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Intense ectoenzyme activities associated with *Trichodesmium* colonies in the Sargasso Sea

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ABSTRACT: Ectoenzyme activities of alkaline phosphatase (APA) and leucine aminopeptidase (LAP) associated with *Trichodesmium*, a globally significant dinitrogen (N_2) fixer, were measured on cruises to the Bermuda Atlantic Time-Series (BATS) site. Rates associated with *Trichodesmium* were compared to microbial enzyme activities in natural seawater between August 1992 and November 1997. Colonies of *Trichodesmium* function as 'microsites' for ectoenzyme activity and express high rates of APA (4 to 66 nmol colony^{-1} h^{-1}) and LAP (53 to 389 nmol colony^{-1} h^{-1}). For *Trichodesmium* APA, substrate half-saturation constants (K_m) exceeded surrounding seawater APA by a factor of 20 to 40 times. Overall, the ectoenzyme activity measured in puff shaped colonies of *Trichodesmium* did not differ significantly from the rates associated with the tuft morphology. Ectoenzyme activites measured in the cyanobacterial consortium did not vary as strongly seasonally as was observed in natural seawater. Elemental ratios of *Trichodesmium* colonies showed a molar C:N ratio around the Redfield stoichiometry (mean = 6, range 4 to 7) while the C:P ratios were much higher (mean = 513, range 163 to 1044). Calculated per volume seawater, measured uptake of phosphate and leucine were 4 to 6 orders of magnitude lower in *Trichodesmium* (1 to 5 fmol P l^{-1} h^{-1} and 0.1 to 3 fmol Leu l^{-1} h^{-1}) than in microplankton (0.3 to 4 nmol P l^{-1} h^{-1} and 0.002 to 0.02 nmol Leu l^{-1} h^{-1}). At peak abundance, *Trichodesmium* contributed a major part of total ectoenzyme activity in surface waters (81% of APA, 64% of LAP) suggesting that the diazotrophic colonies are significant sites of net nutrient regeneration. We propose that the high rates of regeneration associated with *Trichodesmium* colonies may accumulate dissolved nutrients during the course of bottle incubations, which leads to isotope dilution and an underestimation of P and N uptake in radiotracer studies with these cyanobacteria.

KEY WORDS: *Trichodesmium* · Alkaline phosphatase · Leucine aminopeptidase · Regenerated production · Phosphorus · Leucine · Isotope dilution

INTRODUCTION

Microbial ectoenzymes are important in all aquatic systems, including oligotrophic waters, because they regenerate inorganic and organic substrates from macromolecules. Hoppe (1983) used fluorogenic analogs and demonstrated the significance of ectoenzymatic activity associated with microplankton. Since then, ectoenzyme activity associated with free-living bacteria has been shown to be important in the remineralization of particulate organic matter (POM) and dissolved organic matter (DOM) (Rath et al. 1993, Christian & Karl 1995, Martinez et al. 1996, Christian & Karl 1998). Intense ectoenzyme activity by bacteria attached to aggregates and marine snow can be several orders of magnitude higher than the activity associated with free bacteria (Alldredge & Youngbluth 1985, Amy et al. 1987, Karner & Herndl

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1992, Smith et al. 1992). Therefore, it was suggested that ectoenzyme activity by bacteria attached to particles suspended in the upper water column is an important source of regenerated nutrients to the euphotic zone (Karl et al. 1988, Simon et al. 2002).

Microbial ectoenzyme activity associated with the relatively larger sized *Trichodesmium* colonies, a buoyant pelagic diazotrophic cyanobacterium, can also be a hot spot of microbial activity (Nausch 1996), similar to that found in marine snow (Grossart et al. 1998). The 2 most common species at the Bermuda Atlantic Time-Series (BATS) site are *Trichodesmium thiebautii* and *T. erythraeum* (Orcutt 1999). Colonies of *T. thiebautii* can easily be separated into 2 morphologies, 'puffs' and 'tufts', but they have been found to be genetically identical (Orcutt et al. 2002). The *T. thiebautii* puffs have trichomes arranged radially to form a spherical colony, and the *T. thieibautii* tufts have trichomes arranged in parallel. Ectoenzymatic activity associated with colonies of *Trichodesmium* was reported as early as the 1970s (Yentsch et al. 1972). The multitude of procaryotic and eukaryotic organisms associated with *Trichodesmium* colonies (Sheridan et al. 2002) suggests that a number of organisms can be a source for ectoenzyme activities detected in association with these cyanobacteria. Since alkaline phosphatase (APA) and leucine aminopeptidase (LAP) are associated with both phytoplankton, bacteria and *Trichodesmium* colonies (Perry 1976, Hollibaugh & Azam 1983, Martinez & Azam 1993, Berges & Falkowski 1996, Mulholland et al. 2002, Fu & Bell 2003, Stoecker & Gustafson 2003, Fu et al. 2005), we consider the assembly of organisms a center of ectoenzymatic regeneration of organic nutrients that may benefit all associated organisms within this consortium, including *Trichodesmium* itself.

Several studies have used fluorogenic substrates such as methylumbelliferyl phosphate (MUF-P) (Hoppe 1983, Ammerman 1993, Nausch 1996, Mulholland et al. 2002, Ammerman et al. 2003, Sohm & Capone 2006, Sohm et al. 2008) or *p*-nitrophenylphosphate (PNPP) (Stihl et al. 2001, Fu & Bell 2003) as a model for dissolved organic phosphate (DOP) in *Trichodesmium* APA measurements. Colonies of *Trichodesmium* have also been shown to have the ability to utilize phosphonates from the DOP pool (Dyhrman et al. 2006) and, as such, may provide regenerated nutrients to microplankton in oligotrophic systems (Dyhrman et al. 2009). Wu et al. (2000) reported that surface waters of the subtropical North Atlantic has 1 to 2 orders of magnitude lower dissolved inorganic P (DIP) levels than found in the Pacific, suggesting that a P-depleted environment exists for the Sargasso Sea. Cross-basin comparisons between the North Pacific and North Atlantic using MUF-P substrates have also demonstrated higher enzyme rates for the North Atlantic, indicating a P-limited environment (Sohm et al. 2008). With the exception of Carlson et al. (2002), a great number of reports have suggested P limitation associated with bacteria (Cotner et al. 1997, Rivkin & Anderson 1997, Caron et al. 2000, Ammerman et al. 2003, Obernosterer et al. 2003) and phytoplankton, including *Trichodesmium*, in the North Atlantic (Sañudo-Wilhelmy et al. 2001, Mills et al. 2004). The DOP pools at BATS are an order of magnitude higher (80 nM) than DIP pools (5 to 10 nM; Wu et al. 2000, Cavender-Bares et al. 2001, Ammerman et al. 2003). Therefore, microbial APA may be an important process to regenerate inorganic P from DOP pools in this low nutrient environment. With the exception of Nausch (1996), who studied microbial activities on *Trichodesmium* colonies, little work has been done on *in situ* LAP activities associated with *Trichodesmium* colonies. An earlier study by Elardo et al. (1994) reported elevated LAP activity associated with *Trichodesmium* colonies and suggested that the ectoenzyme activity may be important on a microscale level, similar to elevated enzyme activities associated with marine aggregates such as marine snow (Alldredge & Youngbluth 1985, Smith et al. 1992, Azam & Long 2001).

