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# Changes in Proteins Associated with Nitrogen Fixation and Iron Nutrition in the Marine Cyanobacterium *Trichodesmium*

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## ABSTRACT

An abstract of the thesis of Karen Marie Elardo for the Master of Science in Biology: presented November 9, 1994.

Title: Changes in proteins associated with nitrogen fixation and iron nutrition in the marine cyanobacterium *Trichodesmium*.

This investigation tested the hypothesis that iron, as a micronutrient, will affect proteins in *Trichodesmium* and therefore affect nitrogen fixation. Changes in proteins that are a result of iron enrichment were compared to naturally occurring diel changes. Alterations in the iron protein of nitrogenase were compared to nitrogen fixation rates using the acetylene reduction technique. The observed changes in proteins were compared in *Trichodesmium* colonies from the Caribbean Sea and the Sargasso Sea.

*Trichodesmium* colonies were monitored for protein and iron content over a diel period on two cruises. The changes in protein and iron content in *Trichodesmium* colonies were variable but at times showed a cyclic diel pattern. Changes in protein bands on SDS-PAGE showed consistent changes in the banding pattern of a low molecular weight protein that responded to iron nutrition and time of day (Elardo and Rueter 1990). These changes were similar to changes in the iron protein of nitrogenase which also responded to changes associated with iron nutrition and time of day (Elardo 1991).

*Trichodesmium* appear to alter certain proteins which appear as changes in banding patterns in response to environmental factors such as nutrients, temperature and light.

My research shows that the pattern of modification of the iron protein of nitrogenase differs in colonies from the Caribbean Sea compared to those from the Sargasso Sea (Elardo 1991). The Caribbean Sea population in February had a clear pattern of active and inactive forms (day vs. night) of the enzyme. The Sargasso Sea population of *Trichodesmium* spp. had both forms of the enzyme at all times of the day during April and May when  $\text{NO}_3^-$  is present in the euphotic zone due to recent mixing. These differences between the two populations may be due to different environmental conditions since the Caribbean Sea is permanently stratified, warmer and nutrient-depleted throughout the year. The Sargasso Sea undergoes seasonal breakdown of the thermocline during winter months, resulting in an injection of nitrate from deeper water, and minimum temperatures of  $18^\circ\text{C}$ .

CHANGES IN PROTEINS ASSOCIATED WITH NITROGEN FIXATION  
AND IRON NUTRITION IN THE MARINE CYANOBACTERIUM  
*TRICHODESMIUM*

by

KAREN MARIE ELARDO

A thesis submitted in partial fulfillment of the  
requirements for the degree of

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in  
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## INTRODUCTION

Marine nitrogen fixation may be an important source of new nitrogen to oligotrophic oceans (Karl et al. 1992). The supply of nutrients such as nitrogen to the euphotic zone represents one of the main controls of the ocean carbon cycle. Iron may be a limiting nutrient for nitrogen fixation in *Trichodesmium* (Rueter 1988). In his study he found that natural populations of *Trichodesmium* had increased rates of nitrogen and carbon fixation rates when incubated with iron. Recently, Paerl et al. (1994) concluded that nitrogen fixation in *Trichodesmium* may be limited by iron.

Iron in different forms (soluble and particulate) was used in this study to observe changes in proteins associated with nitrogen fixation and to compare changes to nitrogen fixation rates. The result would determine if changes in structure of the nitrogenase complex occur, when exposed to iron, and if this was reflected in changes in rates of nitrogen fixation.

Since nitrogen fixation in *Trichodesmium* is light-dependent, an attempt to observe an increase in protein content in the dark hours following the nitrogen fixation period was conducted by collecting colonies over a diel period. Colonies were also assayed for cellular iron content to determine what levels of iron the colonies had naturally before iron additions.

Finally, colonies from two different environments, the Caribbean Sea and the Sargasso Sea, were examined for changes in the iron protein of nitrogenase that are a result of different nutrient regimes.

### Literature Review

*Trichodesmium* spp. is a non-heterocystous, filamentous colonial cyanobacterium found in tropical and subtropical seas. The macroscopic colonies of *T. thiebautii* have two distinct morphologies, colloquially termed "puffs" and "tufts" (Figure 1). *Trichodesmium* spp. is considered to be the major nitrogen-fixing organism in the marine plankton (Carpenter 1983a). Nitrogen fixation is the reduction of atmospheric nitrogen, catalyzed by the nitrogenase enzyme complex, into ammonia which is then converted to organic compounds. Nitrogen fixation by *Trichodesmium* was first reported by Dugdale et al. (1961) in the Sargasso Sea using the  $^{15}\text{N}$  tracer method. It was noted in the Dugdale et al. study that nitrogen fixation occurred only in the light and that an enrichment to the incubation flasks of a mixture of phosphate, vitamins and trace metals inhibited nitrogen fixation completely. Dugdale et al. (1961) suggested that their results show primary production in an oligotrophic ocean may not be limited by nitrogen. Menzel and Ryther (1961) suggested that iron may be the element critically limiting primary production in the Sargasso Sea near Bermuda. In a later study, Dugdale et al. (1964) demonstrated high rates of nitrogen previous 1961 study. Goering et al. (1966) reported the first *in situ*



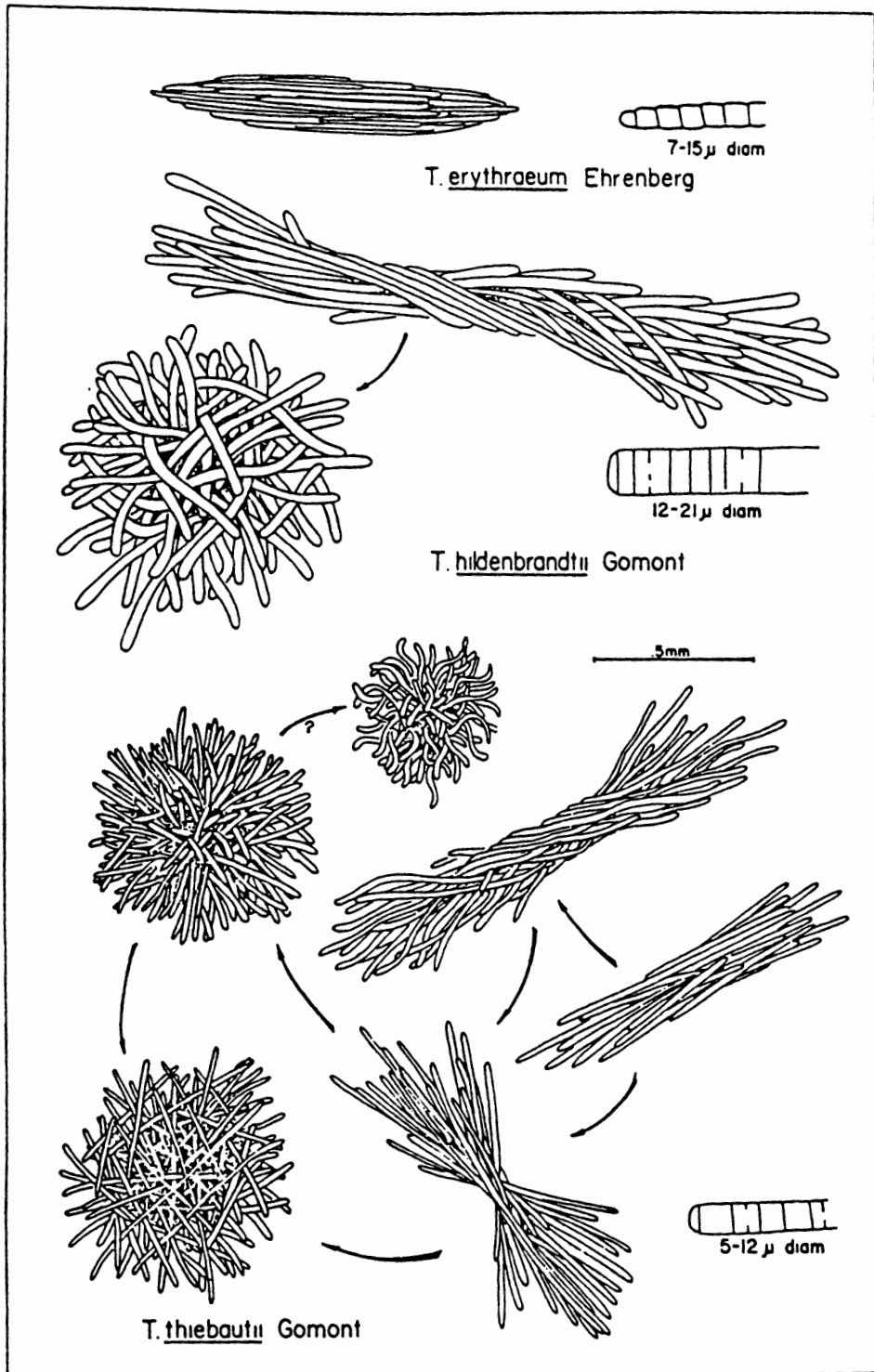


Figure 1. Drawing of *Trichodesmium* puff and tuft colonies. (from Borstad, 1978).

rates of nitrogen fixation, ammonia uptake, and nitrate uptake by *Trichodesmium* in the tropical Atlantic Ocean.

Goering et al. (1966) showed by using the  $^{15}\text{N}$  technique that significant amounts of nitrogen fixation can occur during blooms of *Trichodesmium*. They also showed that *Trichodesmium* can actively utilize ammonia and nitrate concurrently with nitrogen fixation. In a later study, Carpenter (1973) presented data showing rates of nitrogen fixation by *Trichodesmium* in the Sargasso Sea as revealed by the acetylene reduction assay. Although both techniques are valid measurements of the fixation of nitrogen, it has been argued that each technique could result in different errors. Carpenter (1973) suggested that acetylene reduction is a sensitive technique and measures gross nitrogen fixation (all products), whereas the  $^{15}\text{N}$  technique measures net nitrogen fixation (only the  $^{15}\text{N}$  retained in the cell, excluding extracellular losses). In a comparison of both techniques using soybean root nodules, Bergersen (1970) concluded that caution should be observed in the application of the acetylene reduction assay to quantify nitrogen fixation due to the significant variation in the molar conversion ratio. The differences in the two techniques and methods employed by Goering et al. (1966) and Carpenter (1973) resulted in the use of different units to quantify nitrogen fixation by *Trichodesmium*. This has presented a discrepancy when comparing the two techniques and methods applied in the 1960s and 1970s. This discrepancy compounded with the variability in nitrogen fixation rates reported, has precluded an accurate assessment for this organism.

Carpenter and McCarthy (1975) reported that the geographical variation in N<sub>2</sub> fixation over the western Sargasso Sea spanned two orders of magnitude; they attributed this variation to spatial heterogeneity and variable N<sub>2</sub>-fixing activity of *Trichodesmium*. Carpenter and Price (1977) compared nitrogen fixation, distribution and primary production of *Trichodesmium* spp. in the Sargasso and Caribbean Seas. They concluded that *Trichodesmium* is significant to the nitrogen cycle in the Caribbean Sea but not in the Sargasso Sea. Later, Carpenter et al. (1987) reported cellular N turnover times of 1.48 days in the Sargasso Sea and 1.13 days in the Caribbean Sea. By comparing their data with N turnover times reported in the literature, the authors demonstrated the variability of nitrogen fixation rates by *Trichodesmium*.

Thus, variability in nitrogen fixation rates has precluded an accurate assessment for this organism. This variability is compounded by inconsistent use of units to express rates resulting in difficulty comparing studies. Table I illustrates this with selected reported rates for *Trichodesmium* in the Atlantic Ocean.

Marine nitrogen fixation may be an important source of new nitrogen to oligotrophic oceans (Karl et al. 1992). New nitrogen is associated with "new production" which is defined as all primary production associated with newly available nitrogen from outside the euphotic zone (NO<sub>3</sub><sup>-</sup> or N<sub>2</sub>) (Dugdale and Goering, 1967). Carpenter and Capone (1992) have calculated nitrogen fixation rates of massive blooms of the marine diazotrophic cyanobacterium *Trichodesmium* to be 5.4 Tg (5.4 x10<sup>12</sup> g) N yr<sup>-1</sup> fixed. Previous estimates (Carpenter

TABLE I

A SELECTION OF REPORTED NITROGEN FIXATION RATES AND NITROGEN TURNOVER TIMES (td) FOR  
*TRICHODESMIUM* IN THE ATLANTIC OCEAN

Reported rate	td	Author(s)
0.13 $\mu\text{g N}$		Dugdale et al. 1961
0.32 $\mu\text{g N/l}\cdot\text{h}$	825	Goering et al. 1966
0.044 $\mu\text{g N/col}\cdot\text{day}$	47	Carpenter 1973
0.069 $\text{pg N/cell}\cdot\text{h}$	92	Carpenter and McCarthy 1975
0.033 $\text{pg N/cell}\cdot\text{h}$	105	Carpenter and Price 1977
22.1 $\text{fg N}_2/\text{cell}\cdot\text{h}$	31	McCarthy and Carpenter 1979
0.38ng $\text{N/trichome}\cdot\text{day}$	1.48	Carpenter et al. 1987

1983a, Capone and Carpenter 1982) of nitrogen fixation rates were based on non-bloom periods and totaled 4.7 Tg yr<sup>-1</sup>. This underestimate of nitrogen fixation by *Trichodesmium* is due to previous sampling during non-bloom conditions. When bloom and non-bloom fixation rates are totaled, the amount of N fixed is more than double the previous estimates.

The supply of nutrients such as nitrogen to the euphotic zone represents one of the main controls of the ocean carbon cycle. Undersampling of pelagic oceanic environments has obscured reliable estimates of the quantitative importance of nitrogen fixation in new production (Carpenter and Price 1977, Howarth et al. 1988, Capone and Carpenter 1982). Presently, the process of nitrogen fixation has not been included in ecosystem models (Legendre and Gosselin 1989). New nitrogen is most commonly referred to as sources of nitrogen from the upward transport of NO<sub>3</sub><sup>-</sup> from below the euphotic zone (Dugdale and Goering 1967, Eppley and Peterson 1979) and atmospheric deposition (Dugdale and Goering 1967, Knap et al. 1986). Understanding the factors that regulate nitrogen fixation and possible nutrient limitations of this process has become essential in understanding the global oceanic nitrogen cycle and the contribution of this process in primary productivity.

