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Phosphonate metabolism of *Trichodesmium* IMS101 and the production of greenhouse gases

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Abstract

A series of laboratory experiments were conducted to investigate (1) the capacity of *Trichodesmium* IMS101 to hydrolyze phosphonates as a source of phosphorus (P) for growth, (2) the stoichiometric relationship between phosphonate use and biogenic gas production, and (3) the potential inhibition of phosphonate hydrolysis by additions of dissolved inorganic phosphorus (DIP). *Trichodesmium* IMS101 is capable of cleaving the carbon–P bond found in methylphosphonate (MPn) and ethylphosphonate (EPn), and the decomposition of these particular phosphonates results in the stoichiometric production of the greenhouse gases methane (CH_4) and ethane (C_2H_6), respectively. Growth on 2-aminoethylphosphonate (2-AEP) led to modest ethylene (C_2H_4) production. Normalized to rates of *Trichodesmium* carbon (C) fixation, biogenic gas production as a result of either MPn or EPn hydrolysis (0.95 $\pm$ 0.04 mmol CH_4 (mol C)$^{-1}$ and 1.18 $\pm$ 0.11 mmol C_2H_6 (mol C)$^{-1}$, respectively) approximates rates of DIP use (1.11 $\pm$ 0.05 P mmol P (mol C)$^{-1}$) measured in parallel cultures. DIP, MPn, and EPn can be used by *Trichodesmium* IMS101 as a sole source of P with equal metabolic efficiency. Additionally, neither MPn hydrolysis nor the production of CH_4 was significantly inhibited by additions of up to 30 μmol DIP L^{-1}. These results imply that *Trichodesmium* can use multiple P resources simultaneously for growth and that the production of greenhouse gases occurs during decomposition pathways of select phosphonates.

The open ocean harbors a diversity of metabolic strategies for coping with life in a fluid medium often lacking in the requisite macronutrients and micronutrients necessary for microbial growth. Diazotrophs, a group of specialized microorganisms consisting of free-living and highly developed endo- and ecto-symbiotic associations, have the unique capability of reducing dinitrogen gas (N_2) to ammonia (NH_3), a process termed biological nitrogen fixation (reviewed by Zehr and Ward 2002). This ability to directly or indirectly tap into the nearly inexhaustible pool of N_2 dissolved in the surface ocean confers a competitive advantage to diazotrophs and their hosts when confronted with conditions of fixed N limitation. For this reason, the N-poor surface waters of subtropical and tropical habitats are important environments for the proliferation of N_2-fixing microorganisms.

The growth of N_2-fixing microorganisms can have profound consequences, both for the physiology of the microbial consortium and for the structure and function of the ecosystem as a whole. For example, while it is generally agreed that a low ratio of bioavailable nitrogen to phosphorus (N : P) selects for the growth of N_2-fixing microorganisms, provided all other growth requiring nutrients and light are available, diazotrophic productivity eventually leads to an excess of N and of carbon (C), relative to P, in both the particulate organic matter (POM) and dissolved organic matter (DOM) pools (Karl et al. 2001; Capone and Knapp 2007). Karl et al. (1997, 2001) have hypothesized that the anomalously high C : P and N : P molar ratios of suspended and sinking POM observed at the Hawaii Ocean Time-series Station ALOHA (22°45′N, 158°W) are a consequence of a long-term (decade scale) selection for N_2-fixing microorganisms. Among other ecological consequences, the enhancement of N_2-based new production and the subsequent drawdown of "excess" inorganic P appears to be pushing microbial populations residing in the euphotic zone of the North Pacific Subtropical Gyre (NPSG) to an increasingly inorganic P-deplete state (Karl et al. 2001). On seasonal timescales, the relationship between the productivity of diazotrophs and progression of P limitation is also observed in the Sargasso Sea (Cavender-Bares et al. 2001), where winter mixing delivers nutrients to the euphotic zone followed by net removal during spring and summer.

Thus, one consequence of a thriving diazotrophic population is a shift toward P limitation of diazotrophs and other microorganisms alike. Typically, dissolved inorganic phosphate (DIP; also known as orthophosphate [PO_4^{3-}] or soluble reactive P [SRP]) is considered to be the preferred substrate for P nutrition, but the use of dissolved organic P (DOP) as an alternate to DIP for autotrophic growth is well documented in marine ecosystems and in cultured isolates (Thingstad et al. 1993; Björkman and Karl 2003; White et al. 2010). In fact, active DOP use is often cited as evidence for DIP limitation or DIP stress in nature (Dyhrman et al. 2002, 2006; Duhamel et al. 2010). Marine microorganisms can assimilate at least two forms of DOP: ester-linked (C–O–P) and phosphonate (C–P) DOP compounds. While it has been well documented that P esters and phosphonates are bioavailable to some microorgan-

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Table 1. Preconditioning of *Trichodesmium* IMS101 parent cultures. To initiate each experiment, the parent culture was diluted 1 : 10 with fresh medium in a ratio of 1 : 10.

Experiment	P source	P status	Growth phase
I	MPn	replete	exponential
II	DIP	deplete	stationary
III	DIP	deplete	stationary

isms, detailed information is lacking regarding the relationship between DIP and DOP uptake and growth efficiencies, P-resource partitioning by subpopulations of the total microbial assemblage, and the possible simultaneous use of multiple forms of P by a single microbial species or ecotype.

To begin to address a subset of these open questions, we have conducted a series of experiments using the nonheterocystous, filamentous diazotroph, *Trichodesmium*, as a model system (herein and White et al. 2010). *Trichodesmium* spp. are well suited for investigations of P cycling, since cultured isolates are available (Prufert-Bebout et al. 1993), the genome of the most commonly cultured strain has been sequenced, and the P regulation genes have been characterized (Orchard et al. 2003). In addition, a collection of observations made by naturalists and researchers since the middle of the 19th century are available to contextualize the P physiology of this organism (Dareste 1856; LaRoche and Breitbarth 2005). Previous field studies of *Trichodesmium* have documented the potential use of both P monoesters and phosphonates (Stihl et al. 2001; Dyhrman et al. 2006; Karl et al. 2008) under in situ conditions via the presence and activity of alkaline phosphatase and C–P lyase enzymes, respectively. Furthermore, field experiments suggest that the cleavage of select phosphonate compounds results in the aerobic production of greenhouse gases (Karl et al. 2008). Building on this existing base of knowledge, we have conducted controlled laboratory experiments in order to examine (1) the metabolism of select alkylphosphonate compounds (methylphosphonate [MPn], ethylphosphonate [EPn], and 2-aminoethylphosphonate [2-AEP]) by the model diazotroph *Trichodesmium* strain IMS101; (2) the kinetics of growth, cell yields, and gas production via phosphonate hydrolysis; and (3) the potential inhibition of phosphonate hydrolysis via DIP. These experiments complement the work presented in White et al. (2010), which investigates the relationship between P source and the rate and stoichiometry of *Trichodesmium* productivity. The results from White et al. (2010) and this study provide insight into P cycling and resource partitioning in the oligotrophic ocean and the potential physiological and biochemical responses of diazotrophs to DIP limitation.

