

NOTE

EFFECT OF EDTA ADDITIONS ON NATURAL *TRICHODESMIUM* SPP. (CYANOPHYTA) POPULATIONS¹

*James A. Burns*²

Wrigley Institute for Environmental Studies and the Department of Biological Science, AHF 108, University of Southern California, Los Angeles, California 90089-0371, USA

Jonathan P. Zehr

Earth and Marine Sciences A438, Department of Ocean Sciences, University of California, Santa Cruz, Santa Cruz, California 95064, USA

Joseph P. Montoya

School of Biology, Georgia Institute of Technology, 311 Ferst Dr., Atlanta, Georgia 30332, USA

Adam B. Kustka

Department of Geosciences, Princeton University, Princeton, New Jersey 08544, USA

and

Douglas G. Capone

Wrigley Institute for Environmental Studies and the Department of Biological Science, AHF 108, University of Southern California, Los Angeles, California 90089-0371, USA

Despite nearly two decades of intensive research, many questions regarding the physiology and ecology of the marine, non-heterocystous cyanobacterium, *Trichodesmium*, remain unresolved. We note here the effect of EDTA (ethylenediaminetetraacetate) on N₂ fixation by *Trichodesmium*, and the use of EDTA as a means of extending the viability of natural *Trichodesmium* spp. populations. We examined nitrogenase activity (NA) as a function of EDTA concentration, time of collection, light level, and iron addition. Samples collected early in the day and treated with EDTA maintain a steady rate of activity for hours longer than controls. Furthermore, samples preincubated through the night with EDTA were active the next morning, compared with controls that were inactive. The discovery that (10–50 μM) low concentrations of EDTA prolong the duration of NA of *Trichodesmium* during experimental manipulations without affecting the rate of acetylene reduction allows for longer term manipulative experiments to be conducted.

Key index words: acetylene reduction; diazotroph; EDTA; nitrogen fixation; *Trichodesmium*

Abbreviations: NA, nitrogenase activity

The marine, non-heterocystous cyanobacterium, *Trichodesmium* (Cyanophyta), is an important marine diazotroph (Capone et al. 1997) that has recently gained widespread attention with the recognition of the significance of oceanic nitrogen fixation in the global nitrogen cycle (Capone 2001). *Trichodesmium* has been studied throughout the tropical and subtropical oceans of the world (Capone et al. 1997). In nature, *Trichodesmium* is usually encountered in aggregates of tens to hundreds of trichomes that are readily visible to the naked eye and easily isolated by pipet or inoculating loop. Most physiological studies have been conducted on aggregates. *Trichodesmium* also occurs as free trichomes (Saino and Hattori 1980), although these are more difficult to isolate for experimentation.

Trichodesmium appears to be largely dependent upon N₂ fixation for its cellular nitrogen (Carpenter 1983), although it does have the capacity to assimilate ammonium (Mulholland and Capone 2000). Nitrogen fixation is mediated by the enzyme nitrogenase, an enzyme highly regulated at the transcription, translation, and protein levels (Postgate 1998). *Trichodesmium* spp. exhibits a diel pattern in nitrogenase activity (NA) (Capone et al. 1990), where NA begins in the morning near dawn and ends near dusk, reflecting an endogenous circadian rhythm under transcriptional and translational regulation (Chen et al. 1998). The enzyme requires Fe and Mo as cofactors (Kustka et al. 2003a) and the nutrients that regulate *Trichodes-*

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²Author for correspondence and current address: Quileute Natural Resources, Quileute Indian Tribe, LaPush WA, 98350-0187. E-mail: burns@centurytel.net.

mium growth in the environment are widely debated (Capone 2001).

The use of the trace metal chelator EDTA in media for phytoplankton is commonplace (Johnston 1964, Sunda et al. 2005) and it is a component of the synthetic medium, YBC II, used to maintain *Trichodesmium* spp. (Chen et al. 1996), and appears to stimulate nitrogen fixation and growth in *Trichodesmium* (Paerl et al. 1994).