Most nitrogen-fixing cyanobacteria contain heterocysts; a specialized thick walled cell that is the site of nitrogen fixation. This structure protects nitrogenase from oxygen diffusion. Since *Trichodesmium* colonies do not contain heterocysts, it is an intriguing

question how this organism can perform photosynthesis and nitrogen fixation at the same time. This question has yet to be resolved. Saino and Hattori (1978) demonstrated a diel variation in nitrogen fixation in which nitrogen fixation occurred in the daylight in the presence of oxygen evolved through photosynthesis. This creates a paradox, since exposure to oxygen rapidly and irreversibly inactivates nitrogenase. The basis for this diel variation in nitrogenase activity was investigated by Capone et al. (1990). They concluded that the diel cycle of nitrogenase activity resulted from *de novo* synthesis of nitrogenase each morning and from inactivation and degradation of nitrogenase in the late afternoon and night. Carpenter and Price (1977) proposed that nitrogen fixation was restricted to cells in the center of the colony where they observed reduced pigment in the central cells. Bryceson and Fay (1981) found that nitrogenase activity was directly related to colony bundleness, thus supporting the earlier work of Carpenter and Price. Saino and Hattori (1982) found that single trichomes were able to fix nitrogen, demonstrating that colony formation was not necessary for nitrogen fixation. Saino and Hattori also showed that *Trichodesmium* colonies evolved hydrogen under argon and suggested that uptake hydrogenase protects nitrogenase from oxygen. However, Scranton (1983, 1984) and Scranton et al. (1987) demonstrated that hydrogen production was much slower than nitrogen fixation rates and suggested that nitrogen fixation and hydrogen production were not coupled in this system. Paerl and Bebout (1988) reported direct measurement of O<sub>2</sub>-depleted microzones in the center of the colony

which would optimize nitrogen fixation. Carpenter et al. (1990) re-evaluated this hypothesis. They found that further measurements did not reveal microzones and that there was no pigment differentiation between the center or the peripheral region trichomes in *Trichodesmium*. These investigators suggested that there is no single oxygen protection mechanism, but that several processes may work together. They also suggested that an internal enzymatic process may be involved in oxygen protection of nitrogenase. Ohki et al. (1991a) using cultures of *Trichodesmium*, presented a model of activation-deactivation of the iron protein of nitrogenase that may be involved in regulation of nitrogenase.

The nitrogenase enzyme complex is composed of two components, Component 1 (the Mo-Fe protein) dinitrogenase and component 2 (the Fe-protein) dinitrogenase reductase. Dinitrogenase reductase is composed of two identical subunits whereas dinitrogenase is a tetramer composed of two different types of subunits. The nitrogenase complex is irreversibly destroyed by oxygen and different strategies are utilized by cyanobacteria to protect nitrogenase from inactivation (Gallon 1981, Gallon 1989, Gallon 1992, Fay 1992). These strategies include temporal separation of photosynthesis and nitrogen fixation, spatial separation by forming heterocysts, reducing the concentration of oxygen by high metabolic rates, balanced destruction of nitrogenase by continual synthesis of the enzyme and modification of the enzyme so it is oxygen resistant. Evidence by Ohki et al (1991a, 1991b and 1992) and Zehr et al. (1993) as well as diel studies presented here show

that *Trichodesmium* has the capacity to modify dinitrogenase reductase.

Modification of dinitrogenase reductase has been observed in the photosynthetic bacterium *Rhodospirillum rubrum* (Preston and Ludden 1982). This modification involves the ADP ribosylation of one of the subunits of dinitrogenase reductase (Ludden and Burris 1978, Pope et al. 1985). Modification of dinitrogenase reductase in cyanobacteria has been demonstrated in *Anabaena* (Smith et al. 1987, Reich and Boger 1989, Ernst et al. 1990a and 1990b) although the molecular basis for this modification is unknown. When compared to photosynthetic bacteria, Smith et al. (1987) found that modification of nitrogenase in *Anabaena* did not result in the inactivation of the nitrogenase system. Reich and Boger (1989) found that inactivation of nitrogenase with ammonia addition was correlated with equal amounts of modified and non-modified forms of the iron protein in *Anabaena*. Modification of dinitrogenase reductase in *Trichodesmium* has been documented by Ohki et al. (1991a, 1991b and 1992) in cultures and Zehr et al. (1993) in natural populations. This modification has been found in diel studies to occur at night. The exact mechanism for daytime protection against oxygen is yet unclear. Recent work by Bergman and Carpenter (1991) show that nitrogenase activity is confined to certain trichomes (filaments within a colony) which are spatially separated. In their study they demonstrated by immunogold localization and transmission electron microscopy that nitrogenase in *Trichodesmium* is randomly distributed in 10-40% of the trichomes within a colony.



Recent work by Kana (1993) proposes that rapid oxygen cycling may contribute to the protection of nitrogenase by reducing the oxygen tension within the cell or colony. Kana suggests that this rapid oxygen cycling may help explain the low oxygen zones sometimes found in *Trichodesmium* colonies. *Trichodesmium* are unique among non-heterocystous filamentous cyanobacteria in that they perform nitrogen fixation and oxygenic photosynthesis at the same time and appear to use a unique combination of protective strategies.

There is a high requirement for iron in nitrogen fixation, since nitrogenase uses approximately 34 iron atoms to catalyze the reaction of  $N_2$  to  $2NH_3$  (Orme-Johnson 1992). The major source of iron to the ocean photic zone is from atmospheric transport of aeolian dust (Duce and Tindale 1991, Duce et al. 1991, Duce 1986). Duce (1986) calculated that atmospheric input can account for 30% to 96% of the iron input to the Sargasso Sea region and 16%-76% to the North Pacific gyre. Moore et al. (1984) calculated that 10% of the iron from atmospheric dust is leachable and in the biologically usable ferrous form. The low solubility of the stable oxidation state of iron (Fe III) in oxic seawater limits the assimilation of the soluble ionic species by phytoplankton. Iron is essential for many metabolic processes, including photosynthesis, nitrogen assimilation and nitrogen fixation, which creates a high demand for iron by phytoplankton. The concept of iron limitation of primary production has recently gained attention as a result of the work of John Martin and colleagues (Martin and Fitzwater 1988, Martin and Gordon 1988,

Martin et al. 1989, Martin et al. 1991). These investigations concluded that phytoplankton growth in major nutrient-rich waters is limited by iron deficiency.

Menzel and Ryther (1961) determined that iron was the effective component that enhanced  $^{14}\text{C}$  uptake by phytoplankton in the nutrient-poor Sargasso Sea. These authors found that nitrogen and phosphorus alone did not enhance uptake but they did so after samples were previously incubated in iron for 24 hours. This demonstrates Liebig's law of the minimum which states that "growth of a plant is dependent on the minimum amount of foodstuff presented" (Liebig 1840 in Parsons et al. 1984). The idea that the rate of a biological process may be determined by a single limiting factor is more clearly demonstrated in nutrient-rich upwelling areas that are known to have low atmospheric dust loads. Studies in permanently stratified, nutrient-poor areas of the ocean that are readily exposed to atmospheric dust are complicated by the possibility of co-limitation of several nutrients. Ryther and Guillard (1959) pointed out this possibility in their enrichment studies in the Sargasso Sea where they found silicate and one or more of the components in the iron-trace metal mixture to limit photosynthesis rather than nitrogen or phosphorus. Menzel et al. (1963) conducted enrichment studies on Sargasso Sea waters and found that the final growth rate of a population of phytoplankton was a function of time. For example, in three-day experiments iron appeared essential; and in seven-day experiments nitrogen and phosphorus alone produced large populations. These workers also found that the final population

of species composition was determined by the addition of specific nutrients and that separate enrichments of aluminium produced growth curves similar to those from additions of iron (both elements were in combination with nitrogen and phosphorus). Subba Rao and Yeats (1984) investigated the effect of iron on phytoplankton in the Sargasso Sea and found that incubations of four hours had no effect on photosynthesis but incubations of 72 hours enhanced carbon assimilation.

Elder and Horne (1977) investigated the biostimulatory capacity of dissolved iron and concluded that iron was an important nutrient controlling nitrogen fixation in a freshwater reservoir. These authors found the biostimulatory capacity of iron to be particular to nitrogen-fixing cyanobacteria. Wurtsbaugh and Horne (1983) found that nitrogen fixation in freshwater cyanobacteria could be stimulated as much as 500% above the control levels by iron additions. Wurtsbaugh et al. (1985) found nitrogen fixation in a tropical alpine lake to be stimulated by additions of iron but only significantly in one experiment. Rueter and Ades (1987) concluded in a study on the chlorophyte, *Scenedesmus quadricauda*, that iron plays a crucial role in the bioenergetics of carbon and nitrogen metabolism.

Rueter (1988a) found that natural populations of *Trichodesmium* responded to iron additions (as  $\text{FeCl}_3$ ) with increased nitrogen and carbon fixation rates when incubated for two days. Rueter used concentrations of iron ranging from  $10^{-8}$  to  $10^{-6}$  M and found stimulation of nitrogen fixation at the highest iron

concentration. The lowest iron concentration was not always stimulatory. Rueter et al. (1990) showed stimulation of nitrogen fixation in cultures of *Trichodesmium* NIBB1067 with iron concentrations of  $10^{-8}$  and  $10^{-7}$  M Fe when incubated for 15 days.

More recently, Paerl et al. (1994) demonstrated iron-stimulated growth in nitrogen fixation and growth in natural populations and in cultured *Trichodesmium* spp. Stimulation by iron occurred at 1  $\mu$ M FeCl<sub>3</sub> and EDTA-chelated FeCl<sub>3</sub> in both short term (24-48 hours) and long term (5-11 days) experiments. Concentrations of 1  $\mu$ M Fe do not occur in nature but apparently are required to produce observable levels of nitrogen fixation in this organism. The authors concluded that primary production and nitrogen fixation in oligotrophic waters may be limited by iron.

These investigators have shown that iron is important for nitrogen fixation. This study investigated the response of nitrogen fixation in *Trichodesmium* to near ambient concentrations of iron and to aeolian dust particles. Using the combination of immunoassays and acetylene reduction rates, the influence of iron on nitrogenase was compared to changes that are a result of oxygen protection.

## METHODS

*Trichodesmium* are not commonly cultivated; therefore, field samples were collected for use in the experiments presented here. The Ferrozine Assay was used to determine acid leachable iron content on samples collected during peak nitrogenase activity and on diel time scales. The Pierce Bicinchoninic Acid (BCA) Protein Assay Reagent was used to determine cellular protein of samples collected on a diel time scale. Protein nitrogen was estimated from protein content assuming an average nitrogen content of cell protein of 14% (Dayhoff et al. 1965). Iron and nitrogen ratios were determined for samples collected on diels.

To further observe changes in proteins on a diel time scale, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to detect changes in proteins on diels and with the addition of iron in different forms. Iron-specific stains were used to detect iron storage proteins. Tricine SDS-PAGE was used to resolve low molecular weight proteins. Observed changes in two proteins were analyzed by Western blots to identify proteins involved.

Soluble iron ( $\text{FeCl}_3$ ), chelated iron ( $\text{FeEDTA}$ ) and iron in the form of Saharan dust were added to incubation flasks containing *Trichodesmium*. SDS-PAGE and Western blots were used to determine the effect of iron on the iron protein of nitrogenase and

compare these changes to natural changes in this protein that occur on a diel basis.

Acetylene reduction (AR) assay was used to determine the effect of iron in different forms on the activity of nitrogenase. The combination of SDS-PAGE and AR allows a comparison of structural changes in the iron protein of nitrogenase and the activity of the nitrogenase enzyme complex.

### Field collection

*Trichodesmium* colonies were collected in the eastern Caribbean Sea January and April 1990, January and September 1991 and 1992 (Appendix II). A comparative study was done in the Sargasso Sea near Bermuda in April and May 1991 at the Bermuda Atlantic Time-series site (31°50'N, 64°10'W) and at the Hydrostation S site (32°10'N, 64°30'W). Mainly *Trichodesmium thiebautii* were collected in these experiments as great care was used to avoid collecting *Trichodesmium erythraea*. However, other species may have been collected. The speciation of *Trichodesmium* colonies based on ultrastructure is currently being investigated (Bergman, pers. com.). The experiments in the Caribbean Sea were performed aboard the R/V Columbus Iselin. *Trichodesmium* colonies were collected from 10 meter depths using a plankton net 1 m in diameter and 202  $\mu\text{m}$  in mesh size. To dilute the sample, the content of a plankton tow was poured into a bucket containing surface seawater. *Trichodesmium* colonies were plucked from the plankton tow by using a plastic inoculating loop and placed in plastic beakers with

fresh GF/F filtered surface seawater. The GF/F filtered seawater was used as a rinse to isolate the colonies from grazers and assorted associated organisms. Only tuft forms were collected for experiments in the Caribbean Sea. Puff forms were collected only for comparison purposes using SDS-PAGE. Experiments in the Sargasso Sea were performed on shore at the Bermuda Biological Station for Research and onboard the R/V Weatherbird II. *Trichodesmium* colonies were collected from the surface waters (1-5 m) using a plankton net 1 m in diameter 330  $\mu\text{m}$  in mesh size. Mainly puff forms were collected in Bermudian waters, although some tufts were collected separately when available for a comparison.

Protein and iron content were analyzed over diel periods consisting of 18, 24 and 42 hours in April 1990 and February 1991 in the eastern Caribbean Sea (Appendix II). In January 1990 daily iron content at approximately 1300 hours was analyzed on four different days. On April 11-12, 1990, colonies were collected approximately every 7 hr over a 24hr period west of St. Kitts. On April 16, 1990, colonies were collected every 6 hr over an 18hr period west of Saba and the British Virgin Islands. On February 6-7, 1991, colonies were collected every 6 hr for a 42hr period west of Guadeloupe. On February 12-13, 1991, colonies were collected west of St Croix and St. Thomas every 6 hr for a 24hr period.

#### Bicinchoninic Acid Protein Assay Reagent for Total Cellular Protein

The Bicinchoninic Acid (BCA) Reagent available from Pierce Chemical was used for the spectrophotometric determination of

protein in colonies of *Trichodesmium*. The assay was performed as described in Hutchins (1989) for *Trichodesmium* colonies and the assay itself as described by the manufacturer for the "Enhanced Protocol". The BCA reagent creates a complex with protein and  $\text{Cu}^{2+}$  (the biuret reaction) that results in a colorimetric detection for reduced copper ions ( $\text{Cu}^{1+}$ ). This reagent is compatible with biochemical reagents such as sodium dodecyl sulfate which is required in this assay to solubilize cellular and membrane bound protein. The Enhanced Protocol has a protein concentration range of 5-250  $\mu\text{g}/\text{ml}$ . Of the three protocols given by the manufacturer, the Enhanced protocol has a greater color stability.