Methods

Culture conditions—*Trichodesmium erythraeum* IMS101 parent cultures were maintained as described in White et al. (2010) and grown on either MPn (experiment I) or DIP (experiments II, III) as the sole P source (Table 1). Parent and experimental cultures were grown under a 12 : 12 h light : dark cycle using a sinusoidal light curve characterized by a daily maximum of 500 μmol quanta m^{-2} s^{-1} and a daily integrated irradiance of 14 mol quanta m^{-2}.

Table 2. P additions for each treatment in experiments I–III.

	Substrate (μmol L^{-1})						
Treatment	DIP	MPn	EPn	G6P	AMP	2-AEP	Total P*,†
Experiment I							
1	0.50	0	0				0.50
2	0	0.50	0				0.50
3	0.25	0.25	0				0.50
4	0	0	0.50				0.50
5	0	0.25	0.25				0.50
Experiment II							
1‡	0	5					5
2	1	5					6
3	5	5					10
4	10	5					15
5	20	5					25
6	30	5					35
7§	30	0					30
Experiment III							
1		0.50		0	0	0	0.50
2		0.25		0.25	0	0	0.50
3		0.25		0	0.25	0	0.50
4		0.25		0	0	0.25	0.50
5		0		0.50	0	0	0.50
6		0		0	0.50	0	0.50
7		0		0	0	0.50	0.50

* Experiment I parent culture was originally inoculated with 0.50 μmol L^{-1} MPn, which may contribute to final P concentration after the 1 : 10 dilution.

† In addition to substrate added, there is an additional 0.15 μmol L^{-1} DIP contamination from the inorganic salts used in the YBCII medium.

‡ At 3.5 d, treatment 1 was spiked with 30 μmol L^{-1} DIP.

§ At 3.5 d, treatment 7 was spiked with 5 μmol L^{-1} MPn.

Experimental design—Three separate laboratory experiments were conducted to investigate different aspects of phosphonate metabolism and P regulation in *Trichodesmium* strain IMS101. For each experiment, a 1 : 10 dilution of the parent culture was added to fresh YBCII medium (Chen et al. 1996) amended with the selected P substrate (Table 2) and grown in batch culture mode as described in White et al. (2010). The objectives for the different treatments included (1) the characteristics of MPn and EPn use in the presence and absence of DIP, (2) the regulation between MPn and DIP metabolism, and (3) the regulation of MPn metabolism in the presence of several potentially competing DOP compounds (glucose-6-phosphate [G6P], adenosine monophosphate [AMP], and 2-AEP).

All experiments were carried out in replicate 250-mL borosilicate glass bottles with Viton® septa and aluminum crimp seals to ensure that they were gas tight. At each time point, two replicates per treatment were sampled for gases (methane [CH_4] and ethane [C_2H_6]) and sacrificed for analysis of DIP, DOP, chlorophyll *a* (Chl *a*), particulate carbon, particulate nitrogen, and particulate phosphorus

(PC, PN, and PP, respectively) unless otherwise specified. Because of well-known diel periodicity in *Trichodesmium* metabolism, all experiments began at 20:00 h, with the first set of samples taken at t = 12 h (08:00 h the following day). In this way biomass, dissolved nutrients, and biogenic gases were all assessed following a 12-h dark period (for analytical detail *see* Methods section). Subsequent sampling took place every 24 h. Unless otherwise indicated, all reported gas and particulate matter production rates were calculated during the exponential growth phase of the cultures. The *Trichodesmium* cultures used in these experiments were not axenic. In order to address the potential effect of additional P on heterotrophic bacterial growth, bacterial cell numbers were monitored on occasion and a series of control experiments were conducted (White et al. 2010).

Experiment I was designed to evaluate the potential DOP use and CH_4 and C_2H_6 production by *Trichodesmium* IMS101 grown on MPn and EPn, respectively, in the presence and absence of DIP. All culture media had an initial total dissolved P concentration of 5 μmol L^{-1} (Table 2). The primary objective of experiment II was to characterize the use of MPn by *Trichodesmium* IMS101 as a function of varying DIP concentrations up to 30 μmol L^{-1} (Table 2). In treatments 1 (grown in 100% MPn) and 7 (grown in 100% DIP), one set of replicates was spiked at 3.5 d with 30 μmol L^{-1} DIP and 5 μmol L^{-1} MPn, respectively, and incubated with the remaining nonspiked replicate samples for an additional 2 d (*see* Table 2). The purpose of these spikes was to evaluate the short-term effect of DIP on MPn-metabolizing, CH_4-producing cultures (treatment 1) and to examine the potential for MPn and coincident CH_4 production by cultures growing in DIP-rich medium (treatment 7). Finally, experiment III was designed to explore how the availability of P in the form of G6P, AMP, or 2-AEP affects the metabolism of MPn by *Trichodesmium* IMS101.

Gas measurements—CH_4, C_2H_6, and ethylene (C_2H_4) were analyzed by gas chromatography using a purge and trap system following the design of Swinnerton and Linnenbom (1967). Briefly, a ~ 150-mL sample of seawater was purged for 8 min with ultrapure helium (He) gas at a rate of 100 mL min^{-1}. The sample was first passed through Nafion® tubing to reduce water vapor then through Ascarite® and Drierite® columns to remove carbon dioxide and further dehydrate the sample. The sample was then passed through a 3.2-mm OD stainless steel loop that was packed with Porapak Q (80/100 mesh size) and was submerged in liquid nitrogen to quantitatively trap the gases. After purging, the loop was removed from the cryogenic bath and immersed in a water bath (~ 100°C) for 1 min to release the adsorbed gas. The resulting gas sample was then injected at a rate of 25 mL min^{-1} into a gas chromatograph (GC; Shimadzu GC-8A) equipped with a column packed with Porapak N (80/100 mesh size) fitted with a flame ionization detector (FID). Peaks were integrated on a Chromatopac data processor (Shimadzu C-R8A). The detector response was calibrated using standard gases purchased from Matheson Tri-Gas at concentrations of 100 μL L^{-1} volume in He. Five-point standard curves were derived by injecting known volumes of standard gas into a sample that had already been purged of the hydrocarbons being measured, followed by cryogenic trapping identical to the samples. Standard gases were also periodically injected directly into the GC to ensure that the purge and trap procedure was functioning at 100% efficiency. Purge flow rate was maintained using an adjustable flow controller (Alltech) and monitored throughout the system using a calibrated electronic flow meter (Alltech).