In the present study, we investigated the significance of the hydrolytic activity associated with *Trichodesmium*, by measuring seasonal rates and kinetics of APA and LAP associated with the colonial consortium in a comparison to natural seawater. We also investigated whether the 2 morphologies of *T. thiebautii*, puffs and tufts, expressed significantly different rates of ectoenzyme activity. We propose that colonies of *Trichodesmium* at BATS are active sites of regenerated production of P and N. If not accounted for, these high rates of regeneration can lead to isotope dilution and underestimation of P and N uptake in radiotracer studies.

MATERIALS AND METHODS

The BATS site (31° 50′ N and 64° 10′ W) was visited between August 1992 and November 1997. Fig. 1 and Tables 1 to 5 show the times of enzyme measurements (rate comparisons, saturation kinetics), nutrient incorporation and elemental measurements performed on *Trichodesmium* colonies during this

period. During each cruise, surface seawater samples were collected using a 12 l Niskin water sampler attached to a CTD rosette or by using an 8 l GoFlo bottle attached to a Kevlar wire. *Trichodesmium* colonies were collected by gently towing a plankton net at the surface (1 m diameter, 335 µm mesh size) for 15 to 20 min and the collected colonies were emptied into a graduated 10 l polypropylene bucket. Colony abundance was estimated from 300 to 400 ml aliquot samples from the bucket and normalized to volume of filtered seawater using a General Oceanics flow meter attached to the opening of the net (Orcutt et al. 2001). Colonies of *T. thiebautii* were enumerated based on morphology and separated into the easily recognizable puffs (colonies with trichomes arranged radially) and tufts (trichomes arranged in parallel). Ectoenzyme activity of *Trichodesmium* colonies and natural seawater were compared in 2 different ways: (1) on a volumetric basis using *in situ* surface colony abundance and expressed as the percentage of activity contributed by *Trichodesmium* per total activity (*Trichodesmium* + seawater); and (2) using a volume concentration factor (VCF) calculated as the activity per *Trichodesmium* colony volume divided by the equivalent volume of seawater. The latter is a method used to compare marine snow aggregate rates with the surrounding seawater (Smith et al. 1992). In our comparisons, natural seawater was not normalized to particulate organic carbon (POC), since this would place a bias towards the large fraction of the refractive, non-living C-pool at the BATS site (Gundersen et al. 2001). Similarly, ectoenzyme rates associated with *Trichodesmium* were only normalized to chlorophyll pigments (chl *a*) for comparisons to previous studies.

Ectoenzyme activity associated with *Trichodesmium* colonies

The APA assay measures the fluorescent end product methylumbelliferyl (MUF) that is produced when phosphate is hydrolyzed from MUF-P by the enzyme alkaline phosphatase. The APA assay was conducted in polystyrene tubes containing 10 ml of natural seawater (microplankton) or in freshly GF/F filtered seawater with one *Trichodesmium* colony. The activity of APA in natural seawater was measured in comparison to the activity associated with *Trichodesmium* colonies. The MUF-P substrate was added and the incubation was left in the dark at ambient surface seawater temperature. The APA activity was measured after 1 to 2 h for *Trichodesmium* and 3 to 4 h for natural seawater as described by Ammerman (1993). These relatively long incubation times are necessary due to the low biomass associated with oligotrophic seawater. The fluorescence end product was measured on a Sequoia-Turner fluorometer using filter settings of 360 nm narrow band excitation and 430 nm sharp cut emission (Ammerman 1993). On a routine basis, a control blank consisting of freshly (GF/F) filtered seawater was subtracted from the sample readings. No increase in background fluorescence was observed in boiled seawater with MUF-P added as substrate and pigment autofluorescence from the *Trichodesmium* colonies did not interfere with the assay measurements on the fluorometer. Substrate concentration for the seasonal time-series was 10 µM MUF-P for the APA assay and the kinetic experiments confirmed this to be saturating.

The LAP assay measures the end product 7-amino 4-methylcoumarin (AMC) fluorometrically when leucine is hydrolyzed from L-leucine 4-methyl-7-coumarinylamide hydrochloride (Leu-MCA) by the enzyme LAP. Volumes of 10 ml natural seawater and seawater containing 2 *Trichodesmium* colonies were incubated in polypropylene centrifuge tubes. The Leu-MCA substrate was added and the incubation left at ambient sea surface temperature in the dark for 2 to 4 h for *Trichodesmium* and 4 to 8 h for natural seawater. The fluorescence end product was measured on a Sequoia-Turner fluorometer using filter settings of 360 nm narrow band excitation and 430 nm sharp cut emission (Chróst & Velimirov 1991). Substrate concentration for the seasonal time-series was 308 µM for the LAP assay and the kinetic experiments confirmed this to be saturating.

Enzyme kinetics associated with *Trichodesmium* were investigated and compared to natural seawater assays of both APA and LAP. Substrate concentrations for the kinetic studies ranged from 0.1 to 50 µM (APA) and 39 to 385 µM (LAP) and no less than 5 substrate concentrations were used to generate each saturation curve.

Phosphate and leucine incorporations

Orthophosphate was added using a ^{33}P-phosphate label (New England Nuclear Life Science Products; specific activity 4.78 Ci mM^{-1}) and the *in situ* isotope dilution was calculated. Total amount of ^{33}P-phosphate added to each incubation was 5 nM and the incubation time course lasted 0.5 to 1 h. Cells from the seawater samples were collected on 25 mm,

0.2 µm cellulose nitrate filters, rinsed with filtered seawater, and left to dry in separate scintillation vials. Ethyl acetate (1 ml, concentrated) was added to the dried filters and, following dissolution, 9 ml of Hionic Fluor scintillation cocktail (Packard) was added to each sample. Time zero samples for seawater were filtered immediately after substrate addition and processed as an ordinary sample.