Either 25 or 15 colonies, depending on abundance, were placed on a GF/A filter and stored at  $-20^{\circ}\text{C}$  in 15 ml polypropylene centrifuge tubes. The diel samples were collected every six hours for 24 hours or 48 hours during April 1990 and February 1991. Prior to analysis, the filters were taken from the centrifuge tubes and placed in a Wheaton glass mortar. The colonies and filter were ground with a teflon grinding pestle to solubilize proteins in 2 ml of 1% SDS (w/v). After grinding to a foam and ensuring that entire filter was ground, the sample was poured back into the original 15 ml centrifuge tube and one additional ml of 1 % SDS was used to rinse the mortar, bringing the sample volume to a total of 3 ml. The sample solution was then placed in the refrigerator overnight to ensure complete solubilization of cellular protein. After complete solubilization, the samples were spun at 12,000 rpm in a desktop centrifuge. An aliquot of 100  $\mu\text{l}$  of the sample supernatant was added to 2 ml of



BCA Working Reagent prepared as described in the Pierce BCA Assay Protocol. The enhanced protocol requires an incubation at 60°C for 30 minutes before cooling to room temperature. The absorbance of the purple reaction product was read at 562 nm wavelength on a spectrophotometer. The protein concentration of the sample is determined from a standard curve using purified bovine serum albumin supplied in the Pierce BCA Protein Assay Reagent kit. Because of the dilution factor caused by the addition of three ml of 1% SDS, the value of the sample obtained from the standard curve is multiplied by a factor of three.

#### Acid Leachable Iron by the Ferrozine Assay

Ferrozine, a spectrophotometric reagent for iron, was used to determine acid leachable iron content in field collected *Trichodesmium* colonies. The assay was performed as described by Hutchins (1989) for *Trichodesmium* colonies. The assay is a modification of Stookey (1970) and Gibbs (1979). Ferrozine, a disodium salt, is a chelator that reacts selectively with divalent iron to form a stable magenta complex species with a maximum absorbance at 562 nm (Stookey 1970). A 10% hydroxylamine hydrochloride solution (w/v) is used as a reducing agent to yield total dissolved cellular iron. All assay tubes were acid-cleaned overnight and rinsed six times in nanopure distilled water.

Daily variation of acid leachable iron content was analyzed in January 1990 (200 colonies per sample) at approximately 1300 hours on four different days. Samples for the diel experiments were

collected every six hours for 24 or 48 hours during April 1990 (100 colonies per sample) and February 1991 (50 colonies per sample). *Trichodesmium* colonies were placed in 2 ml 1N hydrochloric acid (HCl) in a 15 ml polypropylene tube using an acid-cleaned plastic inoculating loop. Diel samples were collected every six hours for 24 and 48 hours. The 15 ml polypropylene tubes containing the samples were placed in a VWR heating block for 24 hours at 60°C. After leaching samples for 24 hours in the heating block, the samples were centrifuged in a desktop centrifuge for five minutes at 14,000 rpm. One ml of supernatant (or standard) was mixed with 200 µl each in the following order : buffer (400 g ammonium acetate and 350 ml 58% ammonium hydroxide diluted to 1 liter), hydroxylamine hydrochloride (10%), and ferrozine (5.14 g/l). Samples were incubated for 10 minutes at room temperature and absorbance read at 562 nm wavelength using a spectrophotometer. The absorbance was plotted against the standard curve and the calculated values were multiplied by a factor of 2 as these values represent half of the iron in the original 2 ml leaching solution. Standards solutions (0, 1, 5, 10, 30, and 50 µM) were prepared from 18 mM stock of ferric chloride in 1 N HCl. Absorbance at 0 µM was used as an assay blank and subtracted from the other standards. A blank of 1 N HCl was run with every set of sample replicates and subtracted from the absorbance value of the sample in order to eliminate trace background contamination of iron.

An additional experiment was performed to rinse extracellular iron from the colonies and determine a more accurate

amount of intercellular iron in *Trichodesmium* colonies. After handling the colonies as described above, the colonies were rinsed in a Chelex (CHELEX 100, BIO-RAD) and GF/F filtered sea water (CFSW) rinse and an ascorbate rinse (1 M) were used to exclude extracellular iron. Fifty colonies were plucked from a plankton net tow as previously described and then placed in chelexed filtered sea water as a quick rinse before being transferred to 1N HCl. Another 50 colonies were placed into chelexed filtered seawater that contained 1M ascorbate before being placed in 1N HCl. There were two replicates for each condition. Fifty additional colonies were placed in nitric acid (HNO<sub>3</sub>) without being rinsed in order to observe the leaching capability of HNO<sub>3</sub> compared to HCl routinely used in the Ferrozine assay. Colonies were also assayed for protein content in the rinse experiments to determine if a loss of biomass occurred during these additional treatments.

#### Development of the SDS-PAGE Method

The high proteolytic activity in the colonies makes protein separation by gel electrophoresis difficult. A method using liquid nitrogen and high resolution gels on a Mini-Protean II gel apparatus (BIO-RAD) has been devised to overcome this difficulty with good results.

*Trichodesmium* colonies used for the development of this method were collected in the eastern Caribbean Sea, mainly in Exuma Sound, while aboard the University of Miami research vessel Columbus Iselin January 1990. Plankton tows were retrieved from

depths of 10-15 m. Once this method was developed, it was used on all other cruises. The SDS-PAGE techniques used were modifications from Laemmli (1970).

First attempts at sample preparation involved using a 0.05 ionic strength tris-HCL buffer (for details see Appendix 1). A type HA 0.45 $\mu$ m Millipore filter (HA = a mixture of cellulose acetate and cellulose nitrate) attached to a 12 cc syringe fitted with a Millipore Swinex-13 filter rig clamped vertically on a ring stand was required to remove salt water from the sample (Figure 2). Gentle suction was employed to extract seawater from the sample on the filter. Seawater interferes with the matrix of the polyacrylamide gel; its removal gives better resolution and high molecular weight bands were detected.

In the second attempt (see Appendix 1) the 0.05 I buffer step was eliminated and the SDS sample buffer was added directly to samples on the Millipore filters. However, there was no improvement observed in the gel using this modification.

A 12 % polyacrylamide gel method was used that has a 2% bis-acrylamide stock solution instead of 0.8% and stock solutions of 10% SDS and 50% glycerol were added to the unpolymerized gel solution (Zehr pers. comm.). The 12% gels were run at 200 V which over-heated. Some low molecular weight bands were detected but there was still poor resolution (smearing) and there were no high molecular weight bands detected. The samples were stored at -20 °C after preparation and then thawed prior to gel loading.

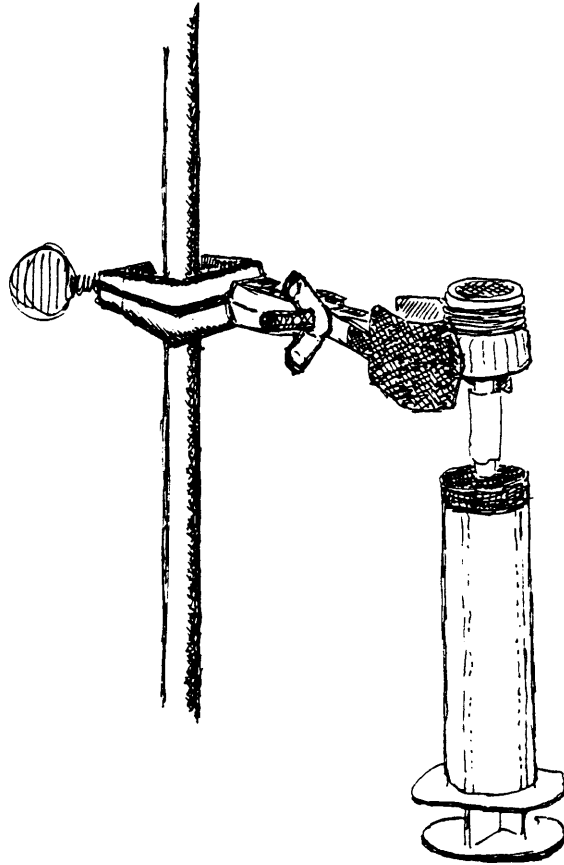


Figure 2. Filtration apparatus.

The following method was tried with good results; sample preparation requires quickly placing colonies onto a Millipore filter with gentle suction from the syringe to remove salt water. Twenty *Trichodesmium* colonies on the Millipore filter were placed in a 1.5 ml Eppendorf tube, frozen in liquid nitrogen and then stored at -20 °C until SDS-PAGE was performed. The SDS sample buffer used for protein solubilization contained PMSF and beta-mercaptoethanol prior to use (Zehr 1990). A 20:40 dilution (20 colonies to 40 µl of buffer) was used and samples were boiled in water and centrifuged in an Eppendorf Microfuge at 8,000 x g. Samples were used immediately after SDS sample buffer. Once SDS sample buffer is added, the samples should be kept at room temperature and never refrozen. *Trichodesmium* are so full of proteases that when frozen with SDS, the SDS precipitates upon thawing and allows for proteolytic activity (Zehr pers. com.). At room temperature the SDS remains coated around the proteins preventing proteolysis. The 12% gel system using glycerol in the gel matrix gives high resolution in the low molecular weight range. This gel system can not tolerate high voltage settings. The solutions used in this method are listed in appendix.

A modification in the preparation of SDS sample buffer was made. PMSF was deleted from stock solution because it did not solubilize well and instead was added later when the stock sample buffer was diluted for sample solubilization to a final concentration of 1 µM. Loading volumes were 10 µl per well, electrode buffer was diluted 1:5 from a 5x stock (Appendix I) and running conditions

were 150 V for two gels for approximately 1.5 hours. The gels were stained in coomassie blue and destained with 10% acetic acid. The results of this method produced good separation of proteins with multiple bands and high resolution.

### Low Molecular Weight SDS-PAGE

In order to detect low molecular weight bands with greater resolution, Tricine-SDS-PAGE technique was used to separate proteins in the range from 1 to 100 kDa (Schagger and von Jagow 1987). This gel system was used to detect phycoerythrin (18kDa) for Western blots. This method was followed as published for a 16.5 % T, 6% C separating gel, a 10% T, 3% C spacer gel and a 4% T, 3% C stacking gel (C denotes the percentage concentration of the crosslinker relative to the total concentration T). Samples were treated as described above for SDS-PAGE with the exception that running conditions of this gel system requires approximately 8 hours with a 30 mA constant.

### Iron and Dust Incubations for SDS-PAGE

Three incubation flasks containing 40-50 colonies of *Trichodesmium thiebautii* were incubated up to 24 hours in 200 ml of GF/F filtered sea water (FSW) consisting of a control (FSW),  $1.8 \times 10^{-8}$  M  $\text{FeCl}_3$  iron, and an addition of 10 mg of Saharan dust to the third culture. The amount of iron contained in the dust was estimated by assuming that 5% of the dust is iron (Rueter pers. comm.) and 10% of the iron is soluble (Moore et al. 1984). Ten mg of

dust added to 200 ml of filtered seawater (FSW) would be approximately 4.48  $\mu\text{M}$  Fe.

Twenty colonies were dissolved in 40  $\mu\text{l}$  of SDS protein solubilization buffer as described above. The samples were run on a 2 12% gels at 150 v for 1 hour. One of the gels was stained with coomassie to observe protein resolution. In order to detect iron containing proteins, the other gel was stained with prussian blue as described in Gaal et al. (1980). The more sensitive bathophenanthroline reagent was also used as described in Uriel and Chuilon (1965) to stain iron proteins in the gel. An equine ferritin standard (100 mg/ml) was used with the bathophenanthroline reagent.

#### Western blot immunoassays

*Trichodesmium* colonies collected were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting for immunoassays (BIO-RAD Immuno-Blot kit). The Immuno-Blot Assay Kit used was goat anti-rabbit horse radish peroxidase conjugate (GAR HRP) and was obtained from BIO-RAD. The method was followed as directed in the instruction manual included in the kit. The primary antiserum used is rabbit anti-dinitrogenase reductase from *Rhodospirillum rubrum* (gift from Dr. Jon Zehr and Dr. Birgitta Bergman) and rabbit anti-phycoerythrin from *Phormidium persicinum* (gift from Dr. Birgitta Bergman). Primary antibodies against dinitrogenase reductase were used at a dilution of 1:5000 and against phycoerythrin at 1:500 (primary



antisera:buffer). The secondary antibody was supplied in the kit and used as instructed in the manual at a 1:3,000 dilution. Colonies were collected when fixing nitrogen during the day and when not fixing nitrogen at night and assayed to determine the presence or absence of the modified form of dinitrogenase reductase. Colonies were also collected to detect any changes in phycoerythrin subunits on a diel time scale. Colonies for iron nutrition experiments were placed in 200 ml of filtered sea water in a 250 ml acid washed polycarbonate erlenmeyer flasks. Three conditions were used: control (no iron added), soluble iron ( $1.8 \times 10^{-8}$  FeCl<sub>3</sub> in 1N HCl) and Saharan dust (10 mg). Colonies were collected in the morning hours, incubated throughout the day and harvested in the evening after "turn-off" of nitrogenase.

#### Acetylene reduction assay

Acetylene was used as a substrate for nitrogenase. This assay is based on the 2 electron reduction of acetylene to ethylene. The product ethylene was measured on a Mini Shimadzu Gas Chromatograph with a flame ionization detector fitted with a 2 m x 1/8" stainless steel column packed with Poropak R, 100/120 mesh. Nitrogen is the carrier gas and hydrogen and compressed air are used for the flame. Colonies were assayed for acetylene reduction during the day-time because nitrogen fixation ceases after late afternoon (Capone et al. 1990). The method was followed as described in Capone (1993) except that 0.5 ml acetylene was used instead of 1 ml. Ten colonies were placed in 5 ml of GF/F filtered

seawater in 9 ml serum vials. The serum vials were capped with red silicone rubber septa and crimp-sealed with aluminum seals. Acetylene was injected into serum vial headspace and 100  $\mu$ l subsamples were removed from the headspace for analysis of ethylene. One hundred  $\mu$ l of an ethylene standard (94.3 ppm) was injected at each time point.

For acetylene reduction assays, *Trichodesmium* colonies were incubated with  $\text{FeCl}_3$  and dust as described for SDS-PAGE with the addition of a fourth flask containing  $10^{-8}$  M FeEDTA. Colonies were either incubated for several hours before acetylene was added or the iron solutions were added directly to the acetylene reduction vial. Colonies were also incubated in  $1.8 \times 10^{-7}$   $\text{FeCl}_3$  and  $10^{-7}$  M FeEDTA and  $10^{-6}$  M EDTA in later experiments for comparison.

