared with cells becoming more and more stressed in stationary phase. A more thorough study of stationary responses in *Prochlorococcus* would provide more insight.

The specific affinity (α_P) can be described as an organism's affinity for taking up a nutrient under limiting concentrations, in other words its 'oligotrophic capacity' with higher values characteristic of organisms better able to survive in oligotrophic environments (Healey, 1980; Button, 1991). Like other marine cyanobacteria (Fu *et al*., 2006), *Prochlorococcus* increases its α_P for PO_4 uptake as growth rate decreases and P becomes more limiting (Figs 2c and 4c). This increase is driven by increases in V_{max} since K_M is fairly constant across P limitation levels. Unlike *Synechococcus* (Fu *et al*., 2006), *Prochlorococcus* has a lower α_P for ATP than for PO_4. Interestingly, *Prochlorococcus* has a limited capacity to acclimate its α_P for ATP, reaching the highest affinity for ATP in P-limited chemostats (Fig. 4c). Like APA, ATP uptake is enhanced under the steady-state P-limited physiological state characteristic of the chemostats and reduced in P-starved stationary phase of batch cultures. A study of the time course of ATP uptake, APA and gene expression as batch cultures move from P-replete into P-starved stationary phase might help to further understand this physiological characteristic. When comparing values of α with other marine cyanobacteria (Table 2), *Prochlorococcus* has a higher α_P under P-limiting growth conditions than the filamentous *Trichodesmium* but is still not as competitive as *Synechococcus* CCMP 1334 based on the study by Fu and colleagues (2006). This is somewhat surprising as the small cell size of *Prochlorococcus* MED4 (diameter ~ 0.4 μm; Ting *et al*., 2007) translates to a higher surface area to volume ratio than *Synechococcus* (assuming a cell diameter of 1 μm; Waterbury *et al*., 1986): 15 μm^{-1} versus 6 μm^{-1}, respectively, which should provide a slight nutrient uptake advantage, although other factors such as P quota and K_M could improve or eliminate the advantage depending on the type of specific cyanobacteria and culture conditions. It is important to note there is only one known strain of unicellular cyanobacteria, marine A *Synechococcus* CCMP 1334 (= WH7803), for which these data are available (Fu *et al*., 2006). Thus, studies of additional strains of marine *Synechococcus* and side-by-side comparisons between *Prochlorococcus* and *Synechococcus* cultures are needed to better understand their relative competitiveness with respect to P uptake kinetics.

Based on our measurements we were able to estimate that the uptake rates for P-limited and P-starved cultured MED4 on a per cell basis are similar to those measured

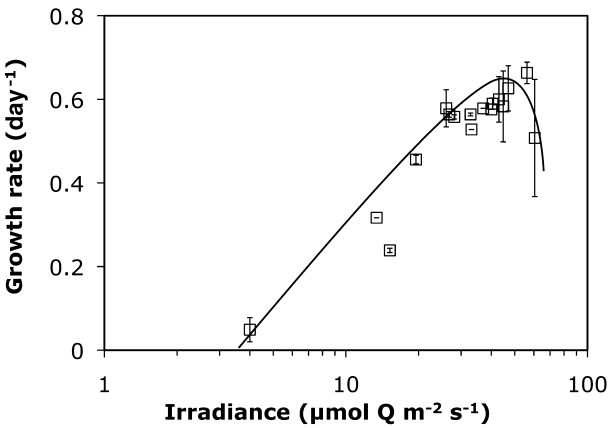

Fig. 7. Growth rate versus continuous irradiance (curve fitted by eye) for *Prochlorococcus* MED4. Symbols and error bars represent means and standard deviations of at least two cultures at any particular light level.