Nutrient, particulate matter, and Chlorophyll a *analyses*—From the ~ 100 mL remaining in the incubation bottle after gas extraction, a small aliquot (< 5 mL) was used to measure Chl *a* variable fluorescence using a water-pulse-amplitude-modulated (PAM) fluorometer (Heinz Walz GmbH Instruments) as described by White et al. (2006). From the remaining volume (~ 95 mL), subsamples were collected onto 25-mm diameter Whatman combusted (450°C, 5 h) and acid-washed (10% HCl, overnight) glass fiber filters (GF/F) for PC, PN, and PP analyses and unprocessed (no combustion or acid preparation) GF/F filters for Chl *a*. The filtrate was used for total dissolved P (TDP) and DIP measurements. Samples for Chl *a* were extracted in 5 mL of 90% acetone (Knap et al. 1996). All samples were stored in the dark at −20°C until analyzed, usually within 1 week and no longer than 3 weeks. Chl *a* was measured fluorometrically using a Turner Designs Model 10-AU fluorometer.

PC and PN samples were analyzed using a Carlo Erba NC 2500 elemental analyzer with a Finnigan MAT ConFlo II coupler with acetanilide (C_8H_9NO) and dried and pulverized plankton used as the standard and check standards, respectively. Samples for PP analyses were combusted at 450°C for 4 h, then extracted in 10 mL 0.15 mol L^{-1} HCl at 60°C for 1 h and allowed to cool before being centrifuged (2800 × *g*, 10 min) to clarify. Aliquots of the supernatant were taken for DIP analysis, as described below. Particulate P values in these experiments correspond to the sum of organically bound P and inorganic polyphosphates produced by *Trichodesmium* during growth.

DIP concentrations were determined colorimetrically as described by Murphy and Riley (1962) using a Cary ultraviolet–visible spectrophotometer while TDP concentrations were measured using a modification of the Valderrama (1981) method (*see* White et al. 2010). TDP values in these experiments correspond to the sum of DIP, organically bound P added to the medium, and DOP produced by *Trichodesmium* during growth. DOP, operationally defined and calculated as the difference between TDP and DIP, and gas production were used to track substrate (MPn or EPn) utilization in the experiments.

Results

Utilization of MPn by Trichodesmium *IMS101*—Prior to the start of the main experiments, we first evaluated whether *Trichodesmium* IMS101 was able to assimilate

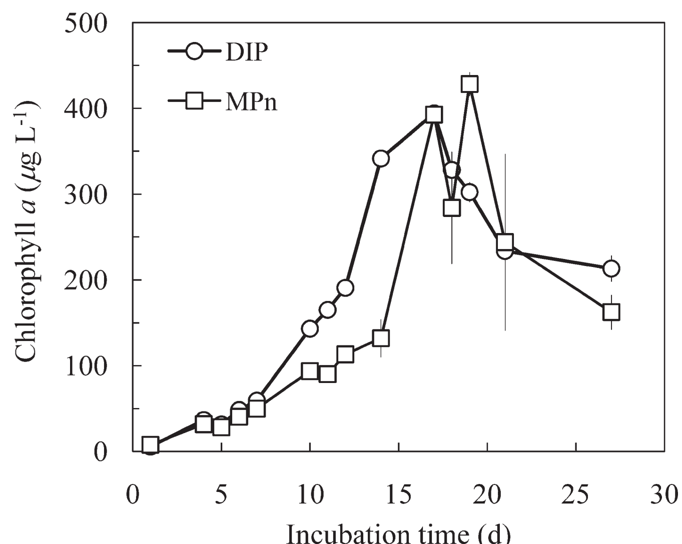

Fig. 1. Growth of *Trichodesmium* IMS101 on DIP or MPn as the sole source of P. Growth rate and cell yield were monitored by discrete measurements of Chlorophyll *a* over the 27-d incubation period.

MPn as a sole source of P. Two batch cultures were prepared with either DIP or MPn (starting concentrations at 5 μmol L^{-1} total P) added as the sole source of P. Growth was tracked by measurements of Chl *a* accumulation over time. The results indicated that (1) *Trichodesmium* IMS101 was able to grow on MPn as the sole source of P, (2) rates of growth (as assessed by accumulation of Chl *a*) were slightly slower for the MPn-only culture compared with growth on DIP-only, and (3) final cell yields (as assessed by maximum Chl *a* concentrations) were indistinguishable between the two treatments (Fig. 1). The subsequent experiments (I–III) were conducted to evaluate in greater detail the physiological controls on phosphonate catabolism.

Experiment I: Phosphonate metabolism and gas production—As in the preliminary experiment, there was no difference in Chl *a*–based, PC-based, or PN-based growth rates among the five P-source treatments (Table 3). Trace levels of CH_4 were produced in the DIP-only treatment and the EPn treatments (Table 3), which was most likely a consequence of the metabolism of MPn introduced from the parent culture (i.e., a 1 : 10 dilution of cells grown in 0.5 μmol L^{-1} MPn). Apparently, MPn decomposition occurs even in the presence of excess DIP (DIP : MPn molar ratio in starting medium is greater than 20 : 1). By comparison, the EPn-only treatment that contained the same amount of MPn from the parent culture produced only ~ 15 nmol L^{-1} CH_4 over the same growth period, while C_2H_6 production was substantial (> 100 nmol L^{-1}; Fig. 2). This result suggests that EPn and MPn can be co-metabolized and that there may be a common transport system since excess EPn appears to decrease the uptake of MPn, relative to the DIP + MPn treatment. Alternately, this finding may be a result of the encounter rate of competing substrates for the active site of the enzyme.

As expected, the highest total CH_4 concentrations measured were observed in the MPn treatment (281 nmol L^{-1}), whereas the highest total C_2H_6 concentrations were observed in the EPn treatment (269 nmol L^{-1}). In the MPn plus DIP equimolar treatment, the CH_4 concentration was 140 ± 7 nmol L^{-1}, and in the MPn plus EPn treatment, there was an approximately equal concentration of CH_4 and C_2H_6 (Fig. 2) of 173 ± 13 nmol L^{-1} and 165 ± 11 nmol L^{-1}, respectively, after a growth period of 10 d. If molar gas production rates, rather than their final concentrations, in the treatments with dual substrates are summed (nmol CH_4 L^{-1} d^{-1} + nmol C_2H_6 L^{-1} d^{-1}), then the total gas production approximates that observed in the treatments with MPn or EPn alone (Table 2). These results suggest that DIP, MPn, and EPn were all used by *Trichodesmium* IMS101 as a sole source of P with equal metabolic efficiency.