## RESULTS

### Protein and Iron Content

Protein content in *Trichodesmium* colonies in April 1990, did not change significantly over a diel period (Figure 3). Considerable variation in samples was observed on April 11th where in one time point samples ranged from 12 to 25  $\mu\text{g}$  protein/colony. Although it is difficult to discern a diel change in protein content per colony because of this variability, colonies were collected at discrete locations for longer periods in time. In February 1991, colonies were collected over a 42 hour diel and showed a trend toward increased protein content in the later hours of the day (Figure 4). However, samples collected one week later did not show this trend over 24 hours. The lack of a diel change in samples may be due to variations in growth rate in different populations of *Trichodesmium*. Colonies undergoing rapid cell division would most likely show fluctuating protein levels.

On April 11-12, 1990, average protein content varied from 10.78 to 18.31  $\mu\text{g}$  per colony (Figure 3). The highest values occurred at 1400 and 1300 hr (Figure 3). On April 16, 1990, average protein content varied from 10.68 to 14.99  $\mu\text{g}$  per colony and highest values occurred at 0400 and 1600 hours (Figure 3). During February 6-7, 1991, average protein content varied from 9.56 to 25.70  $\mu\text{g}$  per colony (Figure 4). In this experiment, there appeared to be a diel

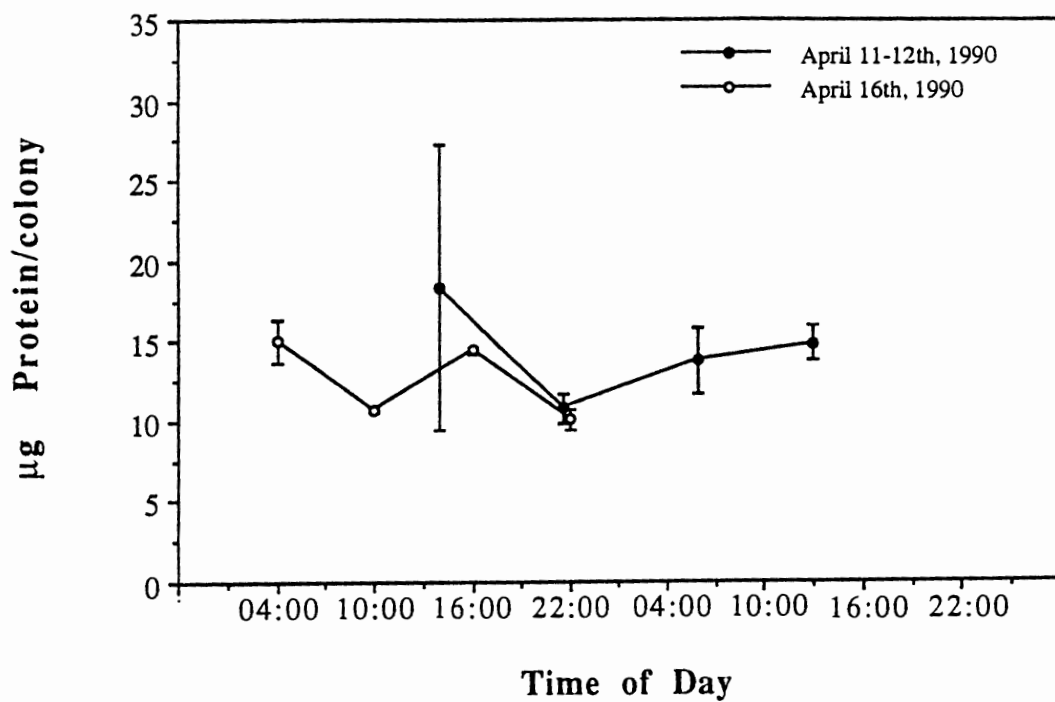


Figure 3. Protein content in *Trichodesmium* colonies as a function of time in April 1990.

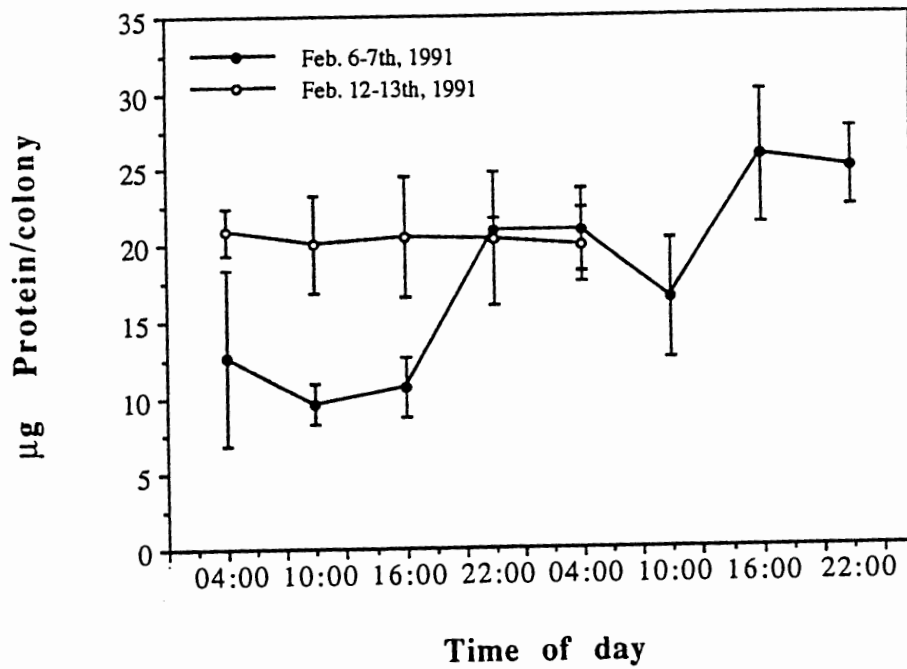


Figure 4. Protein content in *Trichodesmium* colonies as a function of time in February 1991 from the Caribbean Sea.

trend in protein content. During the first 24 hr, highest values occurred at 0400 hours. In the remaining 18 hr of the experiment, highest values occurred 1600 and 2200 hours (Figure 4). On February 12-13, 1991, protein content remained more constant and varied from 19.94 to 20.85  $\mu\text{g}$  per colony (Figure 4).

Daily variation on January 10, 13, 17, and 19 in acid leachable iron content was 0.25 to 0.45 nmol Fe/colony (Figure 5). Daily variation of iron content was plotted against nitrogenase activity measured as acetylene reduction and a correlation between iron content and nitrogenase activity was observed (Figure 6).

Data from April 11-12, 1990, show a variation in average acid leachable iron over a diel period to be 0.18 to 0.47 nmol/colony with the highest value at 1300 hours. On April 16, 1990, average iron content varied from 0.34 to 0.82 nmol Fe/colony with the highest value occurring at 0400 hours (Figure 7). A sharp decrease in iron content was observed in February 1991. On February 6-7, iron content varied from 0.08 to 0.14 nmol Fe/colony (Figure 8). On February 12-13, 1991, iron content remained constant over the 24hr period and ranged from 0.10 to 0.15 nmol Fe/colony.

Acid leachable iron content was compared in different treatments as described in Methods and showed that HCl was superior to  $\text{HNO}_3$  as a leachate for cellular iron. Results from this experiment also show that colonies dipped in chelexed filtered seawater had a lower average iron content than colonies placed in filtered seawater only (Figure 9A). The addition of an ascorbate rinse did not result in even lower values of acid leachable iron

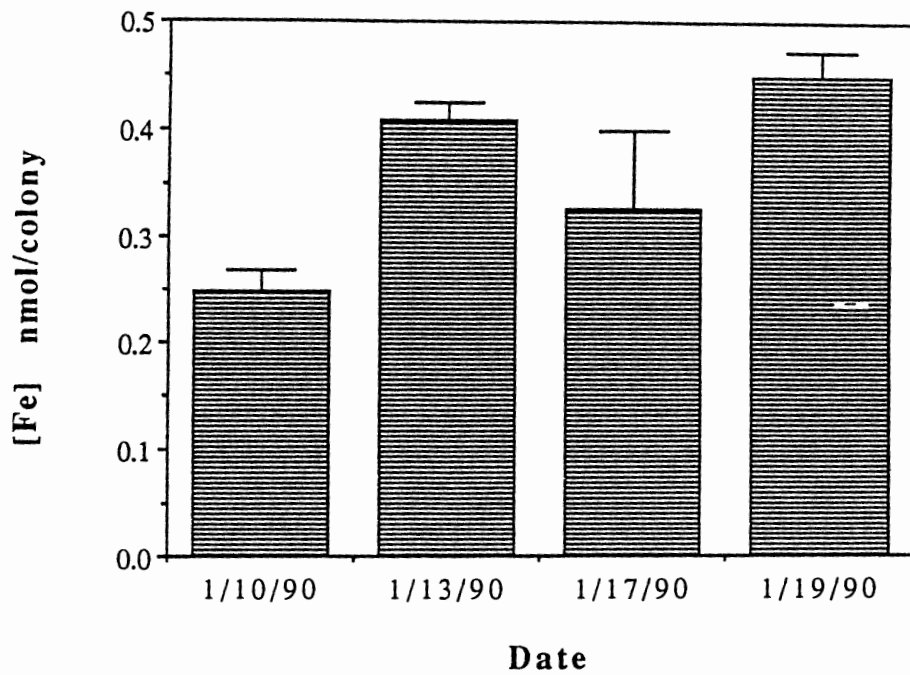


Figure 5. Daily variation of acid leachable iron content in *Trichodesmium* colonies in January 1990 from the Caribbean Sea.

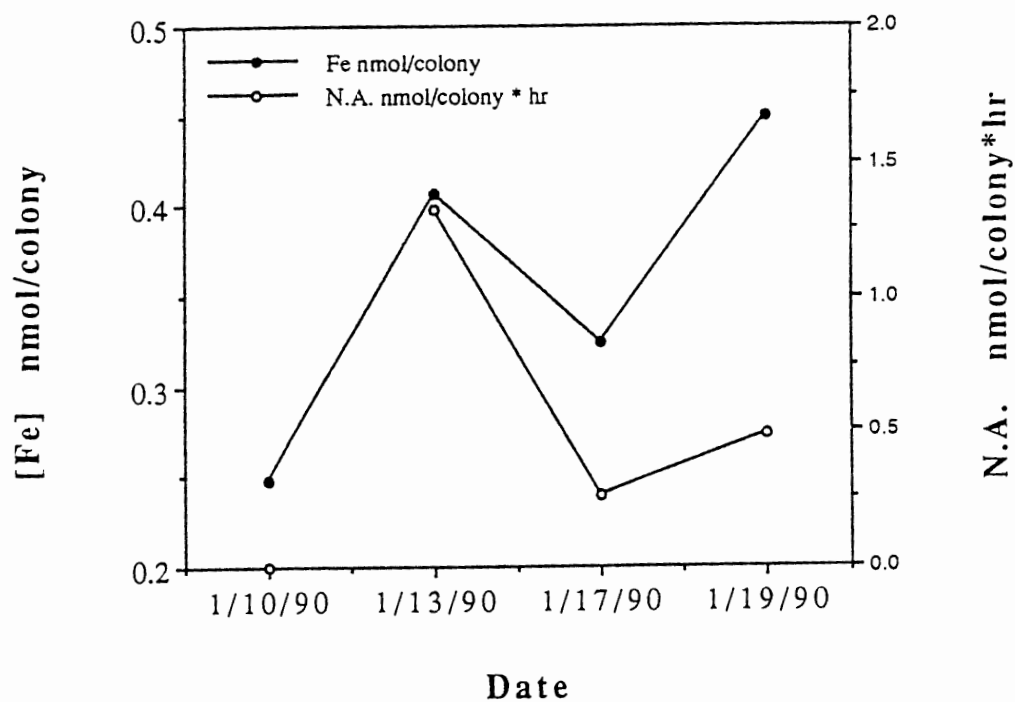


Figure 6. Daily nitrogenase activity (NA) and iron content in *Trichodesmium* colonies in January 1990.