for natural populations at the low PO_4 concentrations present in the North Atlantic oligotrophic ocean. In the North Atlantic oligotrophic gyre, the cell-specific uptake rate for the *Prochlorococcus* population ranged from 0.15 to 0.5 amol P cell^{-1} h^{-1} [estimated from fig. 5b in Zubkov and colleagues (2007)] when the bioavailable PO_4 concentration in the waters was measured at 0.00275–0.005 μM. In the western North Atlantic Ocean, the uptake rate at SRP concentrations of 0.005 μM was ~ 10-fold lower at ~ 0.035 amol P cell^{-1} h^{-1} [from fig. 6 of Casey and colleagues (2009)]. In our MED4 cultures, we would estimate uptake rates of ~ 0.05, 0.05–0.14 and 0.0016 amol P cell^{-1} h^{-1} for cells that are P-starved, P-limited and P-replete, respectively, using an SRP concentration of 6.6 nmol P l^{-1} over 45 min (the incubation time of our uptake experiments), which was calculated from an estimate of SRP flux in the Sargasso Sea of 43 mmol m^{-2} year^{-1} (Lomas *et al*., 2010). From this we can infer that natural *Prochlorococcus* populations in the North Atlantic oligotrophic gyre during the study period were neither P-replete nor P-starved but likely P-limited to some degree, whereas those *Prochlorococcus* in the western North Atlantic Ocean are likely P-starved. The use of genetic markers, such as the expression of *phoR* and *pstS* genes (Nahas Reistetter *et al*., 2013), could further help with understanding the P status of *Prochlorococcus* in these oligotrophic gyres. Another aspect to consider in the interpretation of field measurements is that the dominant *Prochlorococcus* ecotype in the low P surface waters of the North Atlantic is HLII (eMIT9312) type rather than the HLI (eMED4) type (Ahlgren *et al*., 2005; Zinser *et al*., 2006; Malmstrom *et al*., 2010), and P uptake kinetics may differ among different ecotypes and even different strains of *Prochlorococcus*. Thus, additional ecotypes of *Prochlorococcus* should be examined for their uptake kinetics before adopting the uptake kinetic parameters presented in this study for modelling all *Prochlorococcus* populations.

Overall, the results of this study reveal that like other phytoplankton, *Prochlorococcus* MED4 regulates its uptake kinetics in response to P stress, increasing its V_{max} as cells become P-limited. Although V_{max} on a per cell basis is lower than other phytoplankton, the K_M and α_P for PO_4 is competitive with other phytoplankton. We also found that *Prochlorococcus* MED4 exhibits 5′-nucleotidase activity, despite the lack of a known 5′-nucleotidase gene involved in scavenging phosphate from organic molecules such as ATP. Finally, we hypothesize that the timing of different physiological responses to P stress, particularly in P-starved stationary phase, indicates complex regulatory processes that need further examination to fully understand the response of *Prochlorococcus* to conditions of P stress.

Experimental procedures

Culturing conditions

Axenic *Prochlorococcus* MED4 was grown in both batch and continuous (chemostat) cultures at a temperature of 24°C (± 1), under a continuous light level of 50 (± 3) μmol quanta m^{-2} s^{-1}. At this continuous irradiance level *Prochlorococcus* MED4 reached maximum, light-saturated growth rate, as determined by slowly acclimating cultures to increasingly higher continuous irradiances levels until increases in growth rate were no longer observed and a drop in steady-state growth rate occurred (Fig. 7). Cell concentrations and relative chlorophyll fluorescence per cell (as measured flow-cytometrically compared against a 0.57 μm bead standard) of all cultures were monitored using a Becton-Dickinson FACS-Calibur flow cytometer with methods described by Marie and colleagues (2005).