One reason why many basic physiological questions regarding *Trichodesmium*'s capacity to fix atmospheric N_2 still remain open is due to the difficulty of maintaining viable populations long enough to conduct manipulative experiments. *Trichodesmium* has an intrinsically slow growth rate (Carpenter 1983), and confinement of *Trichodesmium* colonies in assay containers required for physiological measurements usually results in cell lysis within 10–12 h (J. Burns and D. G. Capone, personal observation). In this paper, we note the use of EDTA as a means of maintaining the viability of natural *Trichodesmium* spp. populations for physiological studies. We used NA as an index to infer viability. We examined the concentration dependence of EDTA addition in sustaining NA over time, and its interaction with light and iron.

Colonies of *Trichodesmium* spp. were collected with a 1 m plankton net (202 μ m mesh) towed at approximately 1 kt. The depth of collection varied from the surface to 40 m depending on the depth of the population maxima. Sampling was carried out on six cruises: R/V Gyre, May–June 1994; R/V Seward Johnson, March–April 1996, and October–November 1996; R/V Seward Johnson April 1995 in the tropical Atlantic; R/V Roger Revelle, March–April 1999, tropical Pacific; and R/V Ewing, November 1999, north coast of Australia, Townsville to Broome.

Nitrogenase activity was assayed by the C_2H_2 reduction assay on freshly collected colonies as described by Capone (1993). Briefly, individual colonies were picked, using a sterile inoculation loop, into ambient GF/F filtered seawater and placed into acid-washed glass serum vials at a concentration of 1 colony \cdot mL⁻¹ of seawater. Incubations were performed in 14 mL serum vials with 10 mL of seawater. Serum vials were capped, crimp sealed, and injected with 1 mL of C_2H_2 using a gastight syringe. One hundred microliter subsamples of the headspace were taken with a gastight syringe every 30 min to 1 h for 10–12 h beginning at approximately 09:00 local time. All acetylene reduction assays were performed during daylight hours. The C_2H_4 produced from the reduction of C_2H_2 by nitrogenase was quantified using a gas chromatograph with a flame ionization detector. Head-space gases were separated with a Haysep A column (Alltech, Deerfield, IL, USA; 80/100, 6 ft \times 1/8 in). Background C_2H_4 was measured and subtracted from all samples.

Samples were incubated either under ambient sunlight or under halogen lamps with a measured irradiance of 300 μ mol quanta \cdot m⁻² \cdot s⁻¹. Ambient sun light incubations were performed in on-deck incubators.

Light levels were regulated using neutral density filters. Ambient irradiance ranged from 400 to 2000 μ mol quanta \cdot m⁻² \cdot s⁻¹. In both cases, samples were in incubators with flowing seawater at ambient surface temperature, typically about 27° C.

The EDTA (Shelton Scientific Inc., Shelton, CT, USA) was added from a 100 mM stock solution of Na₂EDTA (pH 8) to final concentrations ranging from 5 to 500 μ M. For short-term experiments, EDTA was added to the seawater in the serum bottles just before the injection of acetylene. Preincubation experiments, where colonies were exposed to various treatments overnight before C_2H_2 addition, were performed either in 74 mL glass serum bottles or 250 mL polycarbonate bottles to which EDTA was added directly. During select experiments, iron was added at a final concentration of 10 nM from a 10 μ M FeCl₃ stock containing 0.005 N HCl. Serum bottles for ultra-clean experiments were filled with 50 μ M EDTA and soaked for >24 h to allow EDTA to bind metals loosely sorbed on the glass. Bottles were then soaked in 10% HCl for 24 h and rinsed with deionized water.

For ¹⁵N₂ isotope tracer experiments, 30–50 *Trichodesmium* colonies were placed in a 250 mL pyrex bottle filled completely with filtered seawater (GF/F) and sealed with a septum cap. Bottles were injected with 100 μ L of 99% enriched ¹⁵N₂ gas (Cambridge Laboratories, Andover, MA, USA) and 50 μ L of a 100 μ M solution of NaH¹³CO₃, and then incubated on deck in a flowing seawater incubator under various light conditions using neutral density filters. Experiments were terminated after 4–8 h by gentle vacuum filtration through precombusted 25 mm GF/F filters (Whatman, Maidstone, UK). Filters were stored frozen aboard ship. Once ashore, the filters were treated with fuming HCl to remove inorganic carbon from the filter matrix, dried at 60° C, and stored until analyzed by continuous flow isotope ratio mass spectrometry. The rates of N₂- and CO₂-fixation were calculated from the measured abundance of ¹⁵N and ¹³C using the mass budget approach of Montoya et al. (1996).