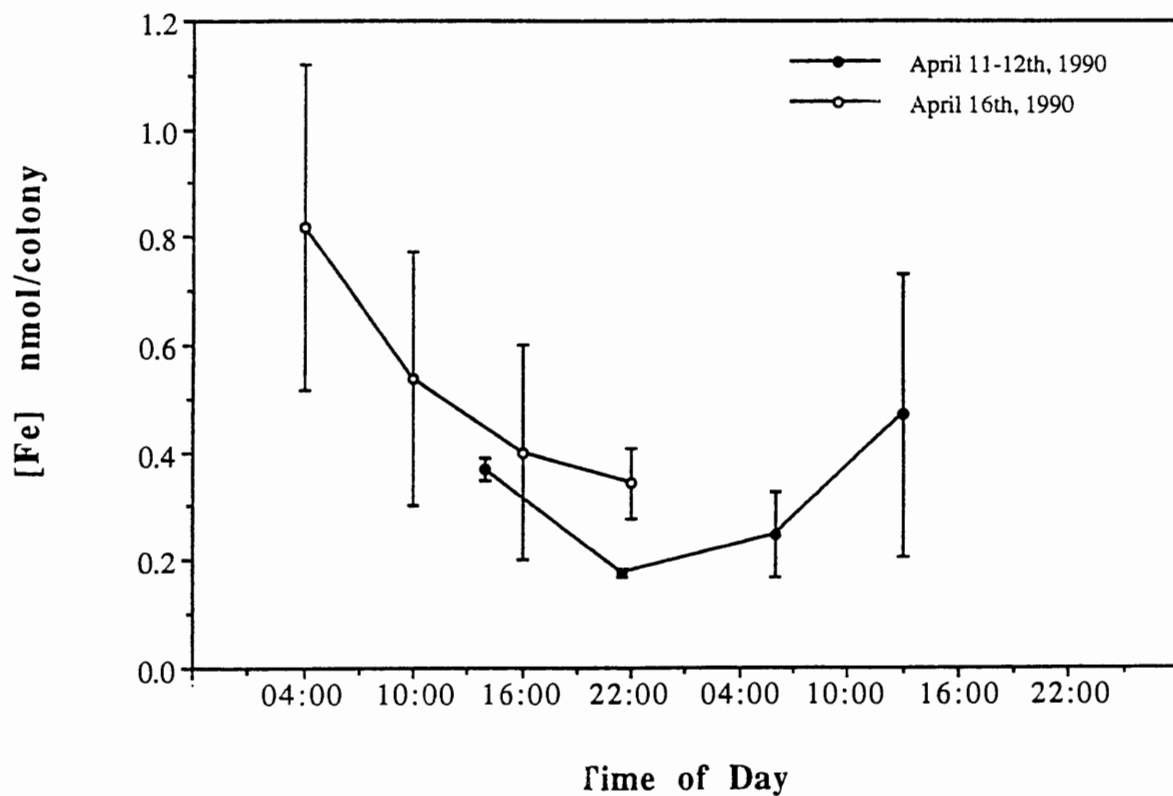
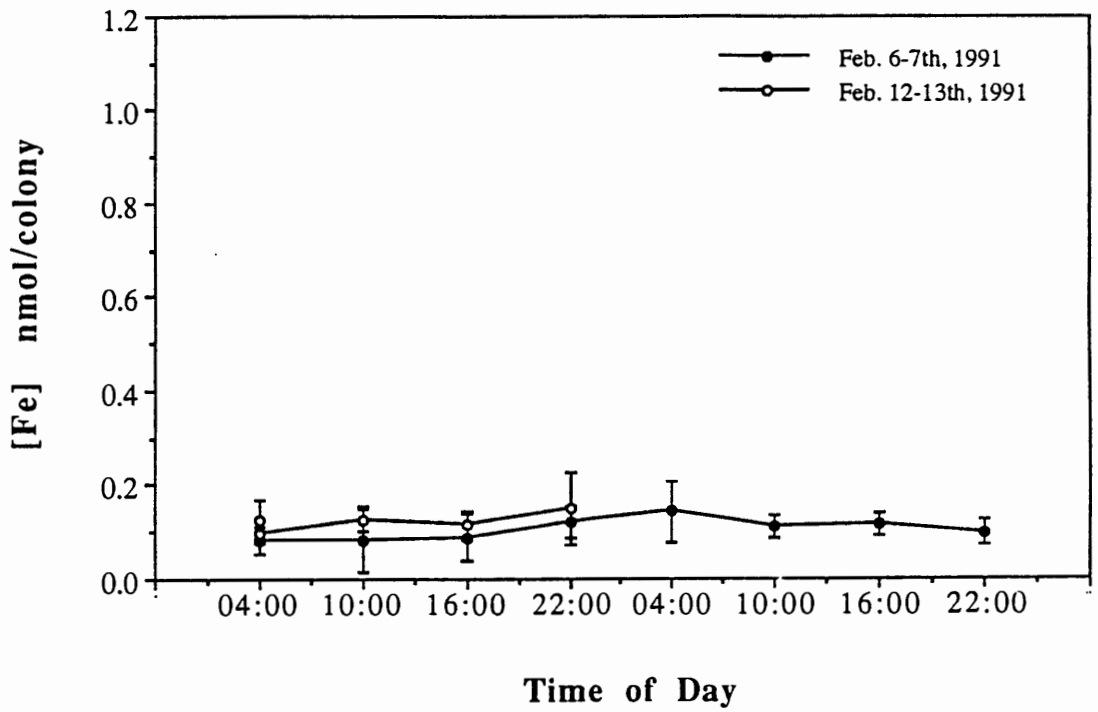
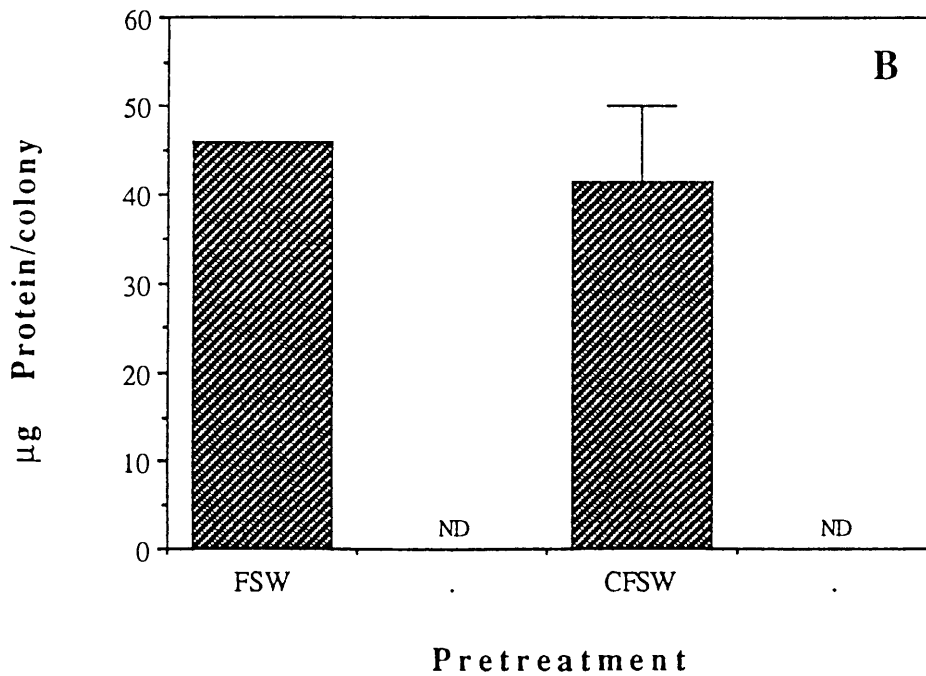
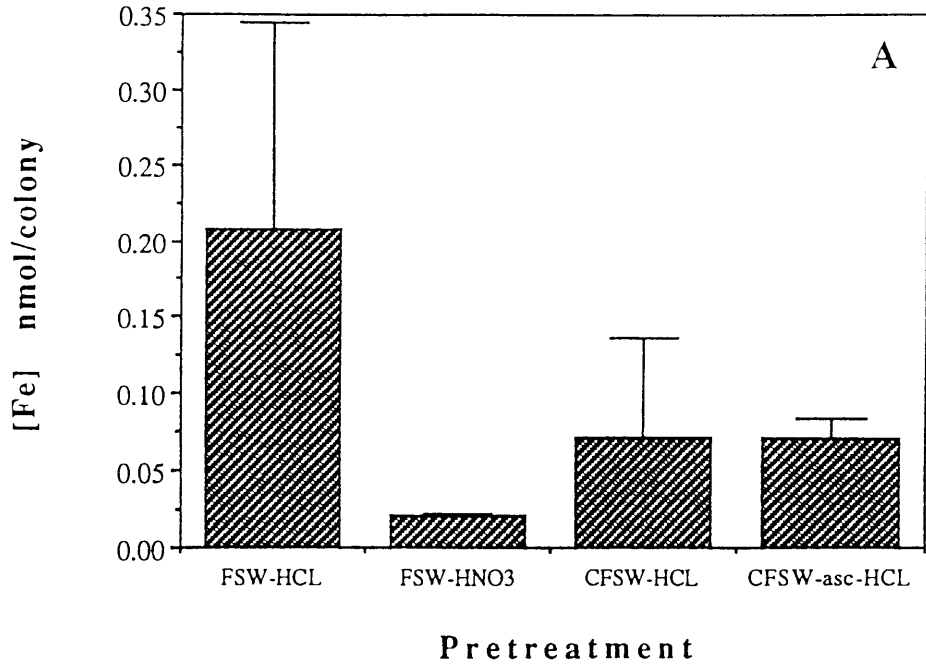


Figure 7. Acid leachable cellular iron content in *Trichodesmium* colonies as a function of time of the day in April 1990.



**Figure 8.** Acid leachable cellular iron content in *Trichodesmium* colonies as a function of time of the day in February 1991.



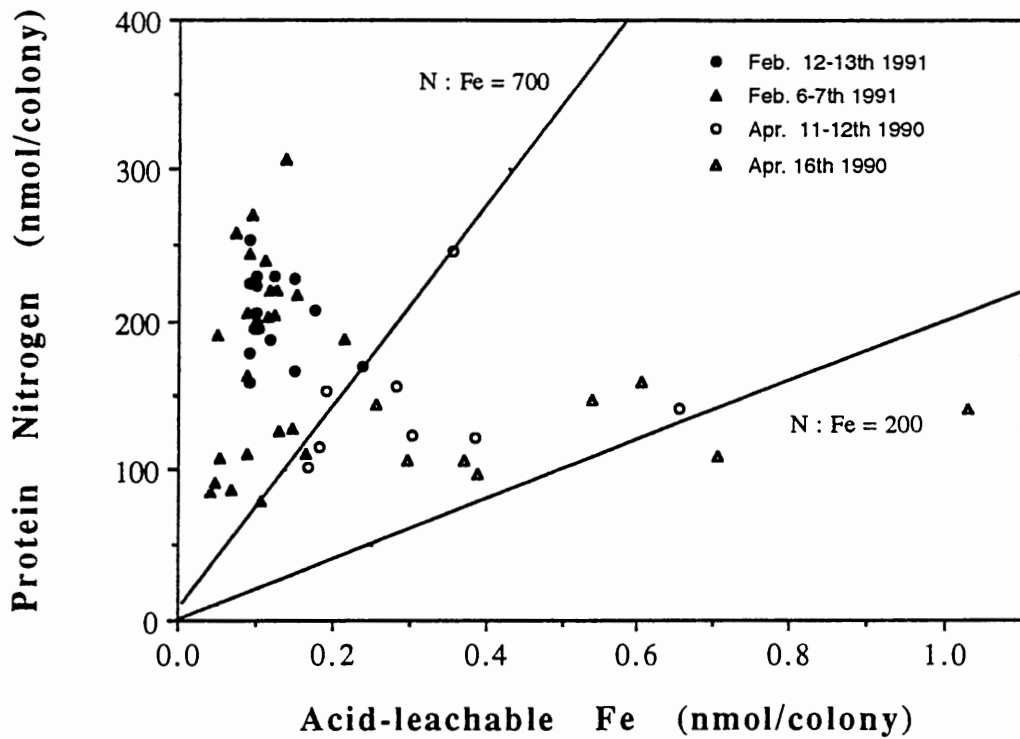
**Figure 9.** A: Pretreatment prior to the Ferrozine assay (A) and the protein content (B) in *Trichodesmium* colonies. FSW-HCl & FSW = Filtered seawater rinse + HCl, FSW-HNO<sub>3</sub> = FSW rinse + HNO<sub>3</sub>, CFSW-HCl & CFSW = FSW rinse + chelated filtered seawater (CFSW) + HCl, CFSW-asc-HCl = FSW rinse + CFSW + Ascorbic acid + HCl.

(Figure 9 A). The results of this experiment indicate that the iron measured in this study is not attributed to extracellular iron. Colonies dipped in chelexed filtered seawater and chelexed filtered seawater with an ascorbate rinse were assayed for protein to determine if the extra handling would decrease the biomass. The analysis of protein/colony did not show a significant decrease in biomass in the Chelex rinse treatment (Figure 9 B). Results for the ascorbate treatment could not be obtained because ascorbate interferes with the protein assay. The Chelex rinse treatment may give a more accurate analysis due to reduction in the amount of ambient iron in seawater.

Due to the considerable variability in protein and iron content per colony, protein-N to iron ratios were calculated from the diel measurements. The mol to mol protein-N to iron ratios were plotted in Figure 10 which includes the boundaries of the upper and lower limits determined previously by Hutchins (1989) for *Trichodesmium* samples in the Caribbean Sea. The data indicate that at times, *Trichodesmium* populations have a higher limit of protein-N to iron ratio of up to 3000. The population in February 1991 had protein-N to iron ratios that exceeded 700 while the population in April 1990 had ratios that lie within the previous limits determined by Hutchins (1989).

### SDS-PAGE

The best results for SDS-PAGE required the use of a gentle



**Figure 10.** Protein nitrogen to iron ratios in *Trichodesmium* colonies from the Caribbean Sea. The N:Fe ratio limits (200 and 700) are taken from Hutchins (1989).

filtration apparatus and millipore HA filter to remove salt from the colonies (Figure 11, lanes 7, 8). Salt apparently interferes with the matrix of the gel or with sodium dodecyl sulfate and prevents complete solubilization of the protein subunits. It was also determined that a dilution of 20 colonies in 40  $\mu$ l of SDS protein solubilization buffer gave the best results (Figure 11, lanes 2-6) and a dilution of 20 colonies in 80  $\mu$ l of SDS protein solubilization buffer was too dilute and did not result in high resolution (Figure 11, lane 9). Also observed on this gel is that puff and tuft colony morphologies have dissimilar protein bands, and that the puff form is more easily solubilized. This observation was observed in subsequent gels.

Changes in an unknown low molecular weight protein(s) between 17 kDa and 27 kDa standards were detected in diel samples (Figures 12, 13, 14). The proteins involved had an apparent molecular weight near 26 and 24 kDa. In Gel #18 (Figure 12) the proteins appeared as two bands at 2200 hours and as a double band with a dominate upper band at 1000 and 1600 hours and a single lower band at 0400 hours. Gel #38 (Figure 13) showed the single upper band at 0400 and the double band at 1030 and 2200 hours. Gel #39 (Figure 14) showed a double band at 0400 and a single upper band at 1000, 1600 and 2200 hours. A diel pattern of these changes in banding pattern was difficult to determine due to the inconsistency of the banding pattern, however a general trend was observed. The upper band appeared dominate during the daylight hours in Gel #18 (Figure 12) and Gel #39 (Figure 14) with a

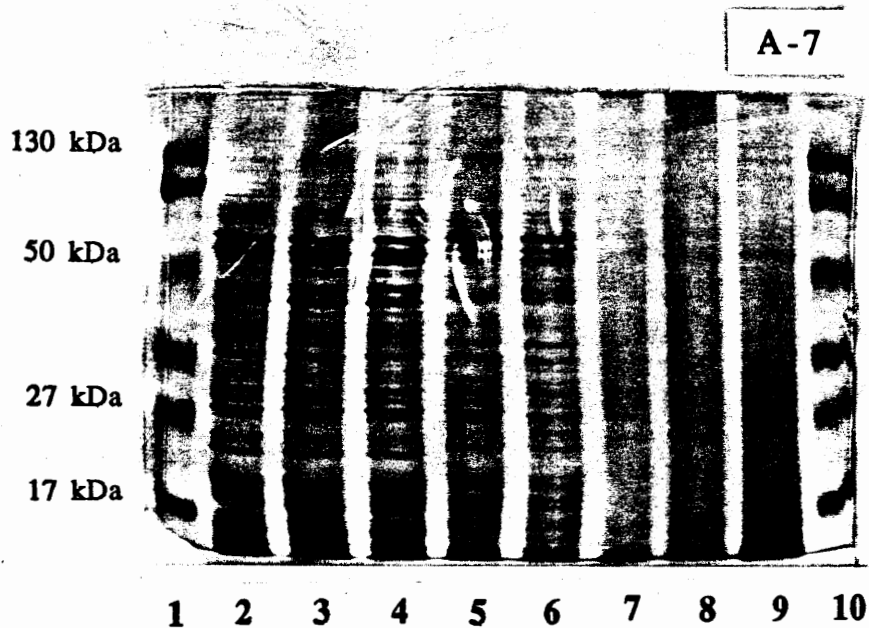
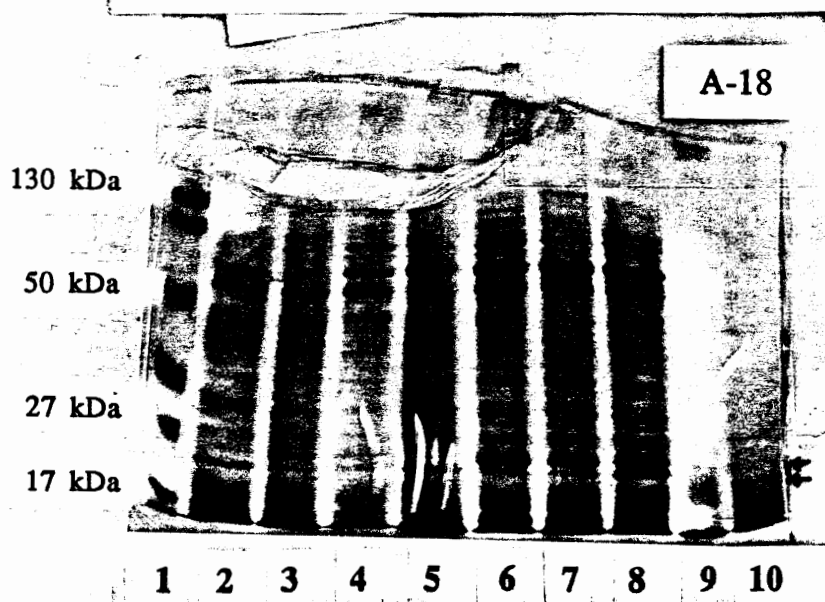


Figure 11. SDS-PAGE (Gel#A-7) of *Trichodesmium* colonies in January 1990 from the Caribbean Sea. 1) molecular weight standard (MW std); 2-4) puffs; 5-6) tufts; 7-8) colonies collected without using the filtration technique to remove seawater; 9) overdilution of buffer relative to the amount of colonies present; 10) MW std.



**Figure 12.** SDS-PAGE (Gel#A-18) of *Trichodesmium* colonies in an iron addition and a diel experiment on 6 April, 1990 from the Caribbean Sea. 1) MW std; 2) control; 3)  $\text{FeCl}_3$  treatment; 4) dust addition; 5) 0400 h; 6) 1000 h; 7) 1600 h; 8) 2200 h; 9) horse ferritin; 10) overdilution of buffer relative to the amount of *Trichodesmium* colonies.



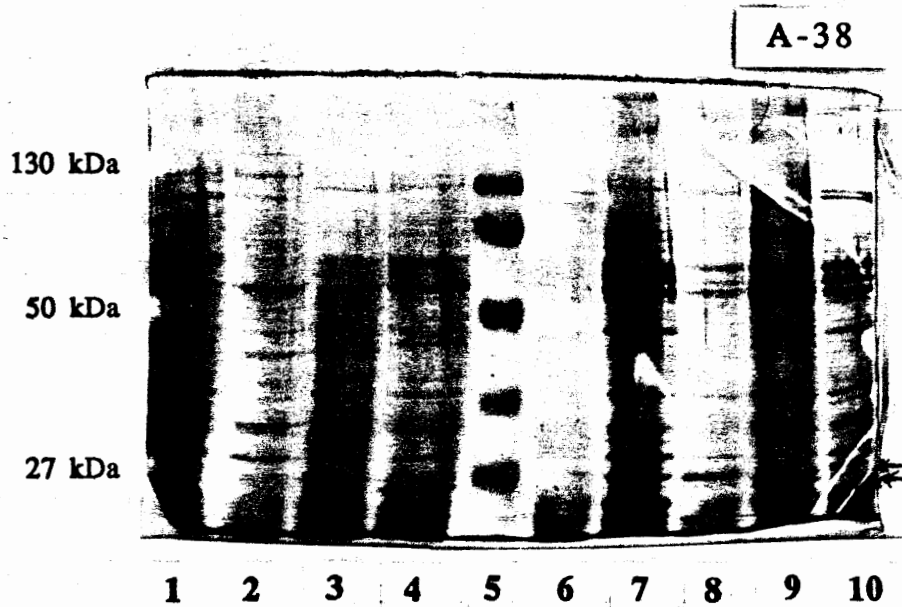


Figure 13. SDS-PAGE (Gel#A-38) of *Trichodesmium* colonies in a diel and an iron addition experiment in February 1991 from the Caribbean Sea. 1) overloaded protein; 2) 0730 h (Feb. 6); 3) 0400 h (Feb. 7); 4) 1030 h (Feb. 6); 5) MW std; 6) 2200 h (Feb. 22); 7) Control with no iron addition; 8) FeCl<sub>3</sub> treatment; 9) dust addition; 10) 2200 h (Feb.7).



Figure 14. SDS-PAGE (Gel#A-39) of *Trichodesmium* colonies in a diel and an iron addition experiment in February 1991 from the Caribbean Sea. 1) 0400 h (Feb. 12); 2) 1000 h (Feb. 12); 3) 1600 h (Feb. 12); 4) 2200 h (Feb. 12); 5) 0400 h (Feb. 13); 6) MW std; 7) control; 8) FeCl<sub>3</sub> addition; 9) dust addition; 10) void.

transition period in the dark hours to a double band. The resolution of the diel samples in Gel #38 (Figure 13) was not as clear as the others and did not show this pattern.

Colonies were incubated in no iron as a control (C),  $10^{-8}$   $\text{FeCl}_3$  (Fe) and in dust (D) for 8, 9 and 26 hours. Gel #18 (Figure 12) shows an 8 hour incubation on April 16th 1990 that started at 0030 and stopped at 0830 hours. The control (lane 2) shows only the lower band and the samples incubated in iron and dust (lanes 3 and 4) show a double band (Figure 12). This was repeated in Gel #38 (Figure 13) in a 9 hour incubation starting at 0030 and stopped at 0945 hours. The control (lane 7) and Fe (lane 8) showed a double band with a dominant upper band and the dust sample showed a strong double band (lane 9) (Figure 13). A longer incubation of 26 hours on February 13th to 14th was started at 0615 and stopped at 0900 hours the next day is shown in Gel #39 (Figure 14). The control has a double band with a dominant upper band and the iron and dust samples show a double band with a dominant lower band (Figure 14).

In order to determine if the unidentified protein was involved in iron storage proteins such as a ferritin-like molecule, gels were stained first with the Prussian blue reaction. Prussian blue did not stain any proteins on the gels. The more sensitive bathophenanthroline stain was used on additional gels containing concentrated dilutions of colonies and a horse ferritin standard resulting in staining only of horse ferritin (see Figure 12 for example of horse ferritin).

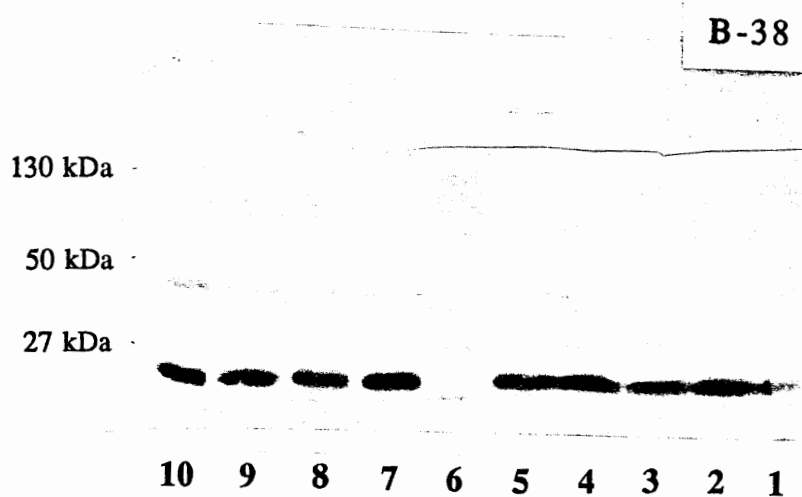
The unidentified protein lies in the same molecular weight region as phycoerythrin. A low molecular weight (LMW) gel was run and resulted in better resolution of low molecular weight proteins including phycoerythrin.

### Western Blot Immunoassays

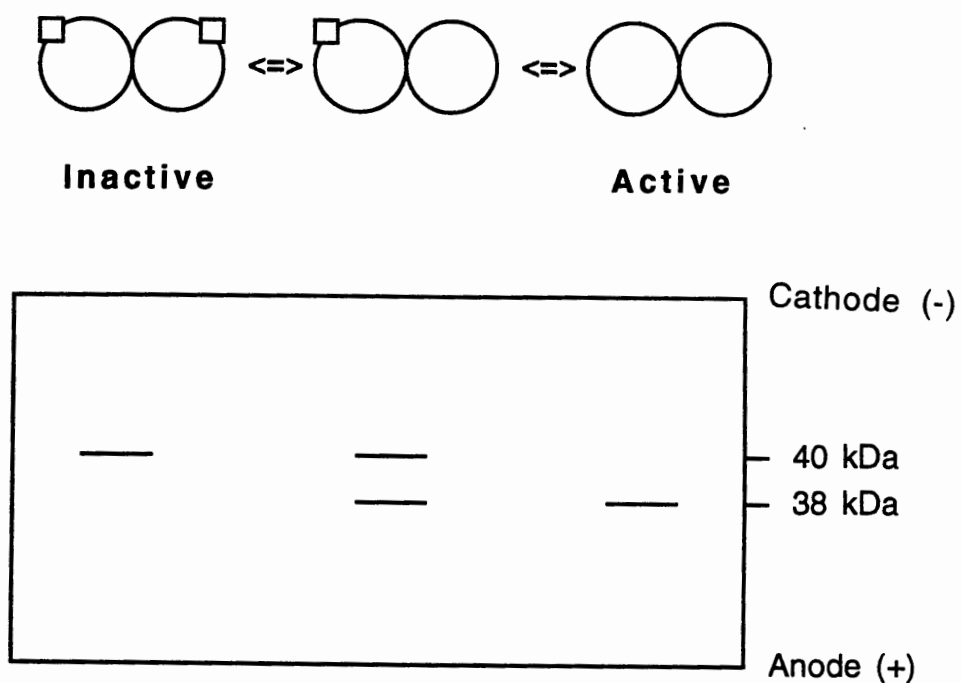
The blot #B38 (Figure 15) and LMW gels were probed for phycoerythrin and resulted in a large band at approximately 18 kDa. Although the molecular weight of the unidentified protein is close to that of phycoerythrin, immunoassay results revealed that it did not cross react with phycoerythrin.

A diel change in the iron protein of nitrogenase was observed in the Caribbean Sea population that corresponds to the activity of the enzyme. Due to the uncertainty of using SDS-PAGE for the detection of molecular weight changes in proteins, caution is used with the term "modification." The iron protein of nitrogenase has an apparent change in molecular weight that may be a result of covalent modification of one or both of the identical subunits. For convenience of descriptive detail in this thesis, the term "modification" will be used however, the mechanism of modification is yet unknown in *Trichodesmium*. The higher molecular weight form is an apparently modified form and is inactive. The lower molecular weight form is unmodified and active. This is more clearly shown in Figure 16.

Results of Western blots against nitrogenase on diel samples and samples incubated in iron and dust from the Caribbean Sea are



**Figure 15.** Immunoassay (anti-phycoerythrin) of *Trichodesmium* colonies in a diel and an iron addition experiment in February 1991 from the Caribbean Sea. 1) 1030 h (Feb. 6); 2) 0400 h (Feb. 7); 3) 1000 h (Feb. 7); 4) 1700 h (Feb. 7); 5) 2200 h (Feb. 7); 6) MW std; 7) control; 8)  $\text{FeCl}_3$  treatment, 9) dust addition; 10) 1600 h (Feb. 6).



**Figure 16.** Schematic diagram of 'modification' (active and inactive form) of the iron protein of nitrogenase (top figure) and how it will appear on a Western blot (bottom figure).

shown in Blot #39 (Figure 17) and #40 (Figure 18). Lanes 1-5 show the changes in the iron protein of nitrogenase over a 24 hour period (Figure 17). The protein is in the inactive, apparently modified, higher molecular weight (approx. 40 kDa) form at 0400 hours. At 1000 hours the protein becomes demodified and is in the lower molecular weight active form (approx. 38 kDa). The iron protein in this blot appears to become modified at 1600 hours (Lane 3) where both forms are seen. Complete modification occurs by 2200 and 0400 and only the modified higher form is seen. *Trichodesmium* colonies were incubated in  $\text{FeCl}_3$  ( $1.8 \times 10^{-8}$  M) and in Saharan dust (10 mg. in 200 ml FSW) for 26 hours until 0900 hours and are seen in lanes 7-9 (Figure 17). The control (no Fe) is predominantly in the upper, inactive form with a small amount of the lower active form. The colonies incubated in  $\text{FeCl}_3$  and dust have both forms of the enzyme which is most apparent in the colonies incubated in dust (lane 9). Blot #40 (Figure 18) shows colonies taken from a 1000 hour tow as time zero for comparison and colonies incubated in  $\text{FeCl}_3$  and dust for 10 hours. Lane 2 and 8 shows the enzyme in the unmodified active form from the samples taken directly from the tow at 10:00. The colonies incubated in iron and dust for 10 hours were prepared at 20:00 hours. Lane 3 shows the control (no Fe) in the inactive higher MW form. Lane 4 and 7 shows the colonies incubated in  $\text{FeCl}_3$  with the enzyme in the inactive higher MW form in Lane 4 and with both forms in Lane 7. The colonies incubated in dust are seen in Lane 5 with both forms of the enzyme.

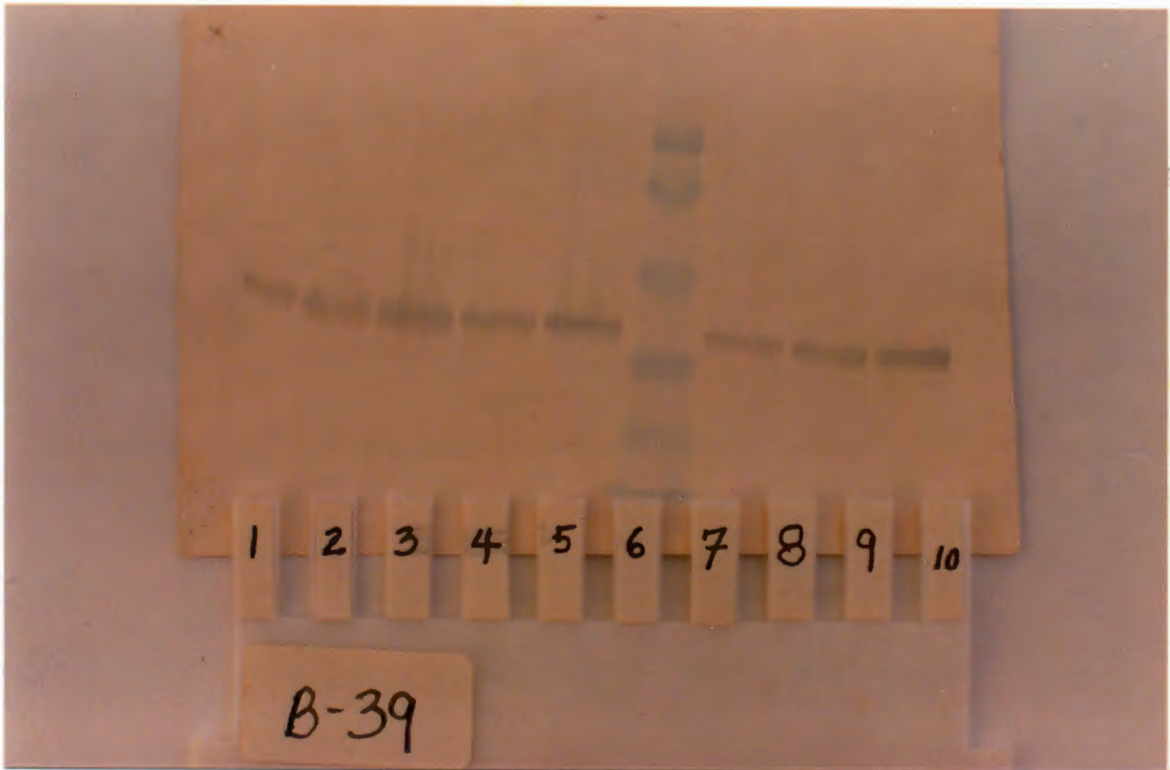
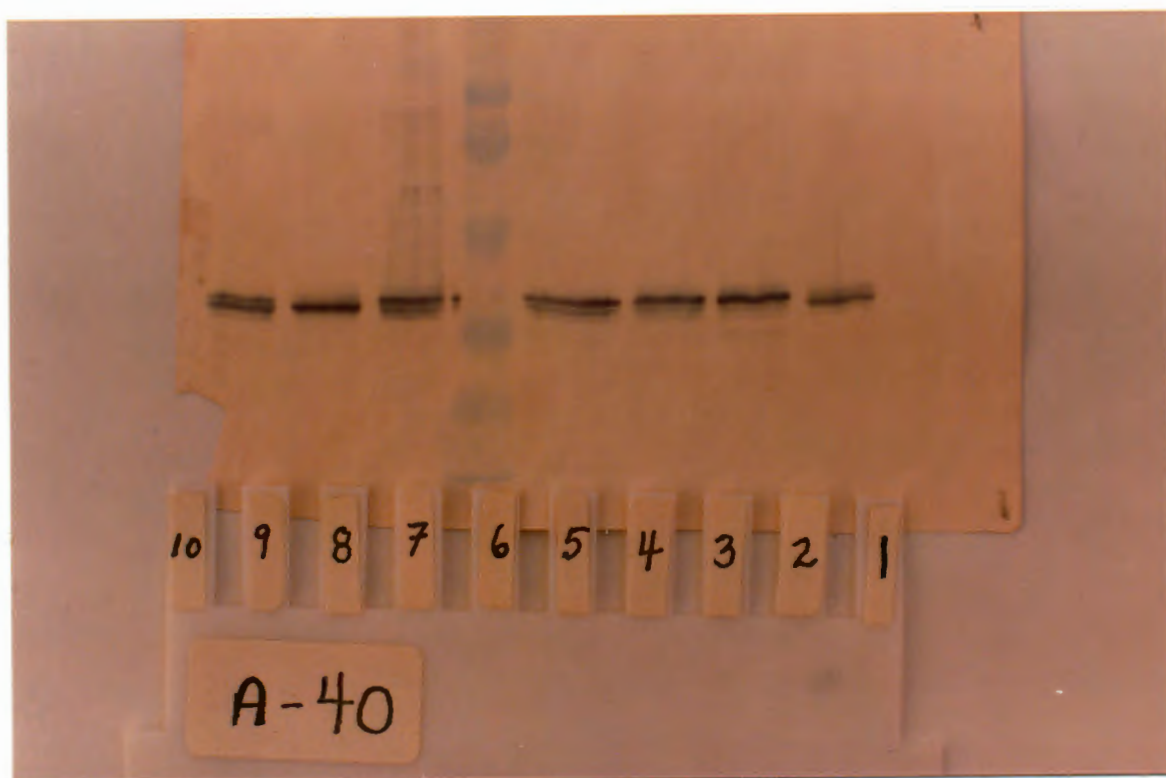


Figure 17. Immunoassay B-39 (anti-dinitrogenase reductase) of *Trichodesmium* colonies in a diel and an iron addition experiment in February 1991 from the Caribbean Sea. 1) 0400 h (Feb. 12); 2) 1000 h (Feb. 12); 3) 1600 h (Feb.12); 4) 2200 h (Feb. 12); 5) 04.00 h (Feb. 13th); 6) MW std; 7) control; 8) FeCl<sub>3</sub> addition; 9) dust addition; 10) Void.



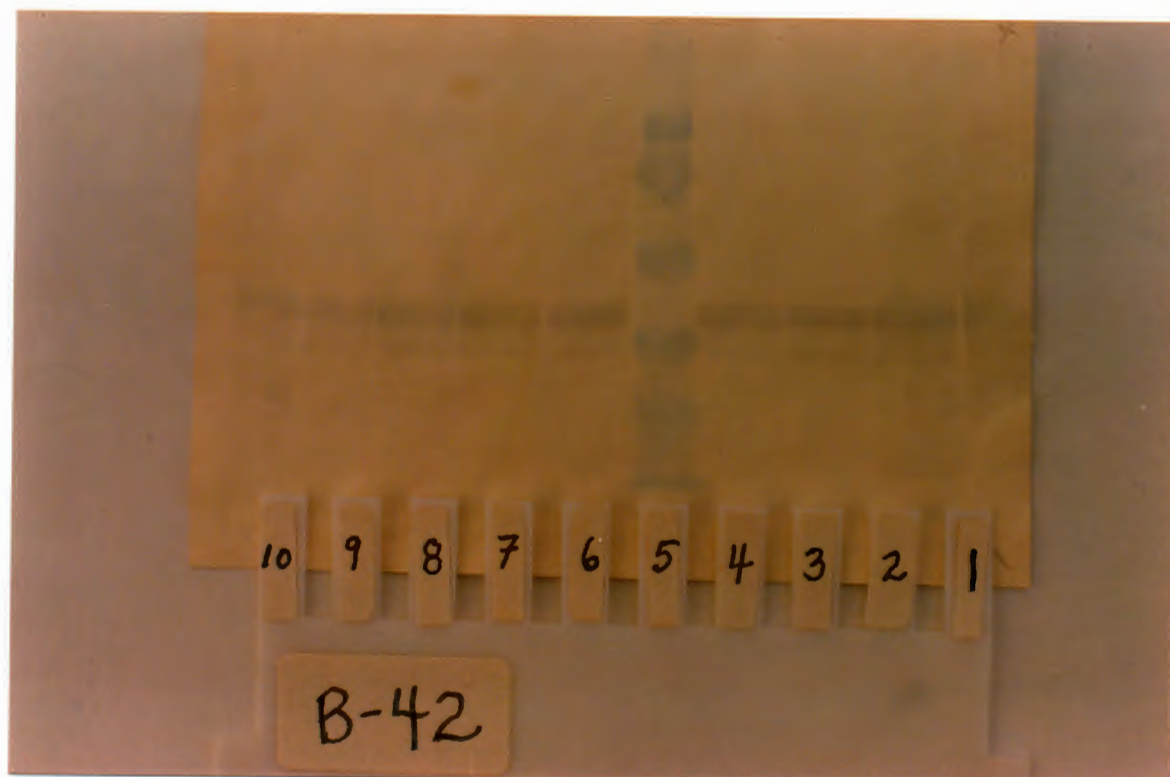


**Figure 18.** Immunoassay A-40 (anti-dinitrogenase reductase) of *Trichodesmium* colonies in an iron addition experiment on 14 February, 1991 from the Caribbean Sea. 1) void; 2) 1000 h time zero; 3) control, 4)  $\text{FeCl}_3$  addition; 5) dust addition; 6) MW std; 7)  $\text{FeCl}_3$  addition; 8) 1000 h time zero; 9) 1000 h time zero; 10) void.

Colonies from the Sargasso Sea did not show a diel change in the iron protein of nitrogenase in April and early May 1991. Blot #42 (Figure 19) shows puff colonies at various times throughout the day and at all times are seen with both forms of the enzyme. Also on this blot (Figure 19) are colonies incubated in no iron (control),  $\text{FeCl}_3$  ( $1.8 \times 10^{-8}$  M) and  $\text{FeEDTA}$  ( $10^{-8}$ M) for approximately 12 hours until 1800 hours in lanes 6-9. The enzyme shows no changes from the samples of puff colonies collected throughout the day and are seen faintly with both forms of the enzyme. Blot #41 (Figure 20) shows samples taken at 1530 hours on April 23, 1991 and show again both forms of the enzyme. Colonies were incubated in no iron,  $\text{FeCl}_3$  and dust for 28 hours are seen in Lanes 4-6. Only Lane 6, the colonies incubated in dust, showed a strong reaction for nitrogenase.

The dominant form of *Trichodesmium* colonies in the Sargasso Sea during the Spring of 1991 were puff forms. Tuft forms were rarer and collected for comparison on Western Blots for the iron protein of nitrogenase. Blot #44 (Figure 21) shows puffs in lanes 1, 4 and 8 with a strong signal for the iron protein of nitrogenase. These colonies were collected at mid-day on May 9, 1991, and are seen with both forms of the enzyme. Tufts are seen in lanes 2 and 9 and show a very weak signal for the iron protein of nitrogenase. Later on May 13, 1991, tufts were collected and run in Blot #45 (Figure 22) in lanes 2, 4 and 9 which show no signal for nitrogenase.

Later, on May 13, 1991, puff colonies appeared to show a diel shift in the iron protein of nitrogenase. This is seen in lane 5 with colonies collected at 1230 hours (Figure 22).



**Figure 19.** Immunoassay B-42 (anti-dinitrogenase reductase) of *Trichodesmium* colonies in a diel and an iron addition experiment in April 1991 from the BATS station in the Sargasso Sea. 1) 2000 h (Apr. 29); 2) 2400 h (Apr. 29);, 3) 0700 h (Apr. 30); 4) 1530 h (Apr. 30); 5) MW std; 6) control; 7) FeCl<sub>3</sub> addition; 8) dust addition; 9) FeCl<sub>3</sub> + EDTA addition; 10) 1800 h (Apr. 30).

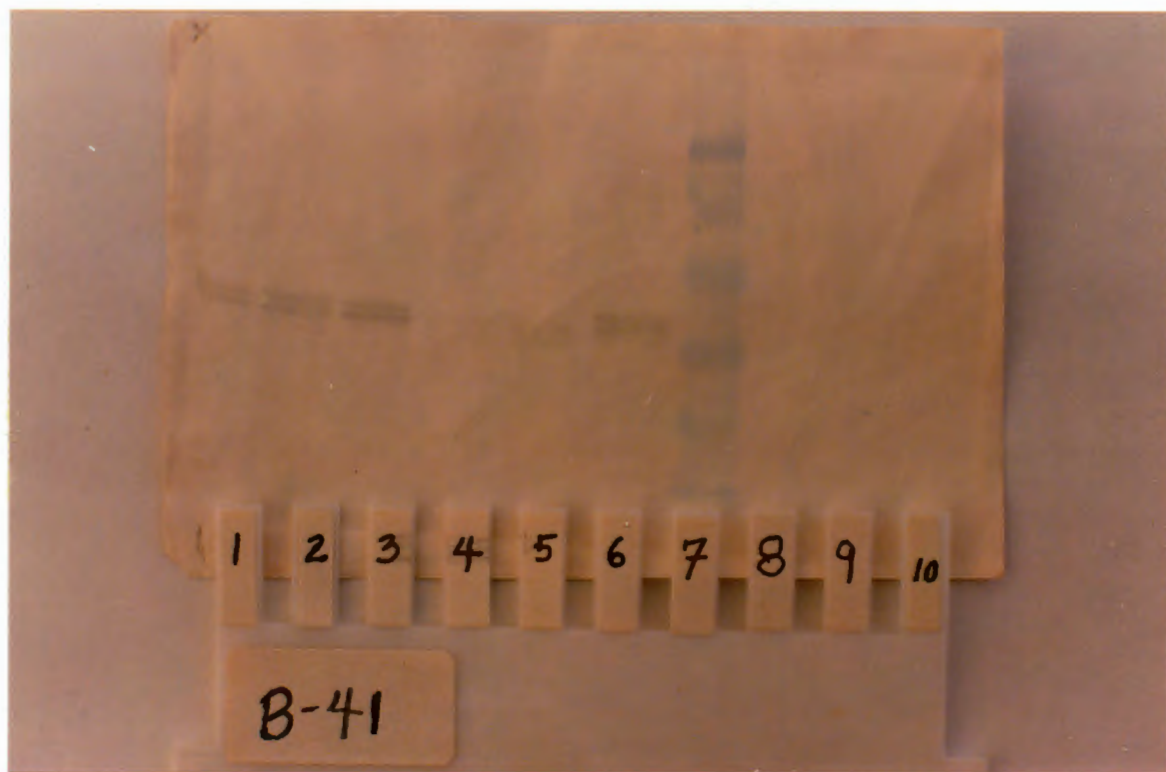


Figure 20. Immunoassay B-41 (anti-dinitrogenase reductase) of *Trichodesmium* colonies in an iron addition experiment on 23 April, 1991 from Hydrostation 'S' in the Sargasso Sea. 1-3) 1530 h; 4) control; 5)  $\text{FeCl}_3$  addition; 6) dust addition; 7) MW std; 8-10) void.

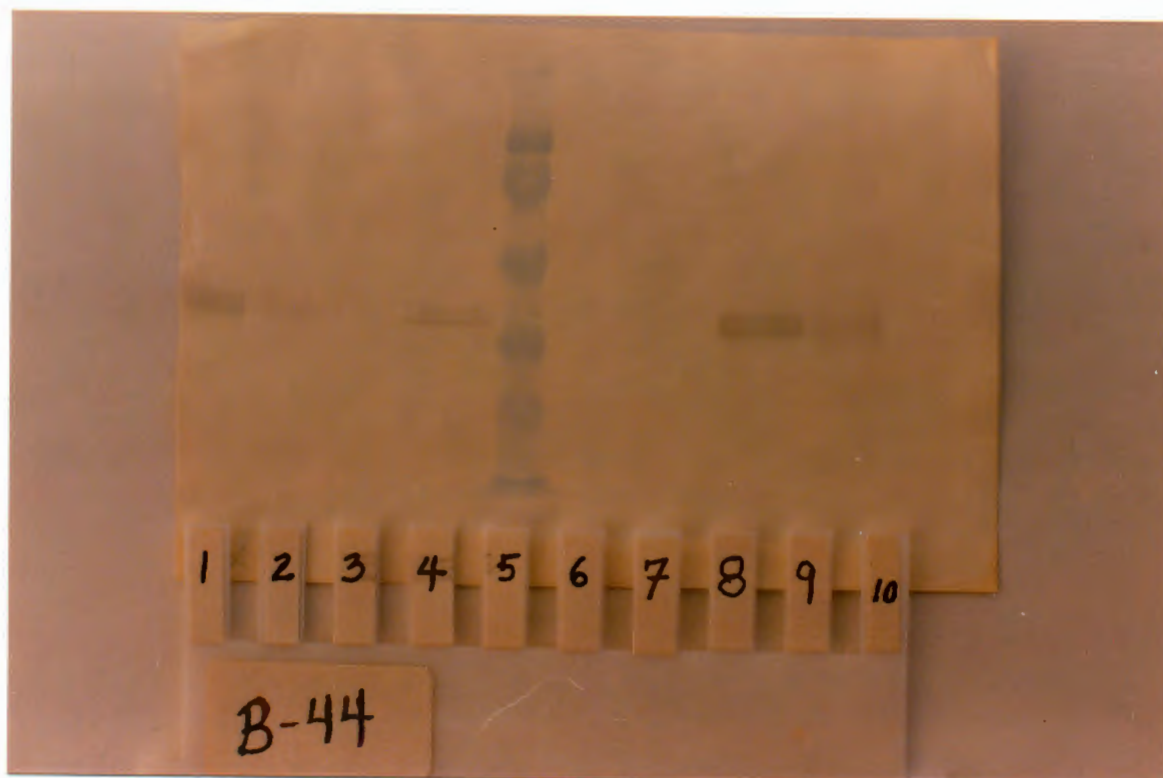


Figure 21. Immunoassay B-44 (anti-dinitrogenase reductase) of *Trichodesmium* colonies (puff and tuft comparison) on 9 May, 1991 from Hydrostation 'S' in the Sargasso Sea. 1) puffs 1200 h; 2) tufts 1200 h; 3) void; 4) puffs 1200 h; 5) MW std; 6-7) void; 8) puffs 1200 h; 9) tufts 12.00 h; 10) Void.

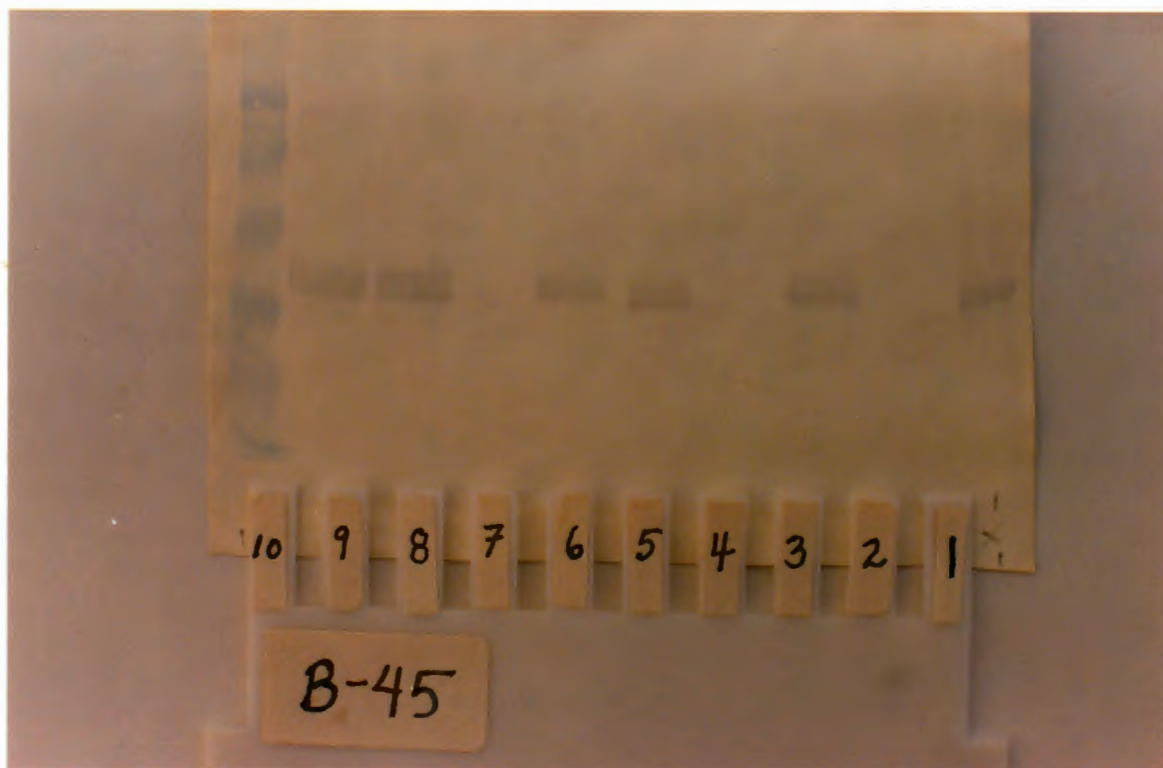


Figure 22. Immunoassay B-45 (anti-dinitrogenase reductase) of *Trichodesmium* colonies (puff and tuft comparison) in a diel experiment in May, 1991 at BATS station in the Sargasso Sea. 1) puffs 1830 h (May 13); 2) tufts 2400 h (May 13); 3) puffs 0030 h (May 14); 4) tufts 1230 h (May 14); 5) puffs 1230 h (May 14); 6) puffs 0500 h (May 14); 7) tufts 0500h (May 14); 8-9) puffs 0500 h (May 14); 10) MW std.

### Hydrography of the Sargasso and Caribbean Seas

During April 1991 in the Sargasso Sea, there was significant nitrate in the euphotic zone from recent mixing. Figure 23 shows nitrate and nitrite in the euphotic zone. Nitrate and nitrite had recently been depleted from the upper euphotic zone and there were significant amounts in the lower euphotic zone. Two weeks later in May there appeared to be a change in the migrating pattern of the iron protein with a shift in molecular weight of the iron protein to the lower active form (Figure 22). There was a concomitant decrease in the nitrate and nitrite levels in the euphotic zone with a corresponding increase in the peak of fluorescence (Figure 24, 25). The CTD profiles in Figure 24 and Figure 25 show the shallow thermocline in the Sargasso Sea that is a result of recent mixing and the deeper thermocline in the Caribbean Sea (Figure 26). Also seen from the CTD profile presented in Figure 26 and Figures 24, 25 is the different temperatures of the Caribbean Sea at 26°C and the Sargasso Sea at 21°C respectively. The hydrographical profiles show the different environmental conditions that were present in the two seas.

### Acetylene Reduction and Iron Additions

Rates of acetylene reduction were highest at midday and decreased in late afternoon (Figure 27). Results from acetylene reduction experiments with iron additions are presented in Table II for the short-term incubations (<5 hrs.) and Table III for the long-term incubations (>5 hrs). Results from April 1990 show a slightly higher average rate of acetylene reduction in colonies exposed to

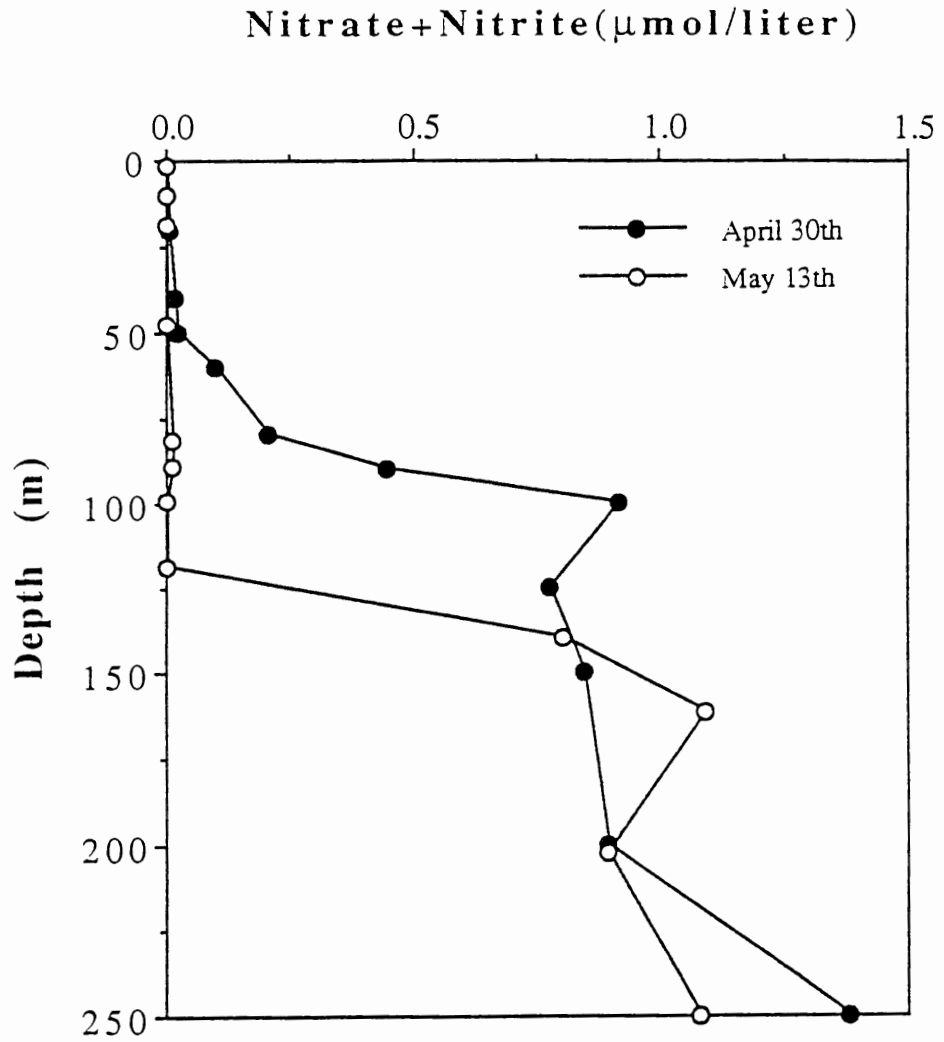
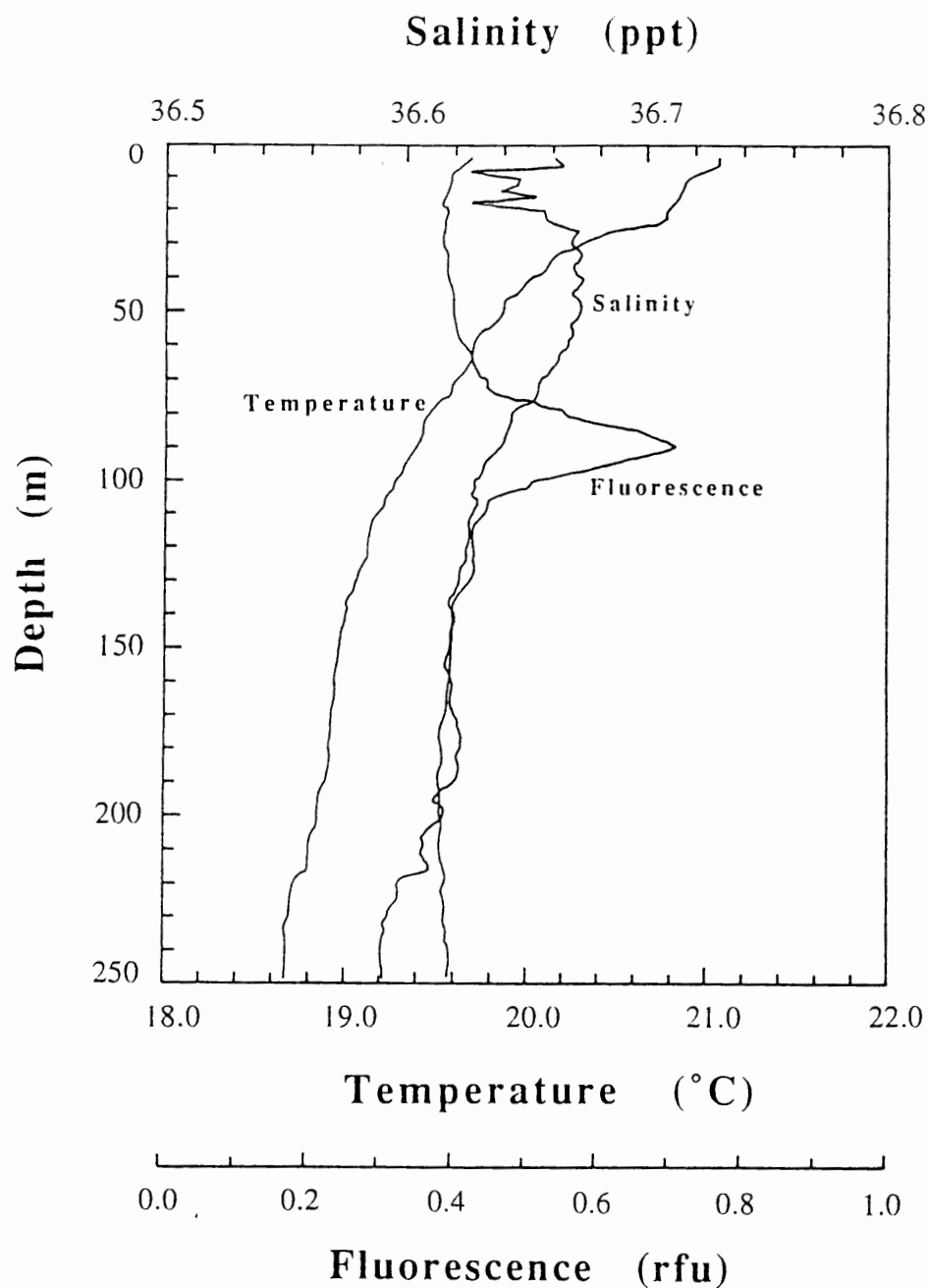