All cultures were grown in 'AMP1C' media, a modification of the artificial seawater-based culture media AMP1 (Moore *et al*., 2007). Modifications include a 10-fold reduction in bicarbonate ($NaHCO_3$, 0.6 mM final concentration) to eliminate precipitation that occurred in the chemostat setup at higher concentrations of $NaHCO_3$, an increased concentration of HEPES buffer (10 mM final concentration, pH 8) to maintain neutral pH and a 50-fold reduction in NaH_2PO_4 (1.15 μM final concentration) creating an N : P ratio of 348 [400 μM final concentration of $(NH_4)_2SO_4$] to invoke P limitation in chemostat cultures and P starvation in batch cultures. The media used for N-limited chemostat controls contained 25 μM $(NH_4)_2SO_4$ and 10 μM NaH_2PO_4, resulting in an N : P ratio of 5:1. All nutrients, buffers and trace metals were sterilized through 0.22 μm syringe filters and added aseptically to autoclaved Turks Island Salt Mix (Rippka *et al*., 2000). All plastic and glassware used for media preparation, batch cultures and all parts of the chemostat setup were acid-cleaned and autoclaved prior to use (Moore *et al*., 2007). All cultures were checked routinely with Marine Purity Test Broth (Bertilsson *et al*., 2003) to verify that they remained free of heterotrophic bacterial contamination. Periodically, samples

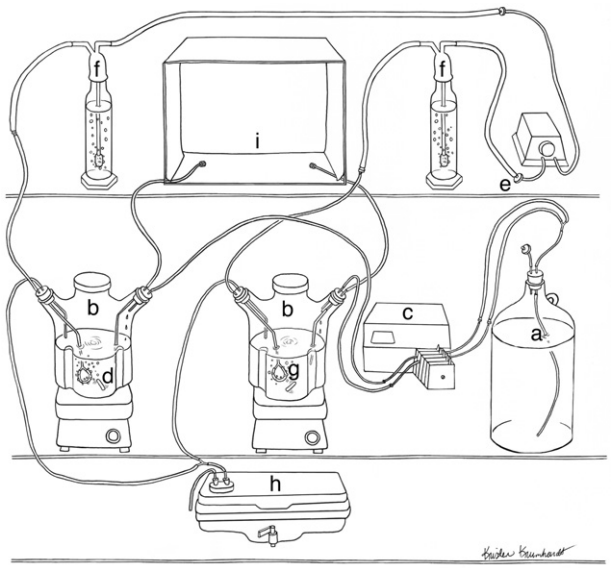

Fig. 8. Replicate chemostat design was composed of a sealed 4 l polycarbonate culture flask containing AMP1C medium (**a**) pumped into two culture flasks (**b**) with a peristaltic pump (**c**) at set rates (*D*) that varied between 0.5 and 0.1 day^{-1}. Cultures were continuously mixed with a magnetic stir bar (**d**) for fast dispersion of media. Fresh air was passed through a 0.22 μm filter (**e**) and then humidified using a gas washing bottle (**f**), bubbled through fine holes in a silicon tubing loop immersed in the culture (**g**), putting the system under positive pressure and forcing excess culture out a glass tube fixed at the 1 l mark into the waste container (**h**). Samples from each chemostat were withdrawn through glass tubes immersed in the cultures with sterile syringes in a sterile area (**i**).

of cultures were stained with 4′-6′-diamidino-2-phenolindole (DAPI) and observed under an epifluorescence microscope (Hobbie *et al.*, 1977) to further verify the absence of heterotrophic bacteria.

Chemostat culturing system

Replicate 1 l chemostat cultures (Fig. 8) were diluted with fresh media at a rate of 0.5 to 0.1 l day^{-1}, depending on the desired growth rate (μ), i.e. extent of P limitation. After altering the rate, chemostat cultures were allowed at least 10 days (and up to 30 days for the 0.1 day^{-1} and 0.2 day^{-1} chemostat cultures) before experimentation to reach steady state, at which point μ = dilution rate (Fig. 1). Small (~ 1 ml) samples were taken every 1–2 days for flow cytometric measurements to ensure steady state (constant culture concentration and constant relative chlorophyll fluorescence) prior to uptake experiments (Fig. 1). Larger samples (> 100 ml) were taken on the day of uptake experiments.