The addition of EDTA to sample incubations of freshly collected *Trichodesmium* during daylight hours did not affect the initial rate (up to 4 h) of acetylene reduction, but resulted in prolonged viability (Fig. 1), defined here as NA measured by the acetylene reduction assay. The analysis of 30 individual experiments in which 20–50 μ M of EDTA were added to the experimental samples displayed no significant difference from the calculated rate in controls (average NA = 0.15 \pm 0.06 and 0.14 \pm 0.04 nmol C_2H_4 \cdot colony⁻¹ \cdot h⁻¹ for pooled EDTA and control treatments, respectively; Student's *t*-test, α = 0.05, *P* = 0.70). The EDTA additions of up to 200 μ M had a similar long-term enhancement effect relative to controls (Fig. 1), although concentrations of 100 μ M and greater appeared to cause a transient initial inhibition of NA (Fig. 1).

The increase in *Trichodesmium* viability with the addition of EDTA (10–50 μ M) allowed for more effective

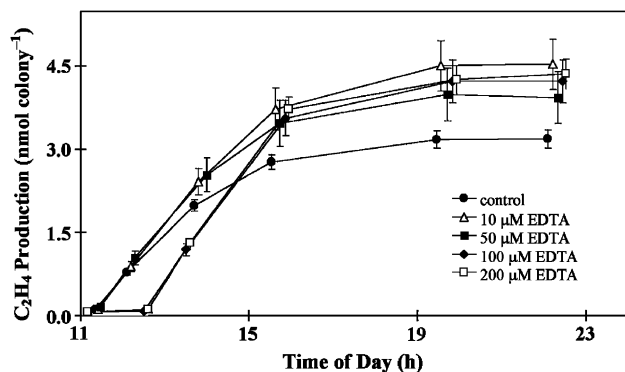


FIG. 1. Nitrogenase activity (acetylene reduction) of *Trichodesmium* spp. incubated with various concentrations of EDTA under halogen lights ($300 \mu\text{mol quanta} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) (bars, \pm SE).

experimental manipulations. For instance, freshly collected *Trichodesmium* placed in bottles (light or dark) and incubated overnight typically showed little or no acetylene reduction activity in the morning (J. Burns and D. G. Capone, personal observation). *Trichodesmium* treated with EDTA, however, did show significant activity in the morning (Fig. 2). The minimum dosage for the effect to occur was approximately $5 \mu\text{M}$ (Fig. 2).

There appeared to be no interaction between light level and EDTA on rates of N_2 fixation over brief time scales, as initial rates of acetylene reduction (up to 6 h) were not significantly different between controls and EDTA treatments at any light level (Table 1). However, the addition of EDTA prolonged fixation relative to the control at all light levels (data not shown). The quality of light was not measured for the various light levels; however, there should be no difference between samples, as the quality of light was not altered by the neutral density filters. This would be true for all wavelengths except UV, which would not be able to penetrate the glass vial and would be negligible in all levels. Similarly, rates of nitrogen and carbon fixation showed

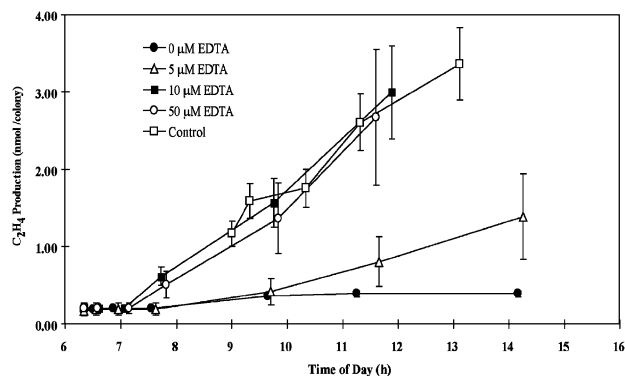


FIG. 2. Nitrogenase activity (acetylene reduction) of *Trichodesmium* spp. incubated overnight with various concentrations of EDTA. Sample labeled as "Control" was collected at 08:30 and not pre-incubated. Sunrise was at 07:00 local time (bars, \pm SE).