At the end of the assay, each *Trichodesmium* colony was transferred to a separate scintillation vial containing 250 µl Milli-Q water. In order to dissolve the *Trichodesmium* colonies, 750 µl Soluene-350 (Packard) was added and left to dissolve, then 9 ml of Hionic Fluor scintillation cocktail was added. After a minimum of 2 d and repeated vortex mixing, the samples were analyzed on a Packard Tricarb Analyzer using the external channel ratio method. In order to correct for ^{33}P-phosphate adsorption to the colonies, time zero samples were made by dipping and immediately removing *Trichodesmium* colonies from the incubation solution.

Leucine incorporation was measured using L-[4,5-^{3}H] leucine (^{3}H-leucine, New England Nuclear Life Science Products) at a final concentration of 21 nM (specific activity 7 to 8 Ci mM^{-1}). In order to obtain total incorporation of ^{3}H-leucine, the samples were not extracted in trichloroacetic acid (TCA), but otherwise followed the method described by Kirchman et al. (1985, 1986). Cells from seawater samples were collected on 25 mm, 0.2 µm cellulose nitrate filters, rinsed with filtered seawater and put in a scintillation vial to dry. Ethyl acetate (1 ml, concentrated) was added to the dried samples and left to dissolve overnight. Hionic Fluor scintillation cocktail (9 ml) was added to each sample before analysis on a Packard Tricarb Analyzer.

Leucine incorporation by *Trichodesmium* was measured with the same final substrate concentration as for seawater and the colonies were collected, processed and time zero corrected as described in the ^{33}P-phosphate incubation.

Elemental cell content and chl *a* in colonies of *Trichodesmium*

Net-tow collected colonies were analyzed for cellular organic carbon (C), nitrogen (N) and phosphorus (P) on selected cruises to the BATS site between August 1992 and November 1997. During the abundance estimation of a net-tow, all enumerated colony morphologies were routinely placed in beakers containing GF/F-filtered seawater. Colonies for C and N analysis (2 to 5 per sample) were transferred to precombusted 7 mm GF/F filters using a 1 µl inoculation loop. The samples were dried at 60°C overnight and fumigated in HCl. The re-dried filters were analyzed on a Control Equipment Corporation 240-XA Elemental Analyzer and the results corrected with empty filter blanks. Colonies for cellular P analysis (3 to 15 per sample) were either placed on a precombusted GF/C filter or left in 250 µl of Milli-Q water in an acid-cleaned scintillation vial. Before the samples were dried at 95°C, 1 ml of 0.017 M magnesium sulfate was added, combusted and finally, hydrolyzed in hydrochloric acid (Solorzano & Sharp 1980). In 1992, the hydrolyzed phosphate was analyzed manually according to Strickland & Parsons (1972). Thereafter, phosphate analysis was carried out using a Technicon Auto Analyzer II optimized for low-level detection of the phospho-molybdenum blue complex (Gordon et al. 1993). The results were corrected with GF/F filter or Milli-Q water blanks as appropriate. Colonies were collected for chl *a* analysis in August, October, November 1997 and in January 1998. Each colony was placed on a 25 mm GF/F filter and stored separate in liquid nitrogen. The samples were extracted in 90% acetone at –20°C and analyzed on a Turner Design 110 fluorometer according to Strickland & Parsons (1972).

RESULTS

Ectoenzyme activity in seawater and associated with *Trichodesmium* colonies

Overall, the rate of APA associated with *Trichodesmium* colonies was not higher for tufts than for puffs (Table 1). Time-series measurements of APA in surface seawater ranged from 0.8 to 4 nmol l^{-1} h^{-1} and showed higher rates in spring and summer (Fig. 1A). Rates of APA associated with *Trichodesmium* puffs in surface waters (Fig. 1B) were an average of 28 nmol colony^{-1} h^{-1} for puffs (range 4 to 66) and, although not unequivocally, showed a similar seasonal trend to seawater microplankton. The half-saturation constant (K_m) for APA associated with *Trichodesmium* colonies was higher than seawater APA (Table 2). Surface seawater APA showed a narrow range in K_m (0.1 to 1 µM) compared to *Trichodesmium* (puffs, 2 to 29 µM; tufts, 2 to 6 µM).

The LAP activity associated with seawater ranged from 0.6 to 17 nmol l^{-1} h^{-1} and showed a strong seasonal pattern with higher rates in spring and late fall (Fig. 1C). Average LAP activity of *Trichodesmium*

Table 1. Comparison of alkaline phosphatase (APA) and leucine aminopeptidase (LAP) rates in *Trichodesmium* puff and tufts colonies at different substrate concentrations. Standard errors (SE) and the number of replicate incubation tubes (n) are shown for all incubations. A Student's *t*-test of difference between 2 sample means was tested for significance at the 95% confidence level (**$p < 0.05$) using degrees of freedom ($df = n_1 + n_2 - 2$) for unequal sample size $n < 30$ (Sokal & Rohlf 1969)

	Substrate (µM)	Enzyme activity (nmol colony^{-1} h^{-1}) Puff	SE	n	Tuft	SE	n	Significance (Tuft > Puff) df	*t*	p
APA										
Aug 29, 1992	0.1	1.01	0.40	6	1.99	0.92	12	16	4.931	<0.05**
Nov 25, 1992	0.1	0.59	0.37	4	0.96	0.37	4	6	1.414	>0.4
Apr 21, 1994	20	20.81	16.68	2	134.35	66.85	2	2	2.330	>0.2
Mar 15, 1996	10	0.85	0.17	3	1.34	0.47	3	4	1.698	>0.2
Jul 9, 1996	10	0.14	0.13	3	1.47	0.71	3	4	3.191	<0.05**
Nov 6, 1996	10	0.32	0.16	3	1.61	0.99	3	4	2.228	>0.1
LAP										
Dec 9, 1993	154	30.95	9.27	2	52.75	0.98	2	2	3.307	>0.05
Jan 24, 1994	308	206.94	24.21	3	240.32	99.22	3	4	0.566	>0.5
Apr 5, 1994	308	205.32	68.01	3	273.95	82.50	3	4	1.112	>0.4
Jul 25, 1995	200	79.97	21.97	2	180.30	85.21	2	4	1.591	>0.2