P uptake experiments with ^{32}P

Short-term P uptake experiments were performed on all chemostat and batch cultures. Concentrations of non-radioactive ('cold') PO_4 or ATP ranging between 0 and 20 μM (typical additions were 0.02, 0.5, 1, 2, 5, 10 μM) were distributed in clear microcentrifuge tubes. Trace amounts of ^{32}P-PO_4 (0.2–3.8 μCi, or 0.06–0.88 pmol, calculated based on specific activity on day of experiment) or [γ-^{32}P]-ATP (0.15–3 μCi, or 0.6–11 pmol, calculated based on specific activity on day of experiment) were then added, followed by 1 ml of culture samples to each tube. Both non-radioactive ATP and [γ-^{32}P] ATP were kept frozen until immediately before use. The same amount of ^{32}P-PO_4 or ^{32}P-ATP was also added to tubes with no cells (containing sterile media) to obtain blanks for uptake. Cultures were incubated at a light level of 50 μmol quanta m^{-2} s^{-1} for 45–60 min for P-limited and P-starved cultures and 120 min for P-replete cultures. Uptake of ^{32}P during this time period was linear as verified with a time course of P uptake at 20 μM PO_4 by P-limited chemostat cultures (data not shown). Uptake was stopped by an excess concentration of cold NaH_2PO_4 (final concentration 1 mM), and cells were harvested by filtration within 30 min.

The amount of ^{32}P taken into the cells was measured by filtering a known volume of the samples through 0.22 μm polycarbonate membrane filters, supported by Whatman GF/F filters pre-soaked with 0.5 mM PO_4 AMP1 media, in order to minimize any non-specific adhesion of ^{32}P onto the filters. In order to eliminate the measurement of ^{32}P adhered to the outside of the cells (i.e. to measure only 'intracellular uptake'), the filtered samples were subsequently followed by a 10 min soak then filtration with a basic oxalate reagent (Tovar-Sanchez *et al.*, 2003). Rinsing cells with oxalate reagent was determined to be sufficient for removing ^{32}P adhered to cell surfaces, as there was no statistical difference in adhered ^{32}P-PO_4 when comparing a high PO_4 rinse (100 μM PO_4) of a P-limited *Prochlorococcus* culture (data not shown). The vacuum was kept low during all filtrations and allowed to run for at least 30 s after filters appeared dry to obtain low blanks. After rinsing, filters were immersed in 3 ml of Optiphase Hi-Safe scintillation cocktail, and radioactivity measured on a Perkin Elmer 1450 MicroBeta scintillation counter.

Velocity of uptake (V in units of fmol P $cell^{-1}$ h^{-1}) was calculated using the following equation, according to the methods of Fu and colleagues (2005a):

$$V = [P(R_f - R_b)]/(R_t tcv),$$

where R_f is radioactivity on filter, R_b is radioactivity of 'no cells' blank and R_t is the total ^{32}P available to cells, all in units of DPM. The concentration of cold PO_4 or cold ATP supplied to cells is given by P (fmol P l^{-1}), time (h) by t, cell concentration (cells l^{-1}) by c and volume filtered (l) by v. The final concentration of cold P in each tube included any measurable residual PO_4 for the P-replete samples but not the P-limited and P-starved cultures since the residual PO_4 was below the detection limit of the method used. V, plotted against cold P concentration, S, was fitted to a Michaelis–Menten equation,

$$V = (V_{max}S)/(K_M + S),$$

and point estimates of uptake kinetic parameters, the Michaelis–Menten constant (K_M) and maximum velocity of P uptake (V_{max}), were obtained using an iterative non-linear regression procedure available on Systat 12.0 (Systat Software, Richmond, CA, USA). The same approach was used for P-specific uptake rates, V_P, which were calculated by normalizing the uptake, V, by the particulate organic phosphorus (Q, see below), from which P-specific maximum

uptake rate, $V_{max,P}$, and P-specific Michaelis–Menten constant, $K_{M,P}$, were determined. Specific affinity (α_P; l μmol P^{-1} h^{-1}) was calculated from P-specific $V_{max,P}$ (= V_{max}/Q; h^{-1}) divided by $K_{M,P}$.

Additional ATP uptake experiments

In order to test whether *Prochlorococcus* MED4 can take up the entire ATP nucleotide, we measured side-by-side uptake of ATP radiolabelled with ^{14}C on its eighth carbon (0.02 μCi, or 426 pmol, based on specific activity on day of experiment) within its carbon backbone versus uptake of ^{32}P-ATP (0.77 μCi, or 328 pmol, based on specific activity on day of experiment) using P-starved and N-starved MED4 batch cultures. If both ^{14}C-ATP and ^{32}P-ATP were taken up, then the whole nucleotide molecule is incorporated into the cell. However, if only ^{32}P-ATP is taken up, then only the PO_4 functional group of the nucleotide is being incorporated. Uptake of ^{32}P-ATP was also measured on P-starved MED4 batch cultures with the addition of various organic P substances in order to determine if alkaline phosphatase (APase) or possibly a 5′-nucleotidase-like enzyme was involved in cleaving the PO_4 moiety from the organic compounds. The organic P substances tested were ATP, cytosine triphosphate (CTP), cyclic 3′, 5′ adenosine triphosphate (cAMP), glycerophosphate (GYP) and glucose-6-phosphate (G6P), all at a 10 μM final concentration. Uptake procedures were followed as described in the previous section.

Measurement of alkaline phosphatase activity

Alkaline phosphatase activity was measured fluorometrically on a Turner TD-700 fluorometer using the fluorogenic compound methylumbelliferyl phosphate (MUFP; Peterson and Jansson, 1978). MUFP has a monophosphate ester bond that serves as a substrate for alkaline phosphatase, which cleaves the PO_4 group from MUFP, producing the fluorescent product 4′-methylumbelliferone (MUF; ~ 360 nm excitation and 449 nm emissions). Standard curves of MUF concentration were measured and used to convert MUF emission to PO_4 that has been cleaved. The rate of PO_4 cleaved was normalized to cell concentration to give units in amol P h^{-1} $cell^{-1}$. Alkaline phosphatase activity was measured on all chemostat and batch cultures on the day that P uptake experiments were carried out.

Determination of dissolved inorganic P and particulate organic P

To obtain residual PO_4 concentrations within chemostat and batch cultures of *Prochlorococcus*, soluble reactive P (SRP) was measured spectrophotometrically on culture media after filtering out cells using a Cary 50Bio UV/Vis-spectrophotometer and the method of Murphy and Riley (1962). The detection limit of the SRP method in our hands was 0.05 μM. Due to a variety of circumstances, we were unable to measure dissolved inorganic P using other more sensitive methods. We measured particulate organic P (= cellular P quota, Q) for many cultures on samples taken concurrently with each uptake experiment rather than estimating P quota using ambient concentration of SRP. Between 25 and 75 ml of culture was filtered onto pre-combusted Whatman GF/F filters and stored at –80°C. Cellular phosphorus was converted to orthophosphate by high-temperature combustion and acid extraction following the particulate phosphorus protocol outlined in Karl and colleagues (1991) with the addition of an additional heated extraction step. Filters were combusted at 450°C for 4.5 h in acid washed glass tubes. Ten millilitres of 0.15 M HCl was added and samples were incubated at 90°C for 30 min. Tubes were centrifuged 30 min at 2800 *g* and 5 ml of supernatant was transferred to clean 15 ml tubes with 0.5 ml of ammonium molybdate mixed reagent and incubated for 1 h at room temperature in the dark. Absorbance at 880 nm was measured using a Molecular Devices SpectraMax M2 spectrophotometer against a distilled water reference. Samples were compared against phosphate standards and procedural blanks.

Acknowledgements

We would like to thank several undergraduate students from USM who helped with sampling during the intense uptake experiments: D. Hardy, M. Ibourk, P. Jacobs and A. Lorrain. We would also like to thank Adam Martiny for helpful discussion. This work was supported by NSF-OCE collaborative grants 0453019 and 0453029 to L. R. M. and G. R. respectively. K. K., K. R. J., T. S. and D. R. were supported by NSF-OCE collaborative grant 0453019; K. C. was supported by a USM Graduate Assistantship; E. A. N. R. by the ARCS Foundation; J. K. S. by an NSF graduate student fellowship; and several USM students were supported with Federal Work Study funds. None of the authors have a conflict of interest.

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