Whereas CH_4 production in the MPn-only treatment occurred within the first 1.5 d of incubation, the drawdown of DOP could not be resolved until day 3.5; thereafter it was accompanied by a steep increase in the rate of CH_4 accumulation (Fig. 3). A rapid increase in gas production concurrent with a lag in DOP drawdown was evident for both MPn and EPn treatments (Fig. 3). For the DIP-only treatment, there was also residual DOP drawdown within the first 5.5 d of incubation. At this point, the DIP concentration was < 50 nmol L^{-1} (data not shown). It is unclear whether the residual DOP is a result of DOP leakage from potentially P stressed or senescing cells. DOP in treatments 2–5 did not drop below 100 nmol L^{-1}. For the MPn- and EPn-only treatments, total DOP drawdown was 330 ± 38 and 340 ± 36 nmol L^{-1}, respectively (Fig. 3). As predicted by a stoichiometric relationship between the uptake of MPn or EPn and the production of biogenic gases, the final concentration of $CH_4 + C_2H_6$ for the MPn-only treatment was 281 ± 6 nmol L^{-1}, and 285 ± 1 nmol L^{-1} for the EPn-only treatment. Thus, nearly the full complement of hydrolyzed alkane (~ 90%) remained in solution following MPn or EPn hydrolysis.

Experiment II: DIP and MPn interactions and substrate preference—The MPn-only (5 μmol L^{-1}) treatment served as the positive control and was predicted to produce the maximum amount of CH_4 in this experimental design assuming DIP is the preferred substrate (*see* Table 2). By comparison, treatment 7 (30 μmol L^{-1} DIP) was the negative control and was predicted to produce no CH_4. Treatments 2–6, each containing 5 μmol L^{-1} MPn and increasing amounts of DIP (1, 5, 10, 20, and 30 μmol L^{-1}), were predicted to produce decreasing amounts of CH_4. CH_4 was not detected for the DIP-only treatment. However, in all cases when MPn was added in the presence of a range of DIP concentrations (5–30 μmol L^{-1}), CH_4 accumulations were detectable and significant. The highest total concentrations of CH_4 were measured in the MPn-only treatment after 3.5 d (Fig. 4). The largest differences were between treatments 1 and 2 and between treatments 6 and 7 (negative control; *see* Table 2); for treatments containing substantial concentrations of both MPn and DIP, CH_4 production did not vary

Table 3. Methane (CH_4) and ethane (C_2H_6) production rates normalized to PC production, specific growth using PC, PN, and Chl *a*, and the average C : N : P ratios for *Trichodesmium* IMS101 cultures. BDL indicates that the gas production was below detection limits.

	Gas production rate per PC production rate			Specific growth (d^{-1})			Average chemical composition		
	CH_4 $(10^{-3}\times PC)^{-1}$	CH_4 $(10^{-3}\times PC)^{-1}$	C_2H_6 $(10^{-3}\times PC)^{-1}$	PC	PN	Chl *a*	PC : PP	PN : PP	PC : PN
Experiment I									
DIP	0.08±0.01			0.23±0.05	0.26±0.03	0.41±0.24	135±39	14.4±4.8	6.6±1.2
MPn	0.95±0.04			0.29±0.02	0.28±0.02	0.47±0.03	125±31	23.3±5.8	5.4±1.1
MPn+DIP	0.55±0.02			0.36±0.05	0.34±0.04	0.40±0.17	113±34	21.7±6.4	5.2±1.2
EPn	0.06±0.01		1.18±0.11	0.29±0.03	0.26±0.02	0.60±0.12	198±64	34.5±11.1	5.5±1.5
MPn+EPn	0.47±0.02		0.55±0.02	0.27±0.05	0.29±0.03	0.36±0.22	155±43	22.2±6.2	7.4±2.1
Experiment II	Before addition	After addition							
MPn	2.58±0.84	1.27±1.01		0.34±0.07	0.39±0.09	0.39± 0.08	181±25	20.3±2.5	8.8±2.1
MPn+1 μmol L^{-1} DIP	1.08±0.34			0.41±0.15	0.43±0.06	0.21±0.10	112±24	11.3±2.5	16.2±6.4
MPn+5 μmol L^{-1} DIP	0.82±0.29			0.27±0.03	0.36±0.03	0.25±0.11	100±12	8.3±1.0	13.4±3.3
MPn+10 μmol L^{-1} DIP	1.06±0.61			0.26±0.01	0.35±0.03	0.26±0.14	111±11	9.9±1.0	10.7±2.0
MPn+20 μmol L^{-1} DIP	0.77±0.22			0.42±0.16	0.51±0.07	0.20±0.14	99±17	8.5±1.4	11.0±2.7
MPn+30 μmol L^{-1} DIP	1.18±0.50			0.36±0.05	0.47±0.07	0.31±0.07	101±13	8.7±1.1	10.1±2.3
DIP	0.00±0.18	0.38±0.18		0.38±0.05	0.42±0.07	0.29±0.09	99±16	13.7±2.2	6.3±0.8
Experiment III									
MPn	0.74±0.09			0.18±0.01	0.32±0.02	0.37±0.11	294±69	27.8±4.8	10.8±2.6
MPn+G6P	0.36±0.12			0.18±0.02	0.31±0.04	0.36±0.06	306±82	31.2±10.6	10.0±3.3
MPn+AMP	0.34±0.10			0.23±0.02	0.32±0.02	0.43±0.06	276±61	30.0±6.4	9.5±2.4
MPn+2-AEP	0.31±0.07			0.21±0.05	0.33±0.06	0.28±0.13	276±54	26.5±6.9	10.5±2.5
G6P	BDL			0.16±0.06	0.33±0.08	0.46±0.07	259±68	24.8±6.9	12.2±2.3
AMP	BDL			0.16±0.04	0.35±0.02	0.42±0.10	252±61	23.7±5.7	12.3±2.3
2-AEP	BDL			0.21±0.01	0.27±0.06	0.36±0.10	273±60	26.7±6.6	10.4±2.4

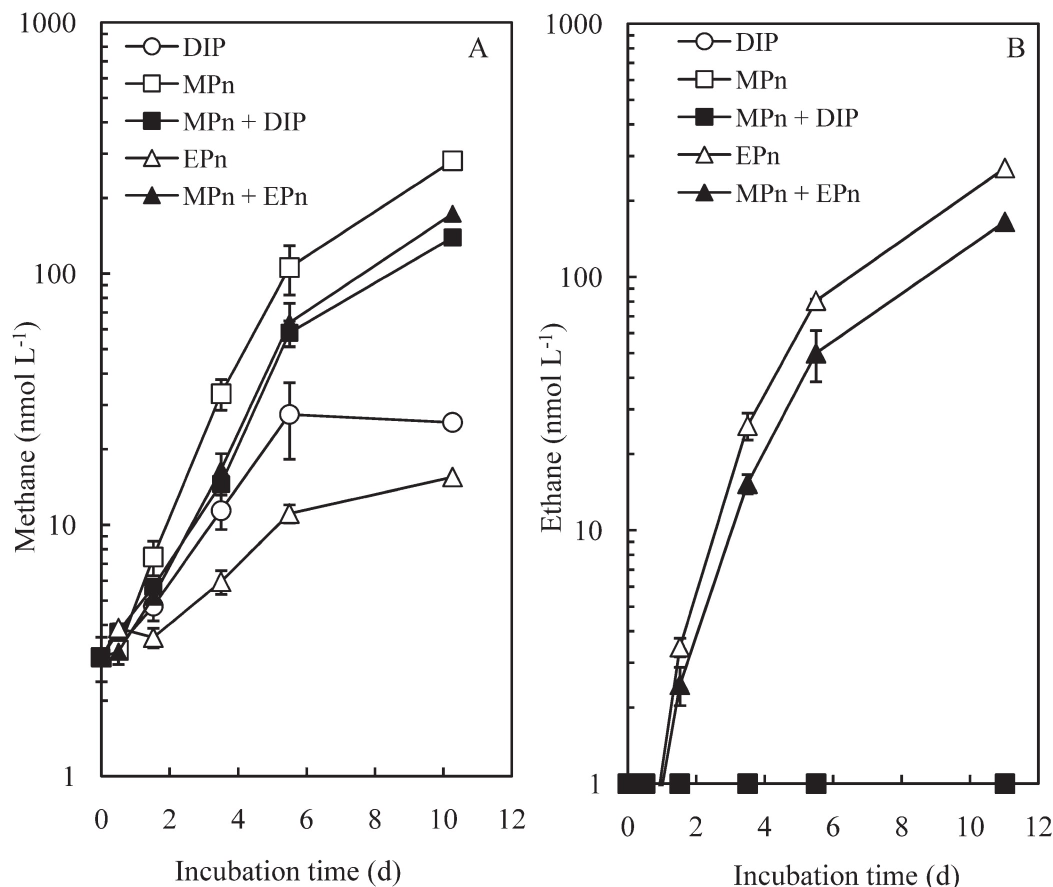

Fig. 2. The aerobic production of (A) methane (CH_4) and (B) ethane (C_2H_6) by *Trichodesmium* IMS101 during growth on MPn and EPn use, respectively, as the sole source of P. C_2H_6 was only detected in treatments with added EPn.

substantially. PC-normalized methane production rates were depressed by the addition of DIP with the greatest reduction observed in the 30 μmol L^{-1} DIP treatment (CH_4 $(PC)^{-1}$ = 46% of the MPn-only treatment); although the rate of CH_4 production in the remaining DIP + MPn treatments (1–20 μmol L^{-1} DIP) ranged from 30% to 42% of the MPn-only treatment (Table 3). These results support the previous observation in experiment I, suggesting that MPn use is not fully inhibited by DIP availability within the timescale of our experiment.

On day 3.5, when the MPn-only treatment was spiked with 30 μmol L^{-1} DIP, CH_4 production decreased slightly, but not significantly, relative to the unamended paired treatments (Fig. 4). In addition, when the 30 μmol L^{-1} DIP treatment was spiked with 5 μmol L^{-1} MPn on the same date, CH_4 production increased significantly (Fig. 4). A mass balance for P was achieved for each treatment when TDP and PP were summed. For all treatments, total P (data not shown) was not significantly different from the sum of the initial PP concentration ($\sim$ 0.58 μmol L^{-1}) and the additions of MPn and/or DIP (*see* Table 2 for P additions).

Experiment III: Substrate competition—As with experiment II, CH_4 production varied predictably among P substrate treatments (Fig. 5). From day 3.5 to 5.5, the CH_4 concentration increased from $\sim$ 50 to $\sim$ 120 nmol L^{-1} in the MPn-only positive control. Treatments 2–4 increased from $\sim$ 40 nmol L^{-1} to a maximum of $\sim$ 65 nmol L^{-1}. This may be a result of mass action since treatment 1 contained 500 nmol L^{-1} MPn, and there was only 250 nmol L^{-1} MPn in treatments 2–4. The rate of CH_4 increase was slightly higher in the MPn-only treatment (Table 3). For all treatments, CH_4 production approximately tripled within the first 12 h of incubation and maintained a relatively constant increase for the duration of the experiment (Fig. 5). However, the rate of CH_4 production for treatment 1 increased from $\sim$ 13 nmol CH_4 d^{-1} in day 3.5 to 38 nmol CH_4 d^{-1} by day 5.5. Treatments 4 and 7, which both contained 2-AEP, produced trace amounts of C_2H_4 (< 1 nmol L^{-1}; Fig. 5). Treatment 7, with 2-AEP only, produced C_2H_4 until day 3.5, where it eventually leveled off. Treatment 4, MPn plus 2-AEP, increased only to 0.11 nmol L^{-1} C_2H_4 at day 3.5, and also leveled off at this point. The production of C_2H_4 from 2-AEP metabolism was not further investigated in this study.

The drawdown of DOP occurred rapidly in all treatments (Fig. 6) and approached detection limits for treatments 2 (MPn plus G6P), 5 (G6P only), and 6 (AMP only). The DOP concentration in treatment 1 (MPn-only, positive control) was below detection by day 5.5, but the CH_4 concentration at this point was only $\sim$ 120 nmol L^{-1}. Total

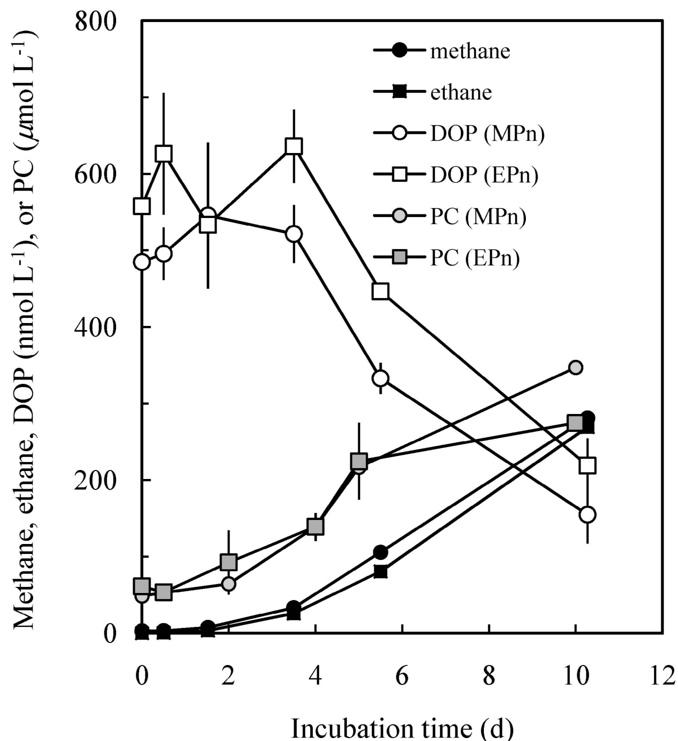

Fig. 3. The production of methane (CH_4) and ethane (C_2H_6), relative to the production of particulate carbon (PC), and the corresponding decrease in DOP during growth on MPn or EPn as the sole source of P.

P (TDP plus PP) was approximately 1.0 ± 0.1 μmol L^{-1} for treatments 1–7 (data not shown). Background DIP contamination (from media carryover and trace salt contamination; White et al. 2010) for some treatments was higher in experiment III (~ 350 nmol L^{-1} SRP) compared with experiments I and II (~ 150 nmol L^{-1} SRP) for reasons that we cannot explain. Initial increases in PP could have been due to DIP uptake resulting in the lower CH_4 production rates than those measured in experiment II (Table 3). For experiments I and II, the MPn-only treatment had CH_4 production rates of 27.9 ± 0.6 nmol L^{-1} d^{-1} and 24.8 ± 7.5 nmol L^{-1} d^{-1}, respectively. In experiment III, the MPn-only treatment had a CH_4 production rate of 12.7 ± 0.7 nmol L^{-1} d^{-1} for the first 3.5 d. These data would suggest that MPn and DIP may be simultaneously used for the initial portion of the incubation period (0–3.5 d).

Discussion

The DIP–DOP "cycle" in the open ocean begins with the uptake of DIP, the end-product of long-term remineralization of P-containing organic matter, and its incorporation into a broad range of cellular compounds of varying molecular weights and complexities. Cell death and autolysis, exudation, viral lysis, and grazing all lead to the release of DOP into the environment, where it can be depolymerized, hydrolyzed, reassimilated, removed by absorption onto sinking particles, or accumulate in the

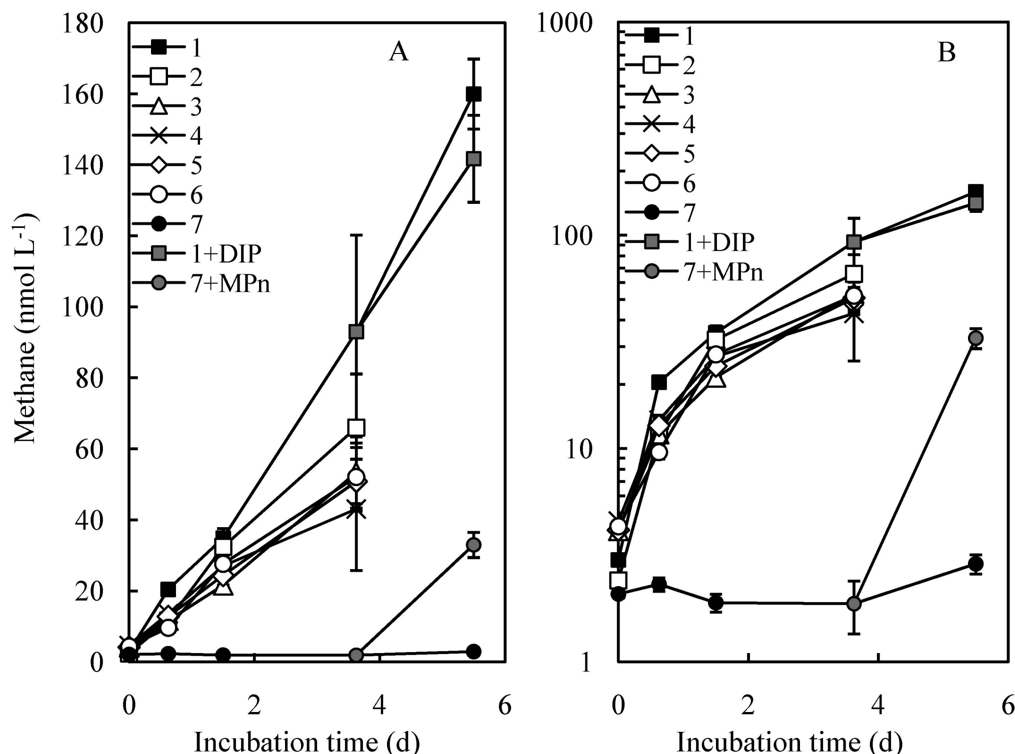

Fig. 4. The production of methane (CH_4) by *Trichodesmium* IMS101 and the effects of DIP additions on MPn use as shown on (A) a linear scale and (B) a log scale. At 3.5 d, treatment 1 was spiked with 30 μmol L^{-1} DIP and treatment 7 was spiked with 5 μmol L^{-1} to determine effect on CH_4 production rates. Symbols and numbers correspond to the treatments presented in Table 2.

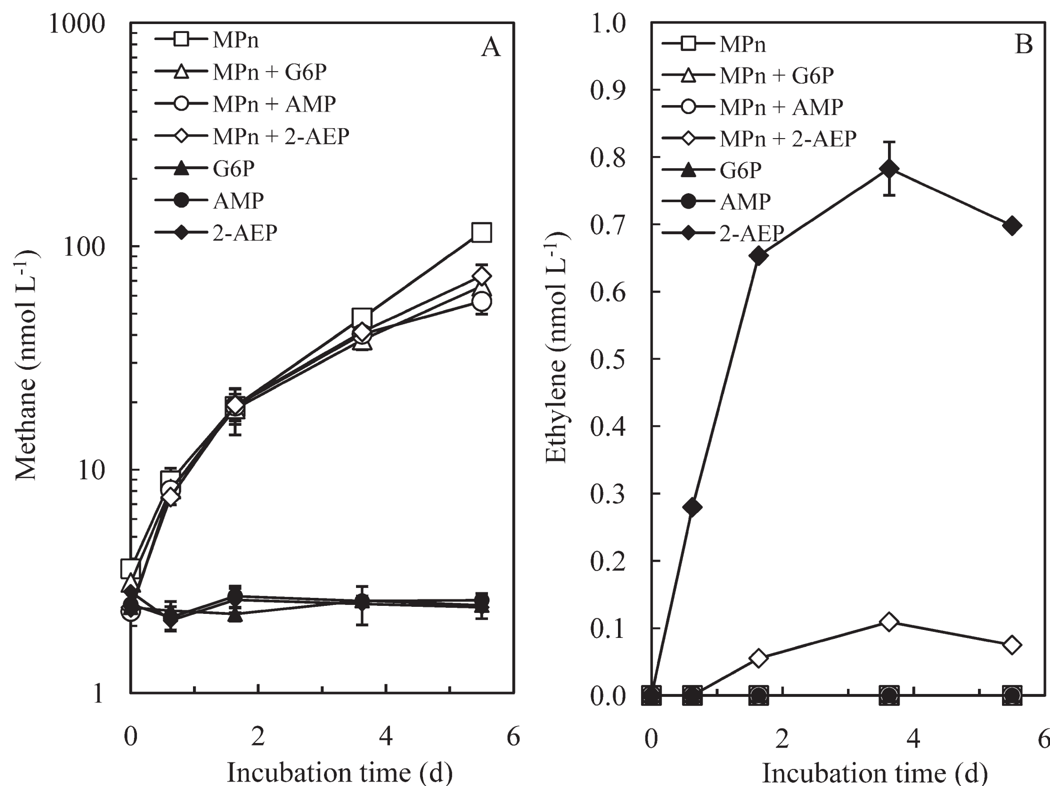

Fig. 5. The production of (A) methane (CH_4) and (B) ethylene (C_2H_4) by *Trichodesmium* IMS101 and the effects of potentially competing organic P substrates on MPn use. C_2H_4 was only detected in treatments with added 2-AEP.

surrounding environment (Karl and Björkman 2002). The plasticity of microorganisms to harvest different molecular forms of P may convey competitive ecological advantages to the organism as well as alter the molecular and elemental composition of particulate and dissolved pools.

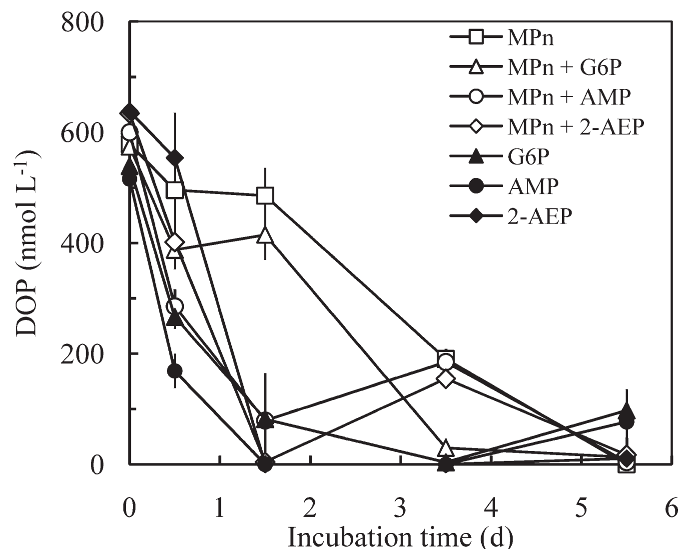

Fig. 6. The drawdown of organic P substrate pools by *Trichodesmium* IMS101 during growth on the respective substrate or mixed substrates (equimolar) as the sole source of P.

In the field, detection of the key enzymes in DOP assimilation pathways, genomic investigations (gene or transcript detection), and physiological approaches (measurement of enzyme activity using substrate analogues or incorporation of radiolabeled model substrates) have all illustrated that a range of microorganisms have the capacity to use select DOP compounds for nutrition and supplement or substitute P obtained from DIP uptake. Thus, it is generally accepted that DOP plays a fundamental role in the growth, metabolism, and community composition of oligotrophic oceanic systems (Dyhrman et al. 2007). Despite this awareness, significant gaps remain in our understanding of P use in nature, particularly the relationship between DIP and DOP uptake for individual microorganisms, the bioavailability and biomolecular composition of DOP, and the growth kinetics and efficiencies for select DOP compounds. Herein, and in a companion paper (White et al. 2010), we have used the model diazotroph *Trichodesmium* to investigate P metabolism and DOP use in order to address a subset of the current uncertainties surrounding our understanding of P cycling in the open ocean.

Like most marine microorganisms studied to date, *Trichodesmium* spp. are able to use DIP as a sole source of P for growth (Mulholland et al. 2002; White et al. 2006); however all evidence suggests they are weak competitors for DIP at low concentrations. Reported half-saturation constants (K_s) for DIP uptake by *Trichodesmium* range

from 0.42 to 9.0 μmol L^{-1} (McCarthy and Carpenter 1979; Fu et al. 2005; Sohm and Capone 2006) with specific affinities (maximum uptake rates K_s^{-1}) consistently lower than values for bulk plankton. These relatively low affinities for DIP suggest that ambient concentrations of DIP in most open ocean habitats are suboptimal for the growth of *Trichodesmium*; hence reliance on alternate forms of P, especially DOP, is inevitable.

There is ample evidence that *Trichodesmium* are active consumers of components of the bulk DOP pool. Numerous studies have documented high rates of alkaline phosphatase (APase) activity as support for use of monophosphate esters by *Trichodesmium* spp. as a reliable source of cellular P under in situ conditions (McCarthy and Carpenter 1979; Stihl et al. 2001; Sohm and Capone 2006). Experiments using "axenic" batch cultures of *Trichodesmium* in the laboratory (Stihl et al. 2001) and cell-specific diagnostic techniques have gone further and confirmed, unequivocally, that *Trichodesmium* express the DIP-regulated enzyme APase under conditions of low external DIP.

More recently, Dyhrman et al. (2006) demonstrated the induction, by DIP stress, of genes in *Trichodesmium* IMS101 that encode for the transport and hydrolysis of phosphonate compounds by the C–P lyase pathway (*phn* cluster). They also documented the expression of two *phn* genes (*phnD* and *phnJ*) in field populations of *Trichodesmium* collected from the western North Atlantic Ocean (Dyhrman et al. 2006). A subsequent study by Karl et al. (2008) demonstrated MPn use by natural microbial assemblages including field-collected *Trichodesmium* colonies. In addition, their study documented that MPn decomposition constitutes a novel pathway for the aerobic production of CH_4 in the sea, a potential solution to the marine methane paradox (Kiene 1991). Results from these two studies (Dyhrman et al. 2006; Karl et al. 2008) suggested, but did not demonstrate, that phosphonates are an important source of P for *Trichodesmium* in oligotrophic marine habitats. Thus, the current state of knowledge regarding *Trichodesmium* P metabolism indicates that natural populations can and are using DOP in situ—a revelation that makes *Trichodesmium* an ideal model system for studies of P metabolism.

In this study, and in White et al. (2010), we sought to examine the substrate specificity of DOP use by *Trichodesmium* IMS101 and describe the P-resource partitioning and metabolic efficiency of P acquisition. Herein, our research was guided by three distinct lines of questioning: (1) To what extent can *Trichodesmium* IMS101 hydrolyze select phosphonates as a sole source of P for growth? (2) Is there a stoichiometric relationship between phosphonate use and biogenic gas production? and (3) Is there evidence for inhibition of phosphonate hydrolysis in the presence of DIP? Our experimental design relied on a batch culture approach to achieve P mass balance and investigate the growth rates and stoichiometry of *Trichodesmium* IMS101 cultures grown on select DOP substrates both in the presence and absence of DIP. Tracking the fate of specific DOP compounds in mixed field assemblages or in the laboratory with cultures of model organisms is probably the most direct way to evaluate the ability of a particular organism to assimilate a specific compound. If growth occurs in defined medium, with DOP as the sole source of P, then the organism can surely use the test compound.

In our experiments, we demonstrated the following: (1) *Trichodesmium* IMS101 can use various phosphonates (MPn, EPn, and 2-AEP) as the sole source of P, (2) metabolism of MPn and EPn by *Trichodesmium* IMS101 produces CH_4 and C_2H_6 as by-products in a stoichiometric relationship with P assimilated, (3) growth efficiency on DOP, defined either as growth rate or biomass yield per mol of P, is comparable to growth on DIP in paired experiments, and (4) cultures that are actively metabolizing MPn and producing CH_4 continue to do so—albeit at reduced rates—even after the pulsed addition of DIP to the medium.

In sum, these results imply that if the supply rate of phosphonates (or at least MPn, EPn, or 2-AEP) in nature was sufficient, *Trichodesmium* would be able to maintain growth efficiencies similar to those observed under comparable DIP supply. White et al. (2010) have elaborated on this result and shown that the rate and ratio of *Trichodesmium* C and N_2 fixation is statistically similar for growth on tested P esters, phosphonates, and DIP alike. Moreover, we have clearly documented DIP–DOP and DOP–DOP co-metabolism at DIP levels significantly higher than observed in the open ocean, suggesting that in nature, *Trichodesmium* may be simultaneously accessing multiple P resources. Additionally, since EPn appeared to decrease the uptake of MPn, relative to the DIP + MPn treatment, our results may also indicate that EPn and MPn share a common transport system or compete for the active site of hydrolytic enzymes. In a conceptual model, Dyhrman et al. (2007) suggest there are three main transport systems—DIP, P ester, and C–P transport—for the assimilation of the dissolved P pools. Our data support this hypothesis. However, our results would also indicate that the bioavailability of the dissolved P substrates should be treated equally since simultaneous use of these pools appears evident.

Another substantial finding of this work is the observation that the enzymatic degradation of the alkylphosphonates MPn and EPn results in the liberation of nearly the full complement of the component alkane, CH_4 and C_2H_6, respectively. Thus, these phosphonates are being used primarily as a P source and not as a source of C. This finding mirrors work with heterotrophic bacteria that also indicates phosphonate hydrolysis as a P capture mechanism rather than a means of acquiring C or C–P (Ternan et al. 1998). The results of 2-AEP metabolism are not as clear. Modest levels of ethylene ($< 1\%$ of the initial 2-AEP on a molar basis) were generated in all treatments with added 2-AEP, suggesting the possibility of a more complex metabolism of the aminoethyl group following cleavage of the C–P bond. It would seem that since the amine group of 2-AEP is already in its most reduced form, 2-AEP could conceivably serve as an N substrate. The specific and inducible transport of 2-AEP has been described for a number of heterotrophic bacteria (Ternan et al. 1998) as a source of C, N, and P. However, because we did not fully constrain the N budget in these experiments (dissolved N

pools were not measured), we cannot identify the fate of the aminoethyl group. So while there is precedent for 2-AEP as a source of N to microorganisms, the degradation pathway for *Trichodesmium* spp. requires further study.

While we have clearly documented phosphonate metabolism and DIP-phosphonate co-metabolism in *Trichodesmium* IMS101 in a laboratory setting, extrapolation of our results to natural populations will require the implementation of carefully designed field experiments that include all naturally occurring and competing P substrates and microorganisms. To date, all research conducted on marine phosphonate metabolism has focused on catabolism, with the exception of one recent study that suggests select species of *Trichodesmium* may also produce intracellular phosphonates (Dyhrman et al. 2009). Phosphonate production pathways, molecular composition, and controls thereof remain unknown at present. Moreover, while phosphonates have been detected in marine ecosystems worldwide, the types of compounds present, their concentrations, and turnover rates have not been measured. Currently, there is no analytical method for detection of specific phosphonate compounds at the concentrations that are likely to be present in seawater (< 0.1 μmol L^{-1}).

Finally, while there remains some controversy regarding the oxidation state of P in phosphonate compounds, it now appears that the valence state is less than + 5, most likely + 3 (White and Metcalf 2007). If phosphonates are confirmed as a significant portion of the marine DOP pool, then the marine P cycle needs to be reevaluated since the current oxidation state of most P-containing compounds in seawater (effectively all; Karl 2007) is thought to be the pentavalent (+ 5) state. If marine microorganisms catalyze an active reduction–oxidation ($+ 3 \leftrightarrow + 5$) cycle in the sea, then there also exists the possibility for coupled energy transduction, especially during phosphonate decomposition and P assimilation. One major enigma, however, is the observation in both laboratory and field experiments (Karl et al. 2008; this study) of the stoichiometric production of CH_4 and C_2H_6 during P assimilation. These reduced carbon compounds could be oxidized to carbon dioxide for an additional energy gain or could be used as biosynthetic precursors for cell growth. The same is true for "energetically wasteful" production of H_2 by *Trichodesmium* as a by-product of the N_2 fixation reaction (Scanton 1983). The collective observations that *Trichodesmium* are not efficient in their use of available energetic resources implies that nutrients are a more probable limiting factor at both the physiological and community level than energy. Resolution of these and related phosphonate metabolic mysteries will require new methodologies and creative future experimentation.

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