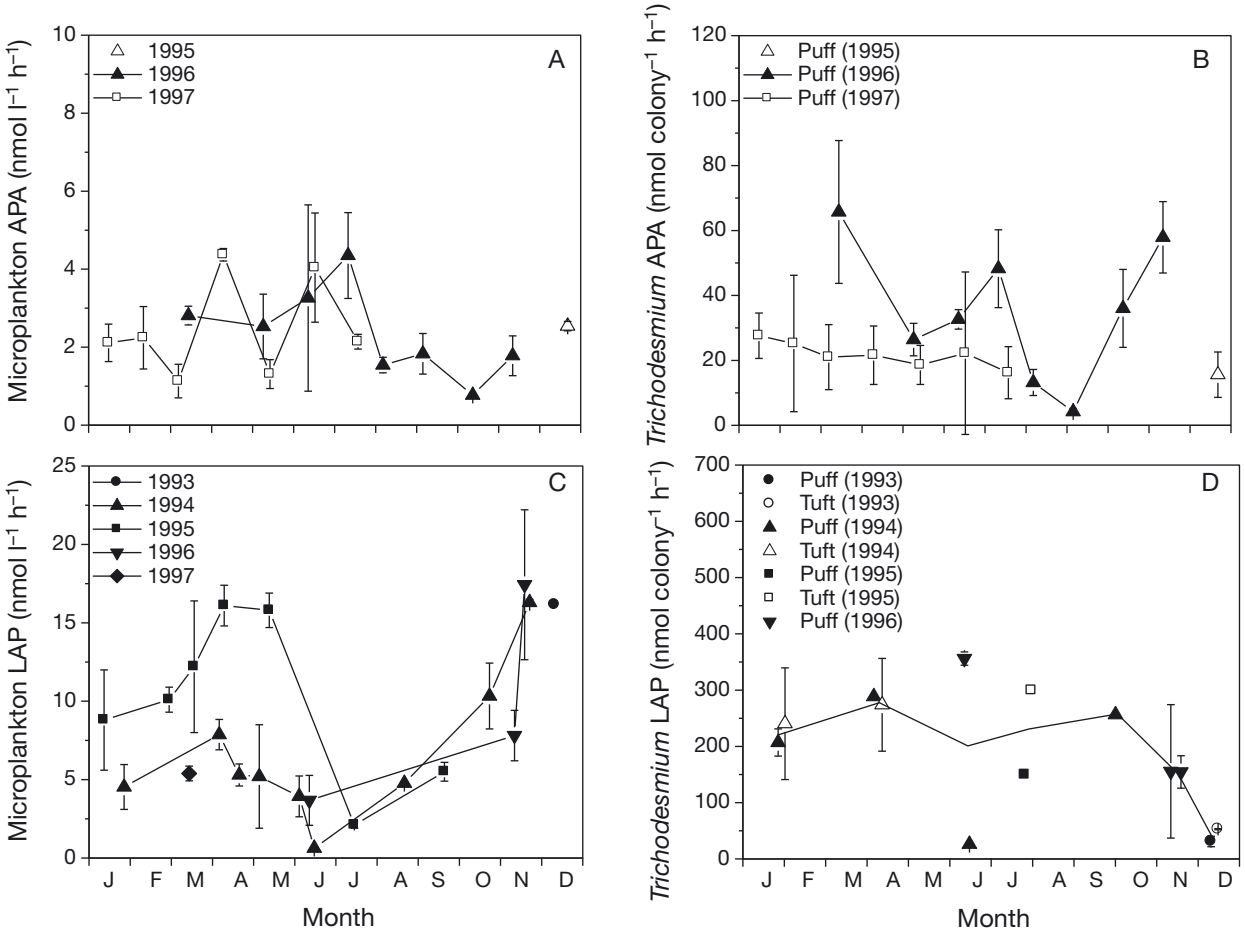

Fig. 1. Ectoenzyme activities measured at the Bermuda Atlantic Time-Series (BATS) site as a function of time within one generic year. Measurements of alkaline phosphatase (APA) were made (A) on microplankton and (B) in association with *Trichodesmium* colonies between 1995 and 1997. Measurements of leucine aminopeptidase (LAP) were made (C) on microplankton and (D) in association with *Trichodesmium* colonies between 1993 and 1997. Number of data points >7 within a year have lines connecting the symbols. The line in D shows calculated combined monthly averages of LAP in puffs and tufts

Table 2. APA and LAP kinetics measured in surface seawater and associated with *Trichodesmium* colonies collected in the vicinity and at the BATS site between August 1992 and July 1995. The half-saturation constant (K_m) and maximum rate of enzyme activity (V_{max}) were calculated for seawater (nmol l^{-1} h^{-1}) and cyanobacteria (nmol $colony^{-1}$ h^{-1}) using a Model I linear regression of a Lineweaver-Burk plot. A log-transformed geometric mean and range was generated from the number of saturation plots (n). Substrate concentrations ranged between 0.1–50 µM (APA) and 39–385 µM (LAP)

	K_m (µM)		V_{max}		n
	Mean	Range	Mean	Range	
APA					
Seawater	0.2	0.1–1	3	1–29	11
Puffs	8	2–29	46	15–104	8
Tufts	4	2–6	145	91–254	4
LAP					
Seawater	171	87–408	14	1–124	7
Puffs	156	45–594	147	54–377	7
Tufts	121	68–175	382	217–578	5

colonies was 201 nmol $colony^{-1}$ h^{-1} for puffs (range 26 to 389) and 216 nmol $colony^{-1}$ h^{-1} for tufts (range 53 to 299). The LAP activity was similar for both puff and tuft colonies and there was no apparent seasonal pattern (Fig. 1D). The K_m for LAP activity associated with *Trichodesmium* colonies and seawater was similar (Table 2) while the average calculated maximum rate of enzyme activity (V_{max}) was much higher in *Trichodesmium* tufts.

The contribution of *Trichodesmium* ectoenzyme activity towards total APA and LAP (compared with seawater on a per volume basis) varied with observed colony abundance (Table 3). At peak colony abundance, *Trichodesmium* constituted more than 3/4 of total APA and approximately 2/3 of total LAP. By using the volume concentration factor to compare marine aggregates with seawater (Smith et al. 1992), *Trichodesmium* APA and LAP activities were 6 to 7 orders of magnitude higher than seawater ectoenzyme activities (Table 3).

Phosphate and leucine incorporation by *Trichodesmium*

The incorporation of P associated with *Trichodesmium* colonies and seawater plankton ranged from 0.3 to 0.7 pmol $colony^{-1}$ h^{-1} and 0.3 to 4 pmol l^{-1} h^{-1}, respectively (Table 4). The calculated P incorporation rate by *Trichodesmium*, normalized to volume of seawater using *in situ* colony abundance distribution, was 5 to 6 orders of magnitude lower than natural seawater plankton (Table 4).

The incorporation of ^{3}H-leucine associated with *Trichodesmium* colonies and seawater plankton ranged from 0.02 to 0.2 pmol $colony^{-1}$ h^{-1} and 0.002 to 0.02 pmol l^{-1} h^{-1}, respectively (Table 4). The ^{3}H-leucine incorporation rates associated with the colonies, normalized to volume of seawater, was 4 to 6 orders of magnitude lower than the incorporation rate measured in seawater plankton (Table 4).

Elemental C, N and P cell content and chl *a* in *Trichodesmium*

The elemental cell content in puffs ranged between 5–12 µg C, 0.9–2 µg N and 0.03–0.2 µg P $colony^{-1}$, whereas the tufts ranged between 6–19 µg C, 1–4 µg N and 0.04–0.06 µg P (Table 5). The elemental molar C:N ratio ranged between 4–7 and the average was similar to the Redfield stoichiometry of 6.6 (Table 5). The molar C:P ratios of *Trichodesmium* colonies were higher than the Redfield stoichiometry of 106 at all times at the BATS site during this study (Table 5) and most apparent in tuft colonies. Average pigment content was 48 ng chl *a* $colony^{-1}$ (range 35 to 62).

DISCUSSION

Ectoenzyme activity generated by the *Trichodesmium* consortium

Microbial activity found in organic aggregations such as marine snow (Alldredge & Youngbluth 1985, Smith et al. 1992, Azam & Long 2001) can be a major biological process in oceanic environments (Azam 1998). Similarly, Nausch (1996) investigated microbial activities associated with *Trichodesmium* colonies and suggested that bacteria play an active role in the degradation of the colonies. Paerl et al. (1989), however, suggested that bacteria associated with *Trichodesmium* colonies are symbiotic or mutualistic in nature, and a great number of other studies have shown that APA and LAP can be associated with marine phytoplankton, bacteria and cyanobacteria (Perry 1976, Hollibaugh & Azam 1983, Martinez & Azam 1993, Berges & Falkowski 1996, Mulholland et al. 2002, Fu & Bell 2003, Stoecker & Gustafson 2003, Fu et al. 2005). Colonies of *Trichodesmium* harbor an assortment of both prokaryotic and eukaryotic organisms (Sheridan et al. 2002) and each colony

Table 3. APA and LAP activities measured in surface seawater and associated with *Trichodesmium* colonies collected in the vicinity and at the BATS site. Seawater plankton (Seawater) enzyme activities were compared to *Trichodesmium* per liter (Abundance × *Trichodesmium*) using known estimates of colony abundance, and the fraction activity contributed by *Trichodesmium* (% Tricho) was calculated as the percentage of total enzyme activity measured (*Trichodemsium* + Seawater). A second set of comparisons were made similar to the Smith et al. (1992) study; the volume concentration factor (VCF) was calculated as the fraction of activity in the volume of a *Trichodesmium* colony, per activity in the equivalent volume of seawater. For the VCF comparison, we assumed an average diameter of 1250 µm of the spherical *Trichodesmium* puff colonies (Carpenter & McCarthy 1975, Post et al. 2002) and calculated an average volume of 1×10^9 µm^3 per colony

Date	Abundance (colony m^{-3})	*Trichodesmium* (nmol colony^{-1} h^{-1})	Seawater (nmol l^{-1} h^{-1})	% Tricho	VCF ($\times 10^7$)
APA					
Dec 1995	4.8	15.6	2.5	2.9	0.6
Mar 1996	0.8	65.7	2.8	1.9	2.3
May 1996	2.8	26.4	2.5	2.8	1.0
Jun 1996	5.0	32.6	3.3	4.8	1.0
Jul 1996	15.6	48.2	4.4	14.7	1.1
Aug 1996	30.1	13.2	1.5	20.5	0.8
Sep 1996	18.5	4.2	1.8	4.1	0.2
Oct 1996	89.2	36.0	0.8	80.7	4.6
Nov 1996	16.4	57.9	1.8	34.8	3.2
Jan 1997	1.0	27.6	2.1	1.3	1.3
Feb 1997	2.4	25.2	2.2	2.6	1.1
Mar 1997	2.3	21.0	1.1	4.0	1.8
Apr 1997	0.2	21.6	4.4	0.1	0.5
May 1997	0.2	18.6	1.3	0.3	1.4
Jun 1997	1.5	22.2	4.0	0.8	0.5
Jul 1997	13.9	16.2	2.1	9.5	0.7
LAP					
Dec 1993	2.5	31.0	16.2	0.5	0.2
Jan 1994	1.3	206.9	4.5	5.6	4.5
Apr 1994	0.5	288.6	7.9	1.9	3.6
Jul 1994	10.7	26.2	0.6	30.7	4.1
Jun 1996	5.0	356.1	3.7	32.6	9.5
Oct 1996	89.2	155.6	7.8	64.0	1.9
Nov 1996	16.4	154.6	17.4	12.7	0.9

should be viewed as a consortium of heterotrophic and autotrophic processes that are difficult to separate. Therefore, *Trichodesmium* should be included as one of the many organisms that may produce ectoenzymes and we cannot assign APA and LAP rates to certain groups within the consortium of a colony as has been done in other studies. Since *Trichodesmium* puffs appear to have more epiphytic organisms associated with the colonies (Borstad & Borstad 1977, Sheridan et al. 2002), we had anticipated higher ectoenzyme rates associated with this morphology. In our study, however, the difference in APA associated with puff and tuft colonies of *Trichodesmium* was most frequently insignificant (Table 1).

Seasonal rates of APA and LAP

In oligotrophic waters, DOM generated from autotrophic phytoplankton can be a major source of substrate for enzyme nutrient regeneration (Azam 1998). Time-series data from the Sargasso Sea gyre show a short-lived bloom of phytoplankton in spring (Lohrenz et al. 1992) that generate a relatively low but significant amount of DOM during this period of time (Carlson et al. 1994, Hansell & Carlson 2001). Since all enzyme activities are substrate resource driven, we would expect higher rates of ectoenzymatic activity during times of high photosynthesis and release of DOM. Therefore, the higher rates of APA and LAP associated with seawater microplankton in spring (Fig. 1) were expected. Peaks in APA could be found well into summer (Fig. 1A) and this was well beyond the release of DOM associated with primary productivity in April-May at BATS (Carlson et al. 1994, Hansell & Carlson 2001). However, the surface seawater APA peaks in summer coincide with peak abundances in free trichomes and colonies of *Trichodesmium* (Orcutt et al. 2001), which may provide a source of DOM for the surrounding seawater microplankton. McCarthy & Carpenter (1979) found high APA rates (0.2 to 0.3 µmol P µg^{-1} chl *a* h^{-1}) associated with *Trichodesmium* colonies using relatively low (0.05 µM) substrate concentrations. By using substrate concentrations equal to 0.1 µM, the APA rates associated with *Trichodesmium* in this study (1.1 to 1.52 µmol P µg^{-1} chl *a* h^{-1}) were similar to the McCarthy & Carpenter (1979) study (Table 6). More recently, Sohm & Capone (2006) used similar substrate concentrations (0.1 µM) and reported APA rates associated with *Trichodesmium* colonies from the western Sargasso Sea (0.003 to 0.01 µmol P µg^{-1} chl *a* h^{-1}) that were almost an order of magnitude lower than the earlier reports from the same region (Table 6). The K_m of *Trichodesmium* APA was higher than seawater (Table 2) and this is indicative of an enzyme adapted to high concentrations of

Table 4. Rates of P- and Leu-incorporation associated with *Trichodesmium* puff colonies and in seawater plankton collected at the BATS site. Seawater plankton incorporation rates (Seawater) were compared to *Trichodesmium* per liter (Abund. × Activity) using known estimates of colony abundance. Note the different units in the volumetric rates. ND: no data

	Trichodesmium			Seawater
	Abundance (colony m^{-3})	Activity (pmol colony^{-1} h^{-1})	Abund. × Activity (fmol l^{-1} h^{-1})	(nmol l^{-1} h^{-1})
P-incorporation				
1996				
Nov 6	16.4	0.27	4.5	0.78
Nov 14	8.3	0.34	2.8	0.26
1997				
Feb 7	2.4	0.50	1.2	0.50
Mar 5	2.3	0.67	1.5	3.96
Leu-incorporation				
1994				
Jul 21	1.0	0.13	0.1	0.002
Aug 1	0.7	0.22	0.2	ND
1996				
Apr 9	5.0	0.10	0.5	0.009
May 7	11.8	0.10	1.2	0.013
Jun 11	25.0	0.02	0.5	0.021
Aug 6	26.4	0.13	3.4	0.015
Nov 6	16.4	0.10	1.6	0.010

Table 5. Elemental carbon (C), nitrogen (N) and phosphorus (P) in *Trichodesmium* colonies collected in surface waters at BATS. Elemental content (µg colony^{-1}) and the molar C:N:P ratios of the colonies are arranged in a composite year. Each SE was calculated from the analyzed means of samples (n) each containing 3 colonies. ND: no data

Date	C	SE	n	N	SE	n	P	SE	n	C:N	C:P
Puffs											
Mar 1993	7.4	0.3	3	1.5	0.2	3	0.071	0.020	6	6	269
Apr 1994	11.6	4.5	9	2.2	0.8	9	0.171	0.075	5	6	175
Jun 1997	7.3	0.7	3	1.9	0.1	3	0.074	0.013	3	4	255
Jul 1997	4.8	2.5	12	0.9	0.5	12	0.076	0.021	3	6	163
Aug 1992	6.5	2.4	23	1.2	0.5	23	ND			6	ND
Nov 1997	9.3	2.8	28	1.8	0.5	28	0.033	0.010	3	6	728
Tufts											
Mar 1993	6.3	5.0	2	1.1	1.0	2	ND			7	ND
Jun 1997	19.0	3.9	4	3.6	0.9	4	0.047	0.012	3	6	1044
Jul 1997	19.4	2.5	3	4.0	0.5	3	0.056	0.008	3	6	895
Aug 1992	9.6	4.1	20	1.7	0.8	20	0.049	0.004	2[a]	7	506
Nov 1997	8.6	4.0	16	1.4	0.9	16	0.038	0.001	2	7	585

[a]Average of colonies collected from surface waters and at 100 m depth

organic P associated with the cyanobacterial colonies. Our results suggest that *Trichodesmium* colonies are able and capable of rapidly hydrolyzing high concentrations of DOP. There is only one other *in situ* study of LAP associated with *Trichodesmium* colonies (Table 6) and the substrate concentration used in that study was more than 3 orders of magnitude lower than the range in our study. Although the measured rates of LAP in this study show a wider range (Table 6), the high range of substrate concentrations, in both *Trichodesmium* and seawater, may suggest that LAP is capable of responding to high concentrations of dissolved organic nitrogen (DON).

Substrate saturation and multiple enzyme kinetics

Increasing substrate concentrations usually yield higher rates of a single ectoenzyme activity until a saturation level is reached. Recent genetic studies, however, have established that *Trichodesmium* contain 2 APAs (phoX and phoA) Orchard et al. 2009) and 3 different APAs (phoX, phoD and phoA) have been found associated with marine bacteria (Luo et al. 2009, Sebastian & Ammerman 2009). Similarly, the Leu-MCA substrate used for LAP measurements is regarded as a model substrate for a number of different proteases (Berges & Falkowski 1996, Obayashi & Suzuki 2005) and, hence, a multitude of different saturation kinetics can be expected depending on the predominant ectoenzymes. Martinez et al. (1996) reported shifts in the enzyme kinetics of ectoenzymes and suggested they were caused by changes in species composition within the microplankton community. Also, Elardo et al. (1994) suggested that different ectoenzymes may become engaged when extreme ranges in concentrations of the APA and LAP substrates were applied in assays. In poorly described pools of ectoenzymes, such as APA and LAP in seawater or associated with colonies of *Trichodesmium*, a multitude of similar enzymes with dramatically different enzyme kinetics may coexist. Therefore, these *in situ* assays may not be as straight forward to interpret as the ones observed in monoculture experiments.

Berges & Falkowski (1996) and Martinez et al. (1996) found LAP in cyanobacteria with a compara-

ble but wider range of substrate saturations (115 to 879 µM) to this study (Table 2), but this is at the higher end of what has been reported from coastal estuaries in central Europe (Somville & Billen 1983) and from the central Pacific Ocean (Christian & Karl 1998). Only APA associated with colonies of *Trichodesmium*, however, exhibited higher K_m when compared to seawater (Table 2) and, hence, the colonies appeared to be only adjusted to higher concentrations of DOP. Karl et al. (1992) found a 3-fold enrichment of DON and more than 27 times higher concentrations of ammonium in water samples collected during a *Trichodesmium* bloom at Stn ALOHA off Hawaii. Therefore, the high rates of LAP found in this study, the amino acid oxidation reported by Mulholland et al. (1998) and reports of the release of recently fixed nitrogen (Glibert & Bronk 1994, Capone et al. 1994), suggest that *Trichodesmium* colonies can be a point source of DON in the sea. The summary of current literature (Table 6) also shows a general agreement that higher substrate concentrations yield higher rates of APA and LAP. We propose that the high rates of APA and LAP associated with colonies of *Trichodesmium* at high substrate concentrations are an adaptive response to the high concentrations of dissolved organic substrates produced by the cyanobacterial consortium.

Colonies of *Trichodesmium* as a source of organic and inorganic nutrients

The molar C:N ratio of the *Trichodesmium* colonies in this study was similar to the Redfield stoichiometry, but the cellular quota for P in the diazotrophs was low (Table 5). Elevated C:P ratios in *Trichodesmium* colonies have also been found in a number of other studies (Mague et al. 1977, Karl et al. 1995, Letelier & Karl 1996, 1998, Sañudo-Wilhelmy et al. 2001, 2004, Mulholland et al. 2002, Fu et al. 2005,

Table 6. Summary of ectoenzyme *in situ* measurements associated with colonies of *Trichodesmium*. Averages (and range) were calculated from a number of single estimates (n) reported from each site. MFP = 3-0-methylfluorescein phosphate; MUF-P = 4-methylumbelliferyl phosphate; PNPP = *p*-nitrophenylphosphate; Leu-MCA = L-leucine 4-methyl-7-coumarinylamide hydrochloride

Sampling site	Substrate	[µM]	µmol µg^{-1} chl *a* h^{-1} Avg	Range	n	Source
APA						
North Atlantic (Sargasso Sea)	MFP	0.05	0.24	0.17–0.30	2	McCarthy & Carpenter (1979)
NE Caribbean	MUF-P	0.05	0.14	0.03–0.35[a]	20	Nausch (1996)
Gulf of Aqaba (coastal)	PNPP	400	0.23	0.15–0.36	4	Stihl et al. (2001)
Gulf of Aqaba (open waters)	PNPP	400	12.2	3.1–31.6	4	Stihl et al. (2001)
NE Caribbean	MUF-P	0.1	0.11	0.03–0.24	9	Mulholland et al. (2002)
Off Northern Australia	MUF-P	0.1	0.004	0.001–0.010	14	Mulholland et al. (2002)
Great Barrier Reef (culture)	PNPP	417	0.002	0.001–0.007[b]	5	Fu & Bell (2003)
Western Atlantic (Sargasso Sea)	MUF-P	0.1	0.006	0.002–0.010	3	Sohm & Capone (2006)
Atlantic (Amazon Plume)	MUF-P	0.1	0.016	0.005–0.070[c]	8	Sohm et al. (2008)
Pacific (SW off Hawaii)	MUF-P	0.2	0.011	0.001–0.012[c]	6	Sohm et al. (2008)
Sargasso Sea (BATS)	MUF-P	0.1	0.05	0.01–0.10[d]	7	Present study
Sargasso Sea (BATS)	MUF-P	1	0.13	0.08–0.17[d]	6	Present study
Sargasso Sea (BATS)	MUF-P	5	0.41	0.31–0.65[d]	3	Present study
Sargasso Sea (BATS)	MUF-P	10	0.56	0.20–2.76[d]	10	Present study
Sargasso Sea (BATS)	MUF-P	20	1.09	0.38–4.13[d]	5	Present study
Sargasso Sea (BATS)	MUF-P	50	1.52	0.57–4.92[d]	6	Present study
LAP						
NE Caribbean	Leu-MCA	0.05	0.038	0.006–0.12[a]	20	Nausch (1996)
Sargasso Sea (BATS)	Leu-MCA	40	0.26	0.21–0.31[d]	2	Present study
Sargasso Sea (BATS)	Leu-MCA	77	1.46	0.21–3.05[d]	6	Present study
Sargasso Sea (BATS)	Leu-MCA	154	1.65	0.65–3.8[d]	7	Present study
Sargasso Sea (BATS)	Leu-MCA	308	4.90	3.22–8.10[d]	8	Present study
Sargasso Sea (BATS)	Leu-MCA	385	5.06	3.20–8.01[d]	2	Present study

[a]Calculated from Table 3 in Nausch (1996) using a substrate concentration of 50 nM (Nausch 1996) and chl *a* content of 32 ng colony^{-1} measured in the same region (Mulholland et al. 2002)
[b]Calculated using APA rate (fmol PNPP cell^{-1} h^{-1}) and chl *a* content (pg cell^{-1}) from Fig. 2 in Fu & Bell (2003)
[c]Calculated from Table 1 in Sohm et al. (2008) using a substrate concentration of 100 nM (Atlantic) and 200 nM (Pacific)
[d]Converted ectoenzyme rates (nmol colony^{-1} h^{-1}) using an average of 48 ng chl *a* colony^{-1}

Krauk et al. 2006, White et al. 2006). Low cellular P in *Trichodesmium* was termed the 'P sparing effect' by Karl et al. (1992) as it was suggested that this is a specific cellular adaptation for growth of these cyanobacteria in P-depleted environments. The significance of *Trichodesmium* ectoenzyme activity in surface waters is high compared with seawater APA and LAP when colony abundance is at its seasonal peak at the BATS site (Table 3). As suggested by Smith et al. (1992) for marine snow, the significance of *Trichodesmium* APA and LAP can also be compared to seawater using a volume concentration factor (Table 3). This comparison assumes that the volume of the interstitial water in a marine snow aggregate is directly comparable to surrounding seawater on a per volume basis. If we apply the same logic to colonies of *Trichodesmium*, the volume displacement of the consortium of organisms associated with a cyanobacterial colony can be compared to the equivalent volume of seawater. The orders of magnitude higher APA and LAP activities associated with *Trichodesmium* (Table 3) suggest that the colonies are 'hotspots' and a source of regenerated P and N in surface waters at BATS.

Inorganic P and Leu incorporation by colonies of *Trichodesmium*

Phytoplankton in P-limiting environments such as the Sargasso Sea depend heavily on regeneration of nutrients to sustain growth (Cotner et al. 1997, Rivkin & Anderson 1997). The high capacity for organic phosphorus and nitrogen hydrolysis (Tables 2 & 6) combined with a low affinity and apparent lack of competitive uptake of inorganic and organic nutrients (Table 4) suggest that nutrient regeneration by *Trichodesmium* colonies may be as important to the surrounding microplankton community as to the cyanobacteria themselves. The low level of both phosphate and leucine incorporation measured in association with the *Trichodesmium* colonies compared to seawater (Table 4) suggests that, on a short-term temporal scale, the colonies are primarily a source of regenerated phosphate and nitrogen (Leu) to the surrounding microbial community. Since high rates of APA associated with the colonies will produce significant amounts of inorganic P, our isotope dilutions may have been severely underestimated by only accounting for bulk dissolved phosphate in surface waters. Sañudo-Wilhelmy et al. (2004) found relatively high concentrations of P adsorbed to the surface of *Trichodesmium* colonies and Sohm & Capone (2006) estimated that an average of 8% of their P-tracer could be accounted for by adsorption. Therefore, in addition to P adsorption, we suggest that the comparatively low P and Leu tracer incorporation rates associated with *Trichodesmium* (Table 4) were caused by high APA and LAP rates diluting the existing P and Leu pools surrounding the colonies. The LAP measurements in this study are based on a model substrate (Leu-MCA) that will account for a number of proteases (Berges & Falkowski 1996, Obayashi & Suzuki 2005). Therefore, Leu can be only one out of a number of possible end products generated by an unknown number of enzymes and this makes it impossible to correct for isotope dilution in incorporation experiments (Table 4). The non-specific organic P hydrolysis by APA produces only 1 end-product, however: DIP. Until recently, P dilution caused by APA regeneration of organic P was not accounted for in isotope tracer studies. Sohm & Capone (2006) and later Sohm et al. (2008) used APA rates to account for radioisotope tracer dilution of the inorganic P pool. These commendable efforts, however, do not take into account the unique APA enzyme saturation kinetics we observed in *Trichodesmium* colonies (Table 2). The mean K_m for *Trichodesmium* APA in this study (Table 2) suggest substrate saturations in the order of 40 to 160 times the concentrations used by Sohm & Capone (2006) and Sohm et al. (2008). Therefore, if higher DOP concentrations are associated with the *Trichodesmium* colonies (higher than can be found in seawater alone) and these are not accounted for, accurate rates of radiotracer isotope dilutions can be severely underestimated. Due to the high rates of P and N regeneration associated with *Trichodesmium* colonies, conventional radioisotope incubations cannot be performed as is typically done in natural seawater.

Ectoenzyme activity, an adaptive response to a changing environment

The V_{max} rates of seawater APA in this study are similar to those in Cotner et al. (1997), who found higher maxima in spring. Cotner et al. (1997) suggested that the increase in seawater APA in spring was due to the intrusion of deep water with lower inorganic P relative to N, resulting in P-limited growth. The highest V_{max} for seawater APA was recorded during spring in the upper 20 m of the water column in March 1993 (data not shown) and was associated with the annual primary production maximum measured concomitantly at the BATS site (Gundersen et al.

2001). The spring bloom production maximum, followed by an increase in DOM excretion (Carlson et al. 1994) may imply that the measured increase in seawater APA is not only a product of P-stress (Hynes et al. 2009), but also a source dependent response to new production and increased availability of organic phosphorus. Recent studies have concluded that DOP, including phosphonates (Dyhrman et al. 2002, 2006), is a major source of P for *Trichodesmium* (Mulholland et al. 2002, Fu et al. 2005). This in turn suggests that APA associated with *Trichodesmium* is not necessarily indicative of a P 'stress' response only, but may also represent a DOP acquisition strategy that has developed in these cyanobacteria as an adaptation to a P-limited oligotrophic environment. The DOP pools at BATS (80 nM) are approximately an order of magnitude higher than the DIP pools (5 to 10 nM; Wu et al. 2000, Cavender-Bares et al. 2001, Ammerman et al. 2003), suggesting that organic P can be a significant source of P for the plankton community. Our measurements of APA associated with *Trichodesmium* colonies support this idea, as there was no seasonal maximum of activity in this changing environment, and the ectoenzyme activity was expressed throughout the year. The wide range in substrate K_m and V_{max} for both enzymes in seawater and in association with the *Trichodesmium* colonies (Table 2) may suggest that a number of different phosphatase and peptidases, with different substrate saturation kinetics, appeared during the course of a year at the BATS site. The variable saturation kinetics may be an adaptative response to a changing pool of organic P and N, or due to changes in the seawater plankton community (Lamy et al. 1999), as well as species variability within the consortium of organisms in the *Trichodesmium* colonies.

Summary

Ectoenzyme activities associated with aggregates such as marine snow can be several orders of magnitude higher than the surrounding seawater (Amy et al. 1987, Karner & Herndl 1992, Smith et al. 1992, Rath & Herndl 1994), which is similar to the level of activity associated with *Trichodesmium* colonies found in this study. Measured ectoenzyme activities associated with *Trichodesmium* colonies are higher than in adjacent waters, and appear to be adjusted to substrate concentrations far exceeding bulk measurements in the surrounding seawater. Thus, the buoyant *Trichodesmium* colonies are an important site for regenerated nitrogen and phosphorus in the sea. Due to the high concentrations of regenerated dissolved N and P that can be associated with the colonies, the pools of nutrients will significantly influence the isotope dilution in conventional radioisotope incubations with *Trichodesmium*. This study suggests that *Trichodesmium* plays an important role in regenerated production of P and N in the nutrient depleted surface waters of the BATS site in summer and fall.

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