TABLE 1. Nitrogenase activity in *Trichodesmium* spp. treated with EDTA at various light levels.

Light level (percent of surface)	Acetylene reduction rate ($\text{nmol C}_2\text{H}_4 \cdot \text{colony}^{-1} \cdot \text{h}^{-1}$)					
	<i>n</i>	20 μM EDTA	SE	Control	SE	<i>t</i> -test (<i>p</i>)
100	6	0.043	0.004	0.037	0.004	0.478
50	7	0.040	0.006	0.035	0.004	0.564
25	7	0.035	0.003	0.035	0.001	0.958

no difference between controls and *Trichodesmium* treated with $20 \mu\text{M}$ EDTA (Table 2) nor did EDTA additions affect the diel pattern of NA (Fig. 3).

The EDTA-chelated Fe did not increase the rate of NA relative to EDTA alone (Table 3). The addition of 10 nM Fe in the presence of $10 \mu\text{M}$ EDTA under our specific conditions ($400 \mu\text{mol quanta} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, 27°C , 36 psu seawater) is expected to increase Fe' (the sum of all inorganic iron species) by $\sim 0.3 \text{ nM}$, an increase in Fe' that yields an increase in steady-state growth rate of Fe-limited cultures of *Trichodesmium* by $\sim 0.08 \text{ d}^{-1}$ (Kustka et al. 2003b). Similarly, no significant differences in acetylene reduction rates were found between non-trace metal clean assay methods ("dirty") and "clean" techniques. Acetylene reduction rate for *Trichodesmium* incubated under clean conditions was $0.7 \pm 0.06 \text{ nmol C}_2\text{H}_4 \cdot \text{colony}^{-1} \cdot \text{h}^{-1}$, while incubations with non-trace metal clean water or bottles amended with $10 \mu\text{M}$ EDTA had rates of $0.6 \pm 0.07 \text{ nmol C}_2\text{H}_4 \cdot \text{colony}^{-1} \cdot \text{h}^{-1}$ (one-way ANOVA, $P = 0.26$) (Table 3).

The ability to maintain colonies of *Trichodesmium* that are biologically active for extended periods of time (24–48 h) is very useful in performing physiological assays. In the past, freshly collected *Trichodesmium* colonies could only be maintained on board ship in a physiologically viable state for about 8–10 h. This was often an insufficient period over which to observe the effect of nutrient additions, given the relatively slow growth rates of this organism. The discovery that 5–200 μM EDTA additions prolong the viability of *Trichodesmium* during experimental manipulations without affecting the rate of NA or carbon fixation greatly enhances our ability to conduct longer-term manipulative experiments (Table 1). This has allowed the study of individual colonies over extended periods of time as opposed to time series of samples drawn from the water column and incubated sequentially. Our data suggest that a $5 \mu\text{M}$ addition of EDTA is the minimum required to maintain viable colonies through overnight

TABLE 2. Stable isotope measurement of N-fixation and C-fixation in *Trichodesmium* spp.

	C or N fixation ($\text{nmol} \cdot \text{colony}^{-1} \cdot \text{h}^{-1}$)					
	<i>n</i>	20 μM EDTA	SE	Control	SE	<i>t</i> -test (<i>p</i>)
^{15}N fixation	8	0.02	0.01	0.01	0.01	0.440
^{13}C fixation	8	9.31	1.64	8.08	1.12	0.546

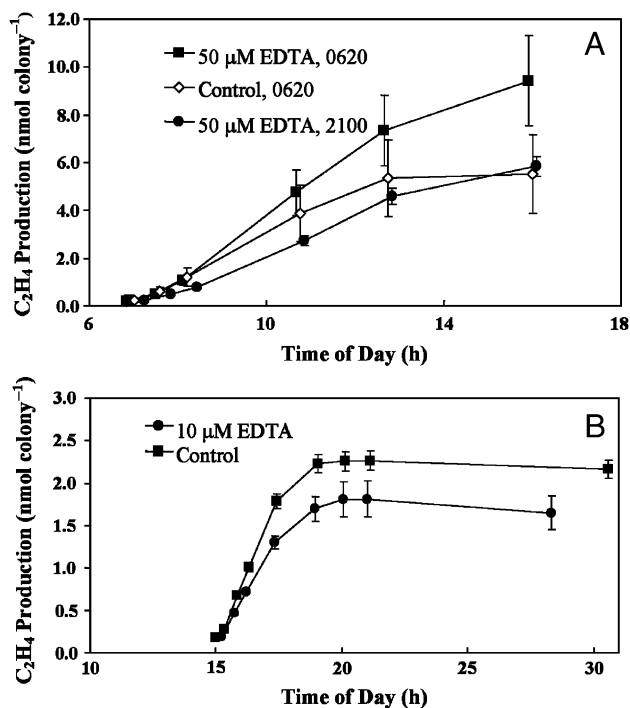


FIG. 3. Nitrogenase activity (acetylene reduction) of *Trichodesmium* spp. incubated with EDTA. (A) Initiation of nitrogenase activity after sunrise (06:00 a.m. local time). *Trichodesmium* collected at 21:00 the evening before and pre-incubated with 50 μM EDTA, *Trichodesmium* collected at 06:20, no EDTA added. *Trichodesmium* collected at 06:20 with 50 μM EDTA addition. (B) Cessation of nitrogenase activity at dusk (sunset was at 18:30 local time). *Trichodesmium* collected at 15:00 p.m., no EDTA addition, *Trichodesmium* collected at 15:00, 10 μM EDTA addition (bars, ± SE).

incubations (Fig. 3) and to prolong activity during daytime experiments (8–10 h; Fig. 1). We routinely used a 20 μM EDTA addition for field assays.

The addition of Fe and EDTA had the same effect as EDTA alone. This, coupled with our evidence that EDTA alone was not simply sequestering Fe from the walls, suggests that the EDTA was not acting by making more Fe available to *Trichodesmium*. Data of Sanudo-Wilhelmy et al. (2001) and Kustka et al. (2003b) suggest that most *Trichodesmium* populations along a central Atlantic transect were not Fe limited. Experiments conducted with EDTA additions from this same cruise showed the same effect, further suggesting that Fe availability does not explain the prolonged activity that we observed. Gerringa et al. (2000) found that concentrations higher than 100 μM EDTA were required before Fe speciation was affected in seawater.

Trace metal ions, such as Cu or Zn, may leach from glass incubation bottles, causing toxic stress in *Trichodesmium*. The additions of EDTA to ambient seawater in incubation bottles may chelate these metals and reduce the bottle effect. Past work showed that EDTA alleviated the toxic stress from copper (Anderson and Morel 1978). With an ambient Zn concentration of ~0.1 nM, and a natural organic high-affinity Zn ligand

TABLE 3. Fe-EDTA experiments conducted on *Trichodesmium* spp., using clean technique and standard assay conditions ($n = 6$).

Treatment	Nitrogenase activity nmol C ₂ H ₄ · colony ⁻¹ · h ⁻¹	SE	One way ANOVA (p)
Ultra-clean technique	0.7	0.06	0.26
"Dirty" technique plus 10 μM EDTA	0.6	0.07	
Control	0.3	0.03	
10 μM EDTA	0.4	0.05	0.22
10 nM Fe + 10 μM EDTA	0.3	0.03	

concentration of 1.2 nM (Bruland 1989), thermodynamic equilibrium calculations (MINEQA2, Allison et al. 1991) predict that, under the following conditions (ionic strength = 0.7, temperature = 27° C, the conservative components present at concentrations found in 36 psu seawater), 50 μM EDTA would sufficiently complex an added component of 10 nM Zn, leaving the Zn' concentration at about 10^{-11.34} M, a ~2-fold increase over an ambient equilibrium Zn' of 10^{-11.7} M. In the absence of EDTA, an added component of Zn of 10 nM would increase the Zn' to 10^{-8.24} M, an ~2000-fold increase.

The mode of action of EDTA on *Trichodesmium* is not clear. The experimental evidence suggests that it is not working in the normal function of a chelator and providing *Trichodesmium* with Fe. However, the possibility that EDTA is chelating some other trace metal is impossible to rule out at this point. Nonetheless, using EDTA during standard N₂ fixation assays has also proven to be useful. Samples collected early in the day continue to fix at a constant rate, consistent with samples collected fresh throughout the day. This allows for experiments involving multiple, overlapping assays throughout the daylight hours.

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