

Production of organic matter by *Trichodesmium* IMS101 as a function of phosphorus source

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Abstract

Herein we present the results of a series of laboratory experiments conducted to delineate the relationship between dissolved organic phosphorus (DOP) utilization and organic matter production by the model diazotroph *Trichodesmium* IMS101. The rate and ratio of *Trichodesmium* carbon (C) and nitrogen (N) production driven by phosphorus (P) derived from select phosphonates and P monoesters were not found to be significantly different than growth rates based on dissolved inorganic phosphorus (DIP). Conversely, despite the observed similarities in C and N fixation rates between parallel DIP and DOP incubations, cultures grown solely on methylphosphonate (MPn) had reduced maximal P content per unit chlorophyll *a* or C relative to cultures grown on P monoesters or DIP. So while *Trichodesmium* IMS101 appears to be able to maintain similar rates of growth on DIP and select DOP substrates, the P uptake efficiency and, hence, the P content per cell is greater for growth on DIP or tested P monoesters relative to growth on the phosphonate MPn. The primary conclusion arising from these results is that neither the observation of P monoester hydrolysis nor highly P-deficient organic matter or expression of phosphonate hydrolyzing genes can be interpreted to indicate P limitation of C or N fixation of *Trichodesmium* spp. under light-saturated, DIP-deficient conditions.

Phosphorus (P) acquisition is required for the growth and maintenance of all living organisms. In the marine environment most, if not all, phototrophs have the capacity to meet their P demands via the active transport of dissolved inorganic phosphate (DIP) across the cell membrane (Cembella et al. 1982). The biochemical basis for DIP acquisition involves the adenosine-5'-triphosphate (ATP)-dependent expression of specific classes of transport proteins (Dyhrman et al. 2007). Under conditions in which the external supply of DIP is low relative to biological demands or uptake potential, microorganisms must invoke alternative pathways for P capture to maintain net positive growth rates. In regions such as the vast oligotrophic gyres of the oceans, where DIP concentrations in surface waters are less than 100 nmol L⁻¹, microbes have adapted to cope with the reduced availability of inorganic P pools. Illustrations of known adaptations to DIP limitation include the reduction of cellular P quotas (Bertilsson et al. 2003; White et al. 2006; Van Mooy et al. 2009), the ability to store inorganic P during periods of excess P availability (generally as polyphosphates) for later utilization (Kornberg 1995), and the up-regulation of enzymatic pathways designed for the hydrolysis of dissolved organic P (DOP) (Yentsch et al. 1972; Hoppe 2003).

Given that DOP concentrations in the open ocean can be approximately five times higher than those of DIP (Karl and Björkman 2002), DOP represents a potentially important P resource for microorganisms. In general,

DOP is a term that encompasses a plethora of P-containing compounds, including nucleic acids, nucleotides, phospholipids, sugar phosphates, and phosphonates. In the marine environment Kolowitz et al. (2001) have found that the high molecular weight (HMW) fraction of DOP is primarily composed of the labile carbon (C)-oxygen (O)-P monoester bond class and highly stable phosphonates having a direct C-P bond. By no coincidence, well-characterized enzymatic pathways have evolved to degrade these predominant components of HMW marine organic matter. Phosphatases, a general class of ectoenzymes able to cleave P monoesters, have been identified in bacteria, cyanobacteria, and eukaryotes (Perry 1972; Cembella et al. 1982; Hoppe 2003). In prokaryotes, hydrolysis of alkyl-linked phosphonate species and the corresponding hydrocarbon production (Karl et al. 2008) are generally considered to proceed via expression of distinct degradative pathways, including the broad-specificity multienzyme system termed C-P lyase (Ternan et al. 1998; Quinn et al. 2007; White and Metcalf 2007). The expression of C-P lyase and active phosphonate degradation has recently been documented in the marine environment (Dyhrman et al. 2006; Karl et al. 2008) using in situ and cultured populations of bacteria and select nitrogen (N)₂-fixing cyanobacteria (e.g., *Trichodesmium* spp.).

Relative to the composition of dissolved P pools, each of the aforementioned strategies for P acquisition (cellular P reduction as well as utilization of internal P stores, external sources of P monoesters, and phosphonates) may have consequences for growth, productivity, and diversity of microbial communities in regions where DIP is limiting. In addition, taxonomic differences in these strategies could explain the coexistence of multiple N₂-fixing photoautotrophic taxa in the surface layers of tropical and

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Table 1. Light acclimation, growth phase, and P status of parent cultures for each experiment (Expt).

Expt	Maximum daily growth irradiance ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)	Integrated daily growth irradiance ($\text{mol photons m}^{-2}$)	Light cycle	Growth phase at initial transfer	P status of parent culture
I	500	13.7	Sinusoidal	Stationary phase	DIP deplete
II	125	5.5	12:12 LD	Stationary phase	DIP deplete
III	325	14.3	12:12 LD	Exponential phase	DIP replete

subtropical regions of the oligotrophic ocean (Church et al. 2009). While recent work has begun to elucidate the composition of organic matter in the ocean and the enzymatic degradation pathways for these compounds, an understanding of the implications of each of these P-acquisition strategies for the production of organic matter is lacking. Herein we seek to investigate the relationship between DIP and DOP utilization and the stoichiometric production of organic C, N, and P using batch cultures of the diazotrophic (N_2 -fixing) cyanobacterium *Trichodesmium* IMS101. This diazotrophic strain was selected for this study because it expresses all of the afore-noted P-acquisition strategies, has a full genome sequence available, and is broadly distributed in the tropical and subtropical gyres of the world ocean (Capone et al. 1997), where DIP can often be at limiting concentrations. Our results have implications for resource partitioning in nature as well as direct utility for efforts to model metabolism in the sea.

Methods

Experimental design—A series of batch cultures was initiated in order to monitor growth of *Trichodesmium* IMS101 on select DOP compounds, compared to growth on DIP. The primary objective of these experiments was to determine the extent to which P sources (inorganic, monoester-linked, or phosphonate sources) affect the production of organic C, N, and P by *Trichodesmium*. The model DOP compounds used in these experiments included the P monoesters D-glucose-6-phosphate (G6P, Sigma-Aldrich G7250, $\geq 98\%$) and adenosine-5'-monophosphate (AMP, Sigma-Aldrich A1752, $\geq 99\%$) as well as the phosphonates methylphosphonate (MPn, Sigma-Aldrich 64259, $\geq 98\%$), 2-aminoethylphosphonic acid (2-AEP, Sigma-Aldrich 268674, $\geq 99\%$), and 2-amino-3-phosphonopropionic acid (phosphonoalanine, Sigma-Aldrich 09247, $\geq 99\%$). The experimental conditions and growth status of all experiments prior to transfer are described in Table 1. For all experiments, P pools (particulate P [PP], total dissolved P [TDP], DOP, and soluble reactive P [SRP, here equivalent to DIP]), chlorophyll (Chl) concentrations, and particulate C and N were monitored until each treatment reached either exponential phase or stationary phase (Table 1), as indicated by Chl fluorescence. The use of batch cultures in this study prevents any assumptions regarding acclimated or steady-state growth. Rather, our experimental design was implemented to achieve P mass balance and to investigate the growth rates and stoichiometry of batch cultures grown on select DOP substrates vs. DIP. The growth stage of experimental inoculants prior to transfer

was monitored in order to address the potential role of luxury P uptake.

An initial experiment (expt I) was designed to test the capacity of *Trichodesmium* IMS101 to utilize 2-AEP, AMP, MPn, and G6P and to ensure mass balance of P pools. *Trichodesmium* biomass was harvested from stationary-phase cultures that had been grown on DIP (initial concentration = 500 nmol L^{-1}) as the sole P source (Table 1). For each treatment, duplicate aliquots of the parent culture were diluted (1:10) into YBCII media (Chen et al. 1996) with no added P. DOP stocks (2-AEP, AMP, or G6P) were then added to achieve final concentrations of 500 nmol L^{-1} . Treatments were incubated in 250-mL borosilicate glass bottles with Viton® septa and aluminum crimp seals in order to achieve a gas-tight seal and to allow for determination of biogenic gases at each sampling point (Beversdorf et al. 2010). Duplicate bottles were sacrificed at each time point for analyses. A second, longer time-scale experiment (expt II) was carried out with three separate treatments, as follows: (1) MPn (initial concentration = 5 mol L^{-1}); (2) DIP (initial concentration = $5 \mu\text{mol L}^{-1}$); and (3) equimolar concentrations of MPn plus DIP (initial concentration = $2.5 \mu\text{mol L}^{-1}$ each). The parent culture for this experiment was harvested from stationary growth (Table 1) in media previously inoculated with a starting DIP concentration of $5 \mu\text{mol L}^{-1}$. *Trichodesmium* biomass was transferred from this parent culture via gentle filtration onto $5\text{-}\mu\text{m}$ -porosity polycarbonate filters, followed by resuspension in media without added DIP to eliminate carryover. Duplicate aliquots of this concentrated biomass were diluted in a 1:10 ratio in 2-liter bottles with YBCII media (no added P). P additions were made (MPn, DIP, or MPn + DIP) to achieve initial TDP concentrations of $5 \mu\text{mol L}^{-1}$.

Lastly, the dynamics of organic matter production (derived from changes in Chl and particulate C, N, and P) of cultures grown on G6P and phosphonoalanine as a sole P source were examined relative to growth on DIP alone (expt III). Duplicate treatments were initiated as described above, with the starting material isolated from a parent culture in exponential growth with DIP as the sole P source (Table 1). In this round of experiments, duplicate batch cultures (2 liters) were grown on G6P, phosphonoalanine, or DIP (all initial concentrations = $5 \mu\text{mol L}^{-1}$) until Chl concentrations stabilized, indicating that populations had achieved stationary phase. Cultures were inoculated with reciprocal substrates (e.g., G6P was added to the DIP treatments and DIP was added to G6P treatments) in order to investigate the role of nutritional history in determining the stoichiometry of organic matter production by *Trichodesmium*. In this last experiment, N_2

fixation was also monitored via the acetylene reduction assay (Capone 1993).

In concert with these experiments, we conducted a series of controls in order to assess the activity of potentially contaminating heterotrophic bacteria. Because there are no axenic cultures of *Trichodesmium* IMS101 available, all efforts were made to minimize the abundance of heterotrophic bacteria. The abundance and growth of heterotrophic bacteria were assessed via enumeration of 4',6-diamidino-2-phenylindole-stained material collected onto an 0.2- μm black Nucleopore filter (Sherr et al. 2001). Additionally, a 5 $\mu\text{mol L}^{-1}$ spike of either G6P or MPn was added to duplicate 1-liter volumes of YBCII media containing no *Trichodesmium* biomass but having heterotrophic bacteria transferred from an actively growing parent culture. DIP and TDP levels were monitored in this negative control in order to assess the potential hydrolysis of DOP compounds via contaminating bacteria. These controls were incubated under identical conditions as experimental treatments. Finally, because of the high sensitivity of the assay, methane production from the decomposition of MPn was monitored over time in treatments with MPn added to the < 2- μm filtrate from exponentially growing *Trichodesmium* cultures (see Beversdorf et al. [2010] for analytical methods for methane determinations).

Experimental conditions—All cultures of *Trichodesmium* IMS101 were maintained in batch culture under controlled temperature (24°C) and irradiance. As a result of practical considerations regarding incubator space and the timing of each experiment, cultures were acclimated to different light regimes for individual experiments (Table 1). A sinusoidal light cycle with a daily maximum of 500 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ was used for expt I. Experiments II and III were grown under a 12:12 light:dark (LD) cycle with fixed irradiances of 125 and 325 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$, respectively (Table 1). Goebel et al. (2008) report the light saturation parameter of the growth vs. irradiance curve for *Trichodesmium* IMS101 to be $73 \pm 29 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. Additionally, Breitbart et al. (2008) find saturation of *Trichodesmium* IMS101 growth rates at 180 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ (albeit growth was not monitored at irradiances between 50 and 180 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$). Neither Breitbart et al. (2008) nor Goebel et al. (2008) observed photoinhibition within the range of light intensities used in our study (125–500 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$). Based on these values for growth saturation, each of the light levels used herein is assumed to be sufficient to achieve maximal growth rates. This assumption can be assessed by evaluating differences in C-based specific growth rates and C:Chl ratios as proxies for potential light limitation of growth and photoacclimation, respectively (MacIntyre et al. 2002).

Growth medium in all cases was an artificial seawater preparation lacking combined N (Chen et al. 1996) and amended with varying P sources, as per the experimental design. Inorganic P contamination of this artificial media is unavoidable as a result of impurities of even the high-grade primary salts. The media used for these experiments

consistently had DIP levels of $184 \pm 43 \text{ nmol L}^{-1}$ ($n = 27$). These values are included in time zero measurements of DIP.

Dissolved and particulate measurements—DIP was measured according to the molybdenum blue method of Murphy and Riley (1962). TDP was determined by the Valderrama method (1981). Simply, dissolved samples were oxidized at 120°C for 40 min in Teflon® digestion bombs, allowed to cool, and run as DIP, as described above. The oxidizing agent used was a solution of potassium persulfate, sodium hydroxide, and boric acid added in a 1:10 oxidant to sample ratio. This method of TDP analysis is sufficient to fully recover simple P monoesters as well as phosphonates (Table 1). DOP concentrations were calculated as the difference between TDP and DIP. DOP values were also corrected for hydrolysis during the phosphomolybdate-blue reaction (Monaghan and Ruttenberg 1999). On average, 2–3% of freshly prepared P monoester stocks were hydrolyzed during the phosphomolybdate reaction. Phosphonate stock solutions were not reactive.

PP concentrations were measured spectrophotometrically as DIP following combustion (450°C, 5 h) and acid hydrolysis (0.15 mol L⁻¹ HCl, 60 min at 60°C), according to the method of Karl et al. (1991). Recovery efficiency was assessed from the analysis of a known dry weight of certified reference material (National Institute of Standards, NIST 1515, apple leaves, certified 0.159% P by weight). Particulate carbon (PC) and particulate nitrogen (PN) were analyzed on a Carlo Erba elemental analyzer using acetanilide (71.09% C and 10.36% N by weight) as the primary standard. All error terms shown represent the standard deviation (σ) for duplicate measures, unless otherwise noted. Error terms for all elemental ratios were calculated using error propagation techniques (Bevington and Robinson 2003). Filter blanks processed during laboratory experiments had an average C, N, and P dry weight of 6.6 $\mu\text{g C filter}^{-1}$ ($\sigma = 2.9$, $n = 24$), 0.55 $\mu\text{g N filter}^{-1}$ ($\sigma = 0.27$, $n = 24$), and 0.07 $\mu\text{g P filter}^{-1}$ ($\sigma = 0.01$, $n = 29$), respectively. Chl was determined fluorometrically following 24-h extractions in 90% acetone (at -20°C in the dark), as described by Strickland and Parsons (1972).

Acetylene reduction and net N fixation—N fixation rates were approximated using the acetylene reduction technique described by Capone (1993). At multiple points of the growth cycle, duplicate 15-mL subsamples of batch cultures growing on G6P, DIP, or phosphonoalanine were placed into 25-mL glass serum bottles, fitted with Viton® septa and aluminum caps, and crimp sealed to prevent gas exchange. Acetylene (C₂H₂) produced from calcium carbide (Aldrich) was injected into each sample bottle at a final volumetric gas to headspace ratio of 10%. Subsamples (100 μL) of the headspace were removed at ~ 2-h intervals over the assay period and analyzed for ethylene (C₂H₄) by flame ionization gas chromatography using a Shimadzu GC-8A. All samples were corrected for C₂H₄ remaining in the liquid phase according to the method of Breitbart et al. (2004) and via use of media blanks, the background levels of C₂H₄ generated by the calcium carbide-water

Table 2. Recovery of select compounds by the Valderrama (1981) method. The number of recovery samples run (n) differs because G6P and MPn were utilized in more experiments than were the other compounds.

Compound	n	% Recovery (σ)
Glucose-6-phosphate monosodium salt (G6P)	13	93.8 (6.4)
Adenosine-5'-monophosphate monohydrate (AMP)	5	97.9 (4.4)
Methylphosphonate (MPn)	18	109.7 (12.8)
2-Aminoethylphosphonic acid (2-AEP)	5	100.4 (4.2)

reaction (Hyman and Arp 1987). Acetylene reduction rates (equivalent to ethylene production) were calculated by applying a linear fit to C_2H_4 evolved over time ($n =$ four time points) in duplicate samples. A conversion factor of 4 mol C_2H_2 per mol N_2 was used to convert acetylene reduction rates to N_2 fixation (Jensen and Cox 1983). Net N_2 fixation was also assessed by measurement of the rate of change of PN concentrations.

Results

TDP analyses and hydrolytic controls—Preliminary experiments confirmed that the variable pH oxidation of Valderrama (1981) allowed for full recovery (Table 2) of each of the organic compounds used herein. This finding is significant, as reports abound of the differential recovery of phosphonates and P monoesters via wet oxidation (Ridal and Moore 1990; Thomson-Bulldis and Karl 1998; Monaghan and Ruttenberg 1999). Parallel incubations were also conducted to assess the potential role of heterotrophic bacteria in the hydrolysis of organic P

Table 3. Phosphorus balance for expts I–III. Total DOP drawdown and PP accumulation reflect the net change in each property. Specific PP-based growth rates (μ) were calculated as the slope of the linear phase of natural log-normalized values. Error estimates are not available for expt I or expt III (stage 2) because maximal PP values were achieved within a single day. In expt III, the phosphonoalanine treatment was not carried over for the second stage.

Treatment (target P additions)	DOP drawdown ($\mu\text{mol L}^{-1}$)	PP increase ($\mu\text{mol L}^{-1}$)	DOP drawdown PP increase $^{-1}$ (%)	μ (d^{-1}) (PP)
Experiment I				
G6P ($0.5 \mu\text{mol L}^{-1}$)	0.54 ± 0.04	0.71 ± 0.19	75 ± 21	0.80
MPn ($0.5 \mu\text{mol L}^{-1}$)	0.58 ± 0.01	0.71 ± 0.16	81 ± 18	0.47
2-AEP ($0.5 \mu\text{mol L}^{-1}$)	0.64 ± 0.02	0.61 ± 0.04	104 ± 9	0.65
AMP ($0.5 \mu\text{mol L}^{-1}$)	0.52 ± 0.04	0.71 ± 0.12	73 ± 14	0.85
Experiment II				
MPn ($5.0 \mu\text{mol L}^{-1}$)	5.00 ± 1.26	4.46 ± 0.44	112 ± 30	0.14 ± 0.01
DIP ($5.0 \mu\text{mol L}^{-1}$)	0.33 ± 0.11	4.66 ± 1.13	7 ± 3	0.18 ± 0.03
MPn + DIP ($2.5 + 2.5 \mu\text{mol L}^{-1}$)	1.68 ± 0.22	4.18 ± 0.61	40 ± 8	0.14 ± 0.04
Experiment III: phase 1 (days 0–16)				
DIP ($5.0 \mu\text{mol L}^{-1}$)	0.03 ± 0.57	4.88 ± 0.27	1 ± 12	0.33 ± 0.02
G6P ($5.0 \mu\text{mol L}^{-1}$)	3.57 ± 0.29	3.68 ± 0.08	97 ± 8	0.32 ± 0.02
Phosphonoalanine ($5.0 \mu\text{mol L}^{-1}$)	0.19 ± 0.31	0.40 ± 0.17	No growth	No growth
Experiment III: phase 2 (days 16–29)				
DIP ($5.0 \mu\text{mol L}^{-1}$)	0.12 ± 0.14	5.10 ± 0.65	2 ± 3	0.79
G6P ($5.0 \mu\text{mol L}^{-1}$)	4.38 ± 0.14	4.35 ± 0.06	101 ± 3	0.92

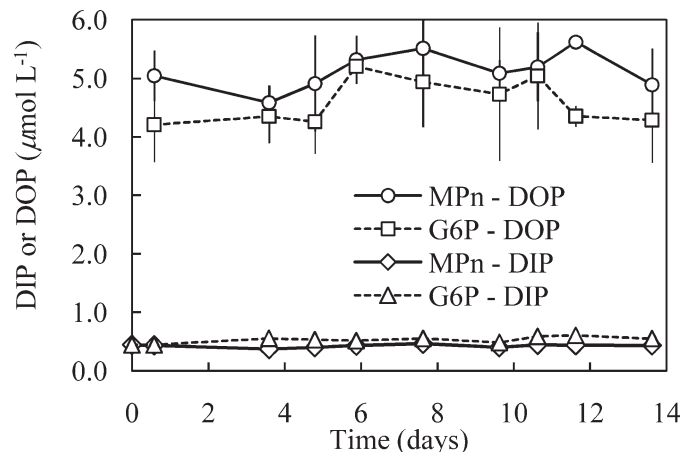


Fig. 1. Control experiments, containing heterotrophic bacteria transferred from the $< 2\text{-}\mu\text{m}$ filtrate from active *Trichodesmium* cultures but with no *Trichodesmium* cells, indicate that neither MPn nor G6P is hydrolyzed. Additionally, DIP accumulation was not evident over the course of a 14-d incubation.

compounds. Bacterial abundance measured over the course of incubations with MPn and G6P was unchanged ($\sim 10^5 \text{ mL}^{-1}$) during the exponential growth phase of *Trichodesmium*. Moreover, when $5 \mu\text{mol L}^{-1}$ MPn was added to the $< 2\text{-}\mu\text{m}$ filtrate of exponentially growing *Trichodesmium* cultures, methane accumulation (CH_4 , the by-product of MPn hydrolysis) over a 40-h time period was negligible ($0.45 \text{ nmol CH}_4 \text{ L}^{-1}$) relative to that of positive controls with *Trichodesmium* ($8.8 \text{ nmol CH}_4 \text{ L}^{-1}$). Lastly, in incubations of media with contaminating bacteria ($1.5 \times 10^5 \pm 3.2 \times 10^4$, $n = 3$) and no *Trichodesmium* biomass, neither DOP hydrolysis nor DIP accumulation was evident

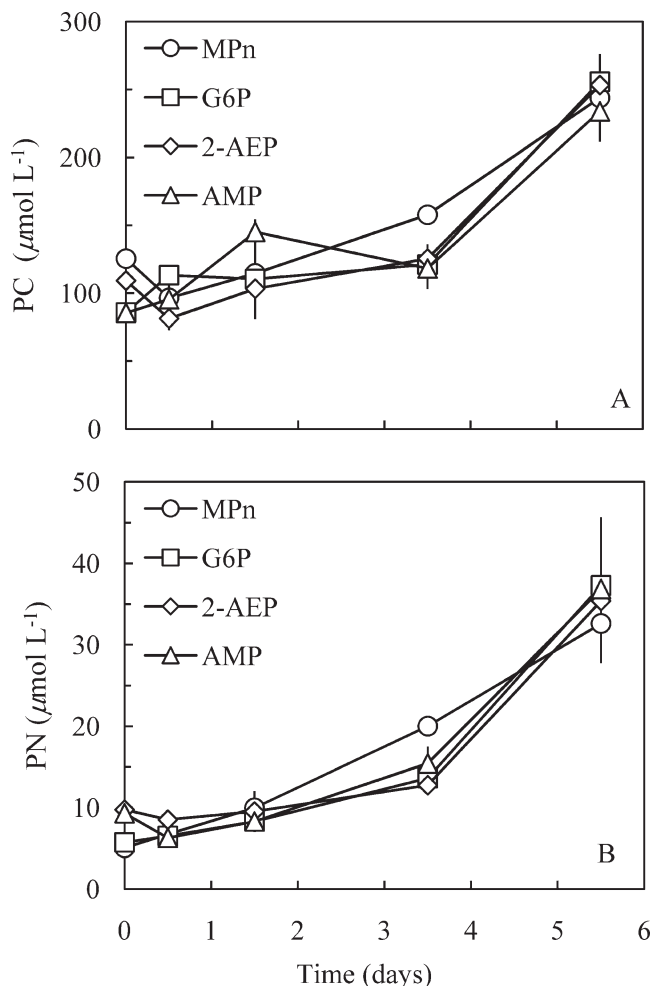


Fig. 2. Time course of (A) PC and (B) PN in *Trichodesmium* IMS101 batch cultures grown on YBCII media containing initial concentrations of 500 nmol L⁻¹ of MPn, G6P, 2-AEP, or AMP.

(Fig. 1). These results and achievement of mass P balance over the course of our experiments indicate that the bulk of observed DOP hydrolysis was attributable to *Trichodesmium*.

Growth and P content as a function of P substrate—With the exception of phosphonoalanine, *Trichodesmium* IMS101 was able to utilize all tested compounds as a P substrate for growth (MPn, G6P, 2-AEP, and AMP; Table 3; Figs. 2, 3). Over the course of all of these batch culture experiments, PP accumulation reflected the draw-down of DOP (Table 3; Fig. 3; note that initial P concentrations differed slightly between treatments for expt II). For expt I, in which initial DOP concentrations were 500 nmol L⁻¹, PP concentrations reached maximal values after a single day of growth (data not shown). For this reason, higher starting P concentrations were used in subsequent experiments in order to compare differences in organic matter production and P utilization.

P accumulation as a function of substrate can readily be compared when PP data are normalized to Chl or PC (Figs. 4, 5). For expt II, cultures transferred from

exponentially growing parent cultures and inoculated with G6P or DIP achieved statistically similar P content per Chl or PC, and these internal pools were utilized at similar rates (Fig. 5A,B). Moreover, PP-based exponential growth rates were statistically similar (*t*-test, *df* = 3, *p* < 0.01; Table 3).

After these cultures had reached stationary phase as a consequence of P-restricted growth (day 16), each treatment was inoculated with the opposite P substrate. In both cases, P content rapidly increased to statistically similar levels of > 5 g P:g Chl or ~ 0.015 mol P:mol C (Fig. 5A,B). The initial rate of increase of Chl-normalized cellular P (Fig. 4) in this second stage (G6P = 1.89 ± 0.36 g P g Chl⁻¹ d⁻¹; DIP = 2.21 ± 0.31 g P g Chl⁻¹ d⁻¹) was significantly higher (*t*-test, *df* = 3, G6P: *p* = 0.02; DIP: *p* ≤ 0.01) than recorded in the previous transfers of P-replete populations (G6P = 0.68 ± 0.42 g P g Chl⁻¹ d⁻¹; DIP = 0.16 ± 0.12 g P g Chl⁻¹ d⁻¹). This result reaffirms previous reports (Romans et al. 1994; White et al. 2006) of luxury consumption of P by *Trichodesmium* and indicates that rates of the hydrolysis and uptake of P monoesters may also occur in excess of the requirements for P-based growth.

In contrast to experiments comparing growth of P monoesters vs. DIP, cultures amended with MPn did not store as much internal P as did those amended with DIP or G6P (expt III; Fig. 5C,D). The maximum P content was significantly higher (*t*-test, *df* = 3, *p* = 0.003 and *p* = 0.02 for Chl and C-normalized P content, respectively) for growth on DIP (5.34 ± 0.43 g P:g Chl and 0.016 ± 0.001 mol P:mol C) relative to MPn (2.54 ± 0.60 g P:g Chl and 0.013 ± 0.001 mol P:mol C; Fig. 5C,D). PP accumulation and, hence, P-uptake rates were slower when growth was based on MPn relative to DIP treatments (Fig. 3D–F; Table 3; PP-based specific growth = 0.14 ± 0.007 for the MPn treatment [over days 5–17] and 0.18 ± 0.03 for the DIP treatment [days 1–10], *t*-test, *df* = 3, *p* = 0.09). While the difference between exponential growth rates is only significant within 90% confidence intervals, the longer lag time (4 d) observed in the MPn treatment and the discrepancies in P:C and P:Chl between MPn- and DIP-based growth (Fig. 5) indicate fundamental dissimilarities in the assimilation kinetics of these compounds.

Examination of the sum of the particulate ratios and dissolved pools reveals differences in the dependency on external and internal nutrient pools over the course of MPn and DIP batch cultures (expt III). For cultures grown on MPn alone, P:Chl and P:C reached baseline ratios of ~ 1 g P:g Chl and < 0.004 mol P:mol C (> 250 mol C:mol P), respectively, after 5 d of growth, indicating that excess internal P pools were fully utilized (Fig. 5C,D) in this initial growth phase. PC and PN accumulation, however, did not plateau until day 18 (Fig. 6), at which time DOP was fully depleted (Fig. 3F), such that growth between days 5 and 18 was regulated by the availability of an external P pool. Notably, MPn treatments maintained minimal P:C levels (0.003–0.005 mol P:mol C or C:P = > 200 mol:mol) during exponential growth. For growth on DIP alone, DIP was fully depleted by day 10 (Fig. 3E), whereas P:Chl and P:C ratios did not reach minimal levels until day 18 (Fig. 5C,D), at which point PC and PN accumulation

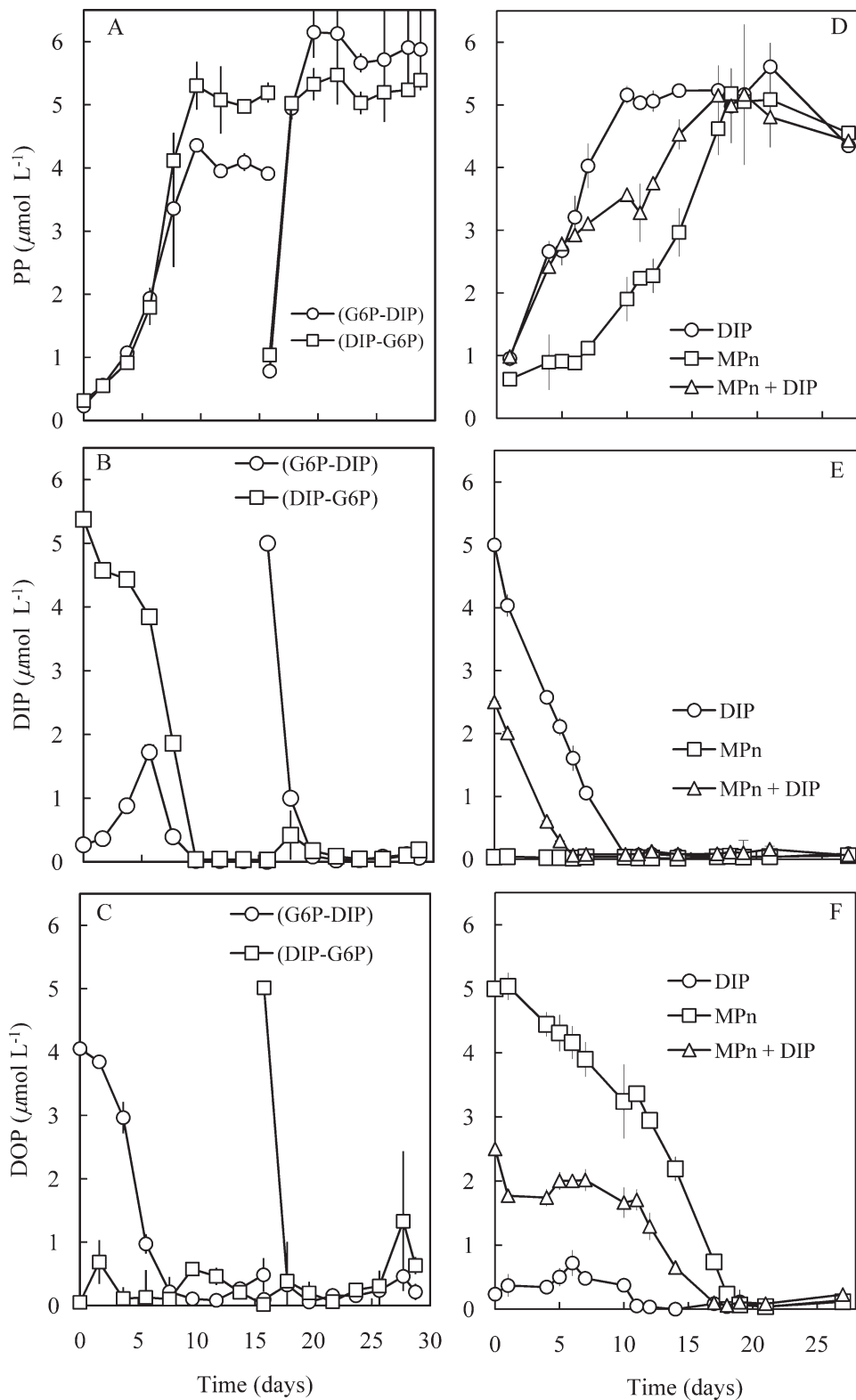


Fig. 3. Time course of (A) PP, (B) DIP, and (C) DOP over the course of two consecutive cycles of *Trichodesmium* IMS101 growth. The first growth cycle (days 0–16) compared growth on G6P and DIP. At day 16, cultures were diluted into fresh media having the alternate P compound (G6P–DIP and DIP–G6P). A subsequent experiment monitored (D) PP, (E) DIP, and (F) DOP trends for cultures grown on DIP ($5 \mu\text{mol L}^{-1}$), MPn ($5 \mu\text{mol L}^{-1}$), and equimolar concentrations of MPn + DIP ($2.5 \mu\text{mol L}^{-1}$ each).

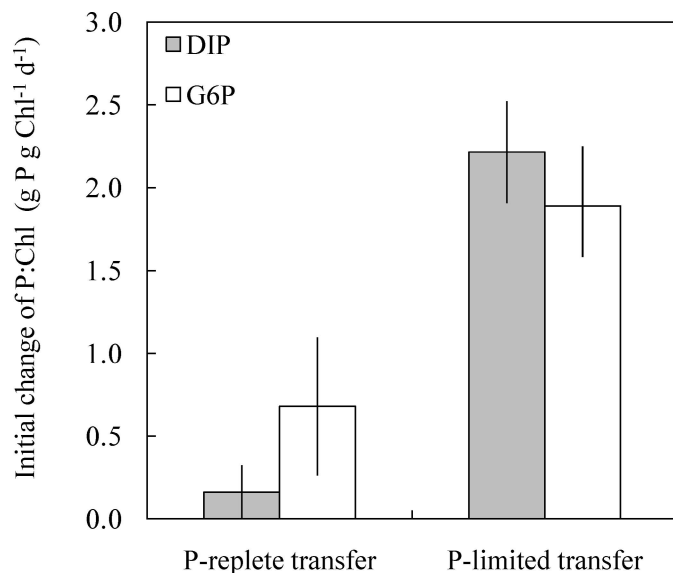


Fig. 4. The initial rate of P uptake per unit Chl by *Trichodesmium* IMS101 following the transfer of exponentially growing P-replete cultures or stationary-phase P-limited cultures grown on G6P or DIP. P uptake per unit biomass is significantly higher in cultures that were P limited at the time of transfer.

stabilized. This observation is consistent with rapid P uptake and subsequent utilization of internal P pools (days 10–18) to fuel growth (i.e., luxury consumption and the temporal uncoupling of growth from external P concentrations). When DIP and MPn were added in equimolar concentrations, DIP was preferentially utilized (Fig. 3E,F), internal P stores were formed (relative to Chl or PC; Fig. 5C,D), and internal P and external MPn were subsequently and concurrently drawn down (days 10–18). The complexities of these results—with P uptake alternate-

ly being regulated by internal vs. external P—make it difficult to parameterize growth simply as a function of either external or internal P pools (Monod- or Droop-type models, respectively; Sommer 1991) in populations having the capacity to utilize a range of organic and inorganic P substrates.

C and N fixation as a function of P substrate—C and N fixation rates as a function of P substrate (MPn, G6P, or DIP) were derived from the accumulation of particulate matter (Figs. 2, 6). A fit was applied to the linear portion of PC and PN increase, and the error of this slope was used to estimate the uncertainty of the derived rate. Results indicate that C and N fixation rates were not significantly different when samples were grown on MPn or G6P, as compared to DIP-based growth under the same experimental conditions (Table 3). Additionally, PC and PN accumulation rates did not differ among treatments in expt I, having initial concentrations of 500 nmol L⁻¹ MPn, G6P, 2-AEP, or AMP (Fig. 2; Table 3). The ratio of C to N fixation ranged from 5.9 to 7.0 mol C: mol N (Table 3). Acetylene reduction rates were also measured over the course of expt III with successive incubations on G6P and DIP. When comparing growth on DIP to growth on G6P, no significant differences were found between Chl-normalized rates of N₂ fixation derived from acetylene reduction (*t*-test, *df* = 17, *p* = 0.91) or PN accumulation (*t*-test, *df* = 29, *p* = 0.99). Moreover, both means of estimating N₂ fixation resulted in similar trends over the course of successive batch cultures (Fig. 7).

Cross-experiment results—Light levels, growth stage, and nutritional history of the parent cultures differed at the initiation of each experiment. For this reason we have previously restricted our analysis to comparisons of treatments within each respective experiment. Some results,

Table 4. C and N fixation rates were calculated from changes in particulate matter inventories over the linear phase of accumulation. Specific PC- and PN-based growth rates (μ) were calculated as the slope of the linear phase of natural log-normalized values. The first phase of expt III (days 0–16) was initiated with cells transferred from exponential phase growth, while the second phase of this experiment (days 16–29) was initiated with cells in stationary phase.

	N fixation ($\mu\text{mol L}^{-1} \text{d}^{-1}$)	C fixation ($\mu\text{mol L}^{-1} \text{d}^{-1}$)	Ratio of C: N fixation (mol: mol)	μ (d ⁻¹) (PN)	μ (d ⁻¹) (PC)
Experiment I					
G6P	7.2±2.1	36±1	5.0±1.5	0.34±0.05	0.16±0.06
MPn	5.7±1.3	32±12	5.7±2.4	0.32±0.02	0.18±0.01
2-AEP	6.5±0.7	37±2	5.8±0.7	0.28±0.07	0.21±0.04
AMP	6.1±0.7	28±4	4.5±0.9	0.35±0.02	0.14±0.07
Experiment II					
MPn	19.8±1.5	117±10	5.9±0.7	0.21±0.02	0.19±0.02
DIP	19.5±0.9	136±10	7.0±0.6	0.23±0.03	0.20±0.02
MPn + DIP	21.3±0.8	126±4	5.9±0.3	0.24±0.02	0.22±0.02
Experiment III: phase 1					
DIP	19.1±1.5	127±14	6.7±0.9	0.38±0.02	0.36±0.01
G6P	20.5±1.6	138±10	6.8±0.7	0.39±0.02	0.39±0.03
Experiment III: phase 2					
DIP	14.6±1.3	102±5	7.0±0.6	0.20±0.02	0.17±0.01
G6P	15.0±0.9	102±2	6.8±0.5	0.19±0.02	0.19±0.01

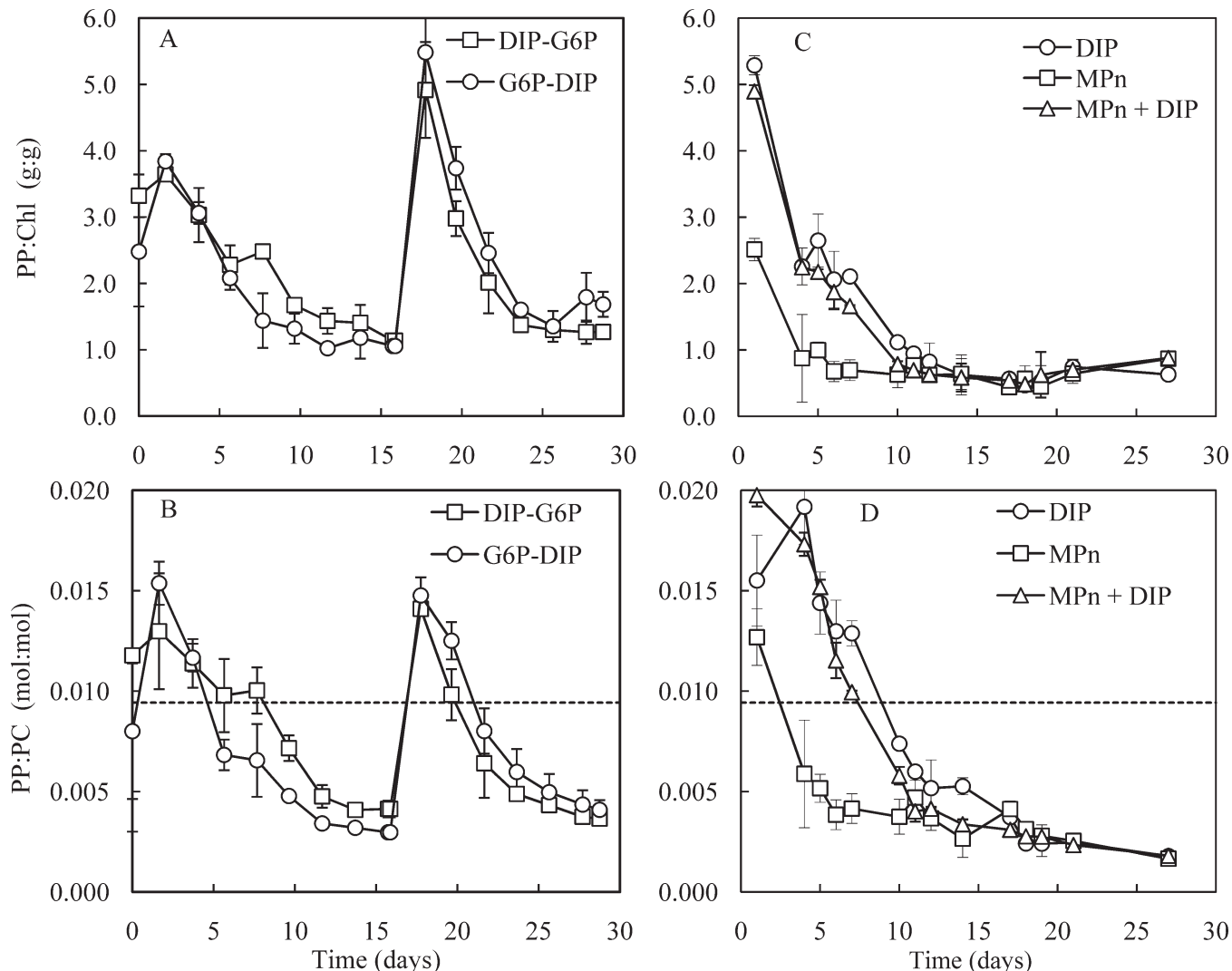


Fig. 5. Time course of PP:Chl and PP:PC for *Trichodesmium* IMS101 treatments grown on (A, B) G6P or DIP and (C, D) MPn, MPn + DIP, or DIP. For the G6P comparison, $5 \mu\text{mol L}^{-1}$ of either G6P or DIP was added at 16 d, resulting in a rapid increase in both PP:Chl and PP:C. The dashed lines in B and D represent the Redfield ratio of 1P: 106C.

however, are notable between experiments. Mean C:Chl ratios for all treatments over the full time course of sampling were $454 \pm 181 \text{ g C:g Chl}$ ($n = 20$) for expt I (sinusoidal light cycle with $500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), $70 \pm 21 \text{ g C:g Chl}$ ($n = 42$) for expt II (12:12 LD, $125 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), and $118 \pm 19 \text{ g C:g Chl}$ ($n = 34$) for expt III (12:12 LD, $325 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). These differences reflect photoadaptation of each culture line to the growth irradiance.

With the exception of the first phase of expt III, each of these experiments was initiated with P-starved stationary-phase cultures. In general, this led to unbalanced growth, with often significant differences in C-, N-, and P-based specific growth rates (Tables 3, 4). Specifically, in expt I, C:N ratios at time zero were $\sim 12 \text{ mol C: mol N}$; however, the ratio of C:N fixation over the course of this experiment (5 d) was, on average, 5.3 mol C: mol N , with a higher N-

based growth rate producing Redfield-like stoichiometry (6.6 mol C: mol N) by the end of the experiment. Comparing growth stages that were initiated from P-limited parent cultures (expts I, II, and the second phase of expt III), PC-specific growth rates were not significantly different (t -tests, $df = 3$, $p > 0.15$; Table 4), despite differences in growth irradiance (Table 1). The light levels used herein ($> 125 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$), the saturation parameters for growth reported by Goebel et al. (2008; $73 \pm 29 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$) and Breitbart et al. (2008), and the statistically similar C fixation rates observed for cultures with similar prior nutritional history indicate that these experiments were all maintained at saturating irradiances. Thus, we can conclude that *Trichodesmium* IMS101 in well-lit, DIP-deficient conditions can sustain growth rates similar to those achieved with DIP alone via the hydrolysis of P bound in G6P, AMP, MPn, and 2-AEP.

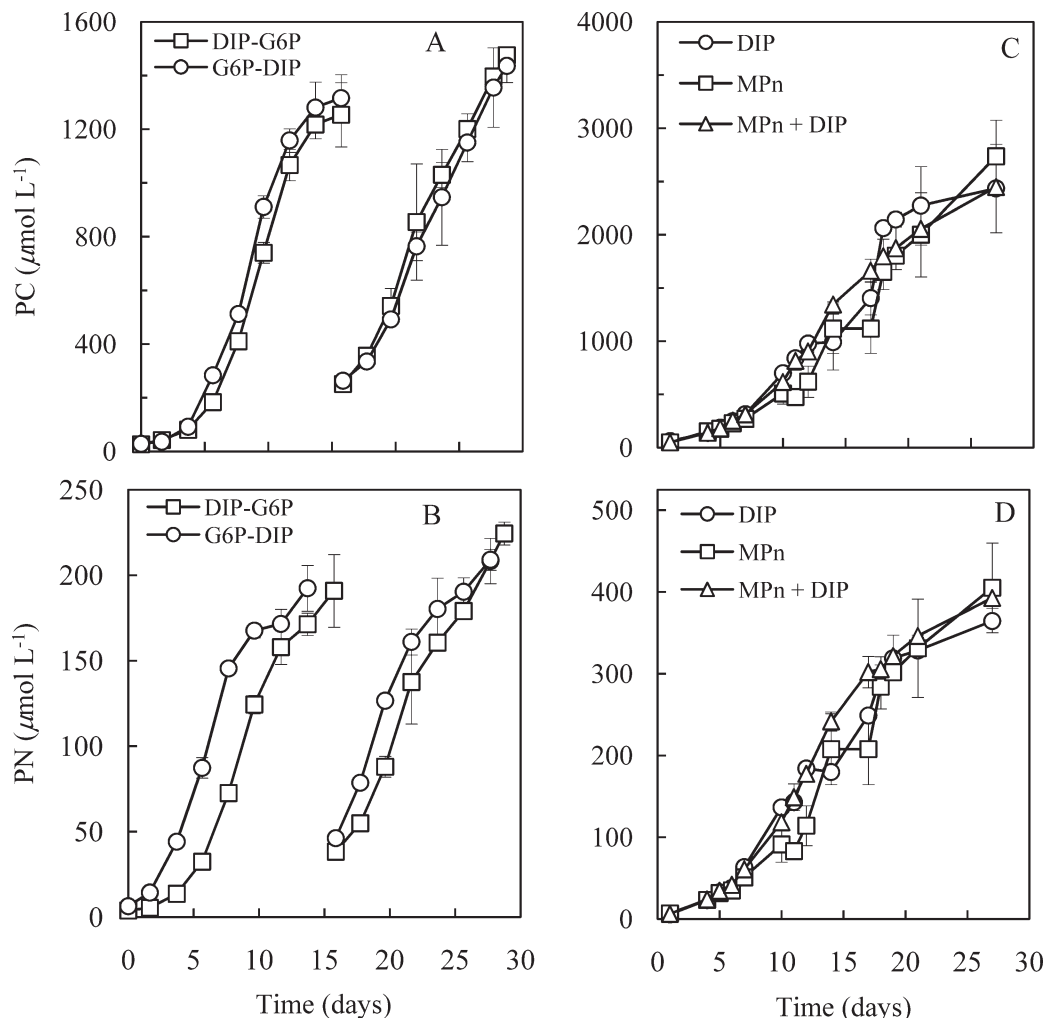


Fig. 6. Time course of PC and PN for *Trichodesmium* IMS101 batch cultures grown on (A, B) G6P or DIP and (C, D) MPn, MPn + DIP, or DIP.

Discussion

An extensive body of work has shown that DOP is an important substrate for microbial growth in the marine environment (Dyhrman et al. 2007; Karl 2007), where DOP is often the dominant P reservoir (Karl and Björkman 2002). The detection of phosphatase and C–P lyase-specific gene sequences, hydrolysis of fluorochrome-tagged organic substrates (Dyhrman et al. 2007), in situ determinations of biologically available P via isotope dilution coupled to ^{32}P -labeling of intracellular ATP pools (Björkman and Karl 2003), and direct observations of DOP uptake in the laboratory support the fact that a wide range of organisms have the enzymatic capacity to hydrolyze DOP compounds. The composition of DOP in marine systems has partially been characterized as a blend of phosphoesters, phosphonates, nucleic acids, phospholipids, vitamins, and nucleotides (Karl 2007). The selective utilization of these compounds can supplement or completely fulfill P requirements for growth, lead to differential success of organisms under low-DIP conditions, and contribute to the regulation of microbial community structure.

The inherent physiological differences between organisms and the substrate specificity of individual enzymatic pathways have direct consequences for growth, productivity, and microbial diversity in DIP-deficient regions of the open ocean. For all living organisms, energy transfer and storage are dependent on phosphorylated compounds. Thus, at a cellular level, the production of organic compounds, the C- or N-based productivity of an organism, is dependent on P. When external concentrations of DIP are low relative to the needs of the organism or when a particular species is a poor competitor for DIP, alternative P capture pathways provide a means for meeting the P demands necessary for maintenance and growth. The relationship between P substrate and productivity is of particular importance when addressing the metabolic activity of diazotrophs, those organisms having the ability to utilize the near-limitless pool of N_2 dissolved in seawater for growth. Diazotrophy allows select organisms to circumvent N limitation in nature and shifts the range of potentially limiting elements to P, iron, essential vitamins (e.g., B_{12}), or perhaps CO_2 (Sañudo-Wilhelmy et al. 2001; Karl et al. 2002; Hutchins et al. 2007).

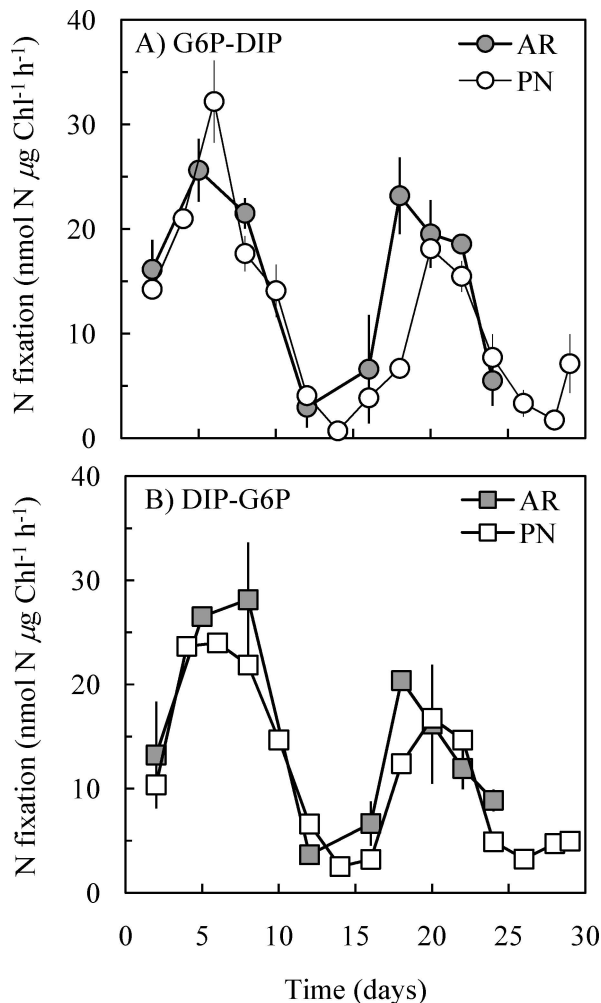


Fig. 7. Comparison of the rate of N₂ fixation derived from acetylene reduction (AR) and PN accumulation normalized to Chl for *Trichodesmium* IMS101 cultures (A) initially grown on G6P and subsequently diluted into media with DIP at 16 d and (B) initially grown on DIP and subsequently diluted into media with G6P.

Like any other functional groups, a differential capacity for DOP utilization exists for individual diazotrophic genera or ecotypes. Specifically, of the major classes of marine diazotrophs in culture, it has been demonstrated that the filamentous, colony-forming genus *Trichodesmium* has the enzymatic capacity to hydrolyze both P-linked monoesters and phosphonates, whereas the DOP utilization of cultured populations of *Crocospaera watsonii*, a unicellular diazotroph that coexists in regions where *Trichodesmium* is found, appears to be limited to select P monoesters (Table 5). Given that phosphonates comprise 25% of the high-molecular-weight DOP pool (Kolowitz et al. 2001), this disparity may confer a competitive advantage to *Trichodesmium* in the sense that a broader spectrum of P resources may be accessed for growth. Intraspecific and ecotype differences in DOP-uptake mechanisms as well as a determination of the production rates and concentrations of bioavailable DOP will be integral to assessing the utility of phosphonate capture in nature. In addition, such proposed resource

partitioning will depend on the yet-to-be-determined chemical composition of DOP compounds in seawater.

In this study, we sought to examine the substrate specificity of DOP utilization by *Trichodesmium* IMS101 and to describe the relationship between P source and the stoichiometry of organic matter production by monitoring growth in cultures with added DOP in the absence of DIP. Results clearly show that *Trichodesmium* can utilize both P monoesters (AMP, G6P) and select phosphonates (2-AEP, MPn) as a sole source of P. Only phosphonoalanine treatments failed to stimulate *Trichodesmium* productivity, indicating that *Trichodesmium* does not possess substrate-inducible C-P hydrolyases (Quinn et al. 2007), and as such, the utilization of phosphonates in nature will be a function of the class of phosphonates available.

A significant finding of these experiments is that there appears to be a greater P cost for growth on MPn to the extent that maximal P content per unit Chl or C is reduced when MPn is the sole source of P (Fig. 5C,D). This finding is not entirely unique. Vera et al. (2008) have shown that the chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans* significantly reduces intracellular pools of polyphosphate, inorganic polymers of P residues linked by high-energy phosphoanhydride bonds, when grown on MPn or ethylphosphonate as compared to growth on DIP. If the variable P-storage capacity observed for *Trichodesmium* also reflects a reduced allocation of P to polyphosphate when cells are grown on MPn, our results have implications for elemental stoichiometry, that is, the ratio of C to N to P, as well as for what Kornberg (1995) terms "life after log." Polyphosphates can not only serve as reservoirs of energy and phosphate that may prolong growth but they can also modulate the cascade of physiological responses that occur as cells enter stationary phase (e.g., the bacterial stringency response; Kornberg 1995; Chatterji and Kumar Ojha 2001). While the precise role polyphosphate plays in the fine-tuning of metabolism in stationary growth is not fully understood and will probably vary by class and species, the differential accumulation of P as a function of P source and the potential regulatory role of polyphosphate for metabolism may alter the ability of *Trichodesmium* and other microbes to prolong survival and adapt to environmental stress.

In contrast to growth on MPn, *Trichodesmium* IMS101 rapidly builds internal P stores when P-starved populations are grown on either DIP or the P monoester G6P. Maximum P content for both treatments was ~ 0.015 mol P : mol C (C : P ~ 66; Fig. 5B), or roughly half the average C : P ratio assumed for phytoplankton, C₁₀₆ : P₁—the Redfield (1958) ratio. Taken together, the fact that highly P-enriched matter was observed soon after P-starved cells were introduced to fresh media and that P reserves were drawn down as dissolved P was exhausted indicates that *Trichodesmium* can form intracellular polyphosphates when exposed to both DIP and P monoesters. Direct measurements of polyphosphate concentrations are needed to verify this assumption; nonetheless, we believe this may be the first indication of the production of polyphosphate via DOP hydrolysis.

Table 5. Summary of our present knowledge of the DOP utilization capacity of *Trichodesmium* and *Crocospaera*.

Functional class—compound	Empirical formula	Metabolizing diazotroph (reference)*
P monoesters (C–O–P bond class)		
Glucose-6-phosphate	C ₆ H ₁₃ O ₉ P	<i>Trichodesmium</i> (4)
Adenosine-5'-monophosphate	C ₁₀ H ₁₄ N ₅ O ₇ P	<i>Crocospaera</i> (1); <i>Trichodesmium</i> (4)
Glycerophosphate	C ₃ H ₇ MgO ₆ P	<i>Crocospaera</i> (1); <i>Trichodesmium</i> (2)
myo-Inositol hexakisphosphate	C ₆ H ₁₆ CaO ₂₄ P ₆	<i>Crocospaera</i> (1)
Phosphonate (C–P bond class)		
Methylphosphonate	CH ₅ O ₂ P	<i>Trichodesmium</i> (3,4)
2-Aminoethylphosphonic acid	C ₂ H ₈ NO ₂ P	<i>Trichodesmium</i> (4)
Ethylphosphonate	C ₂ H ₇ O ₂ P	<i>Trichodesmium</i> (4)
Phosphonoalanine	C ₃ H ₈ NO ₃ P	Unknown

* (1) Dyhrman and Haley (2006); (2) Mulholland et al. (2002); (3) Karl et al. (2008); and (4) this study.

We have further shown that the rate and ratio of *Trichodesmium* organic C and N production driven by P derived from phosphonates (MPn, 2-AEP) and P monoesters (G6P, AMP) are not significantly different than the rate and ratio for that grown on DIP. Both acetylene reduction and PC and PN accumulation rates confirm these results (Figs. 2, 6, 7). In the field, *Trichodesmium* biomass is characterized by conservative PC:PN ratios and variable PP quotas (C:N:P ratios ranging from C₈₀:N₁₄:P₁ to C₉₄₃:N₁₈₂:P₁, with a mean of ~ C₃₀₀:N₅₀:P₁; White et al. 2006) relative to the benchmark Redfield ratio of C₁₀₆:N₁₆:P₁. Similar to that observed in nature, the relative uniformity of C:N fixation rates exhibited in batch culture is contrasted by a large range in P content. In this study, deviations in C:P ratios were driven by the rapid uptake and utilization of intracellular P by P-starved cultures. Independent of P source, following luxury consumption, *Trichodesmium* IMS101 characteristically maintained diazotrophic growth with a highly reduced P quota (< 0.005 mol P: mol C; Fig. 5, equivalent to a molar ratio of > C₂₀₀:P₁) relative to the Redfield ratio. Assuming *Trichodesmium* IMS101 is representative of natural populations, the ability of this organism to maintain optimal productivity with reduced P requirements will preferentially enhance both the C and N components of dissolved and particulate pools, thus favoring community-scale P limitation. This could potentially cascade to affect the stoichiometry of grazer biomass and the export fluxes generated by migrating zooplankton (Hannides et al. 2009). Moreover, these data imply that diagnostic thresholds for P stress derived from C:P or N:P ratios should be applied with great caution and are likely two- to threefold higher than the Redfield ratio, as laboratory isolates maintain exponential growth with C:P ratios of > 200.

The composite results from these experiments indicate that neither the observation of P monoester hydrolysis nor highly P-deficient organic matter or expression of phosphonate hydrolyzing genes can be interpreted to indicate P limitation of C or N fixation of *Trichodesmium* spp. under light-saturated, DIP-deficient conditions. In order to translate these observations to natural systems, it may be necessary to first elucidate the compound-specific distribution of the DOP pool to better understand the potential for DOP to supplement growth requirements. A finer descrip-

tion of DOP composition in marine systems coupled with knowledge of the substrate-specific kinetics of the full range of cultured diazotrophic isolates will facilitate our understanding of resource partitioning and the controls of diazotrophic productivity and diversity. In short, it is clear that a wide range of diazotrophs can and are using DOP in nature and are perhaps maintaining optimal rates of productivity while doing so (provided that trace element or vitamin concentrations are not limiting). Thus, it may now be necessary to focus additional resources and technology on the determination of the supply pathways and chemical composition of bioavailable DOP in order to understand the determinants of the microbial ecosystem structure in oligotrophic marine habitats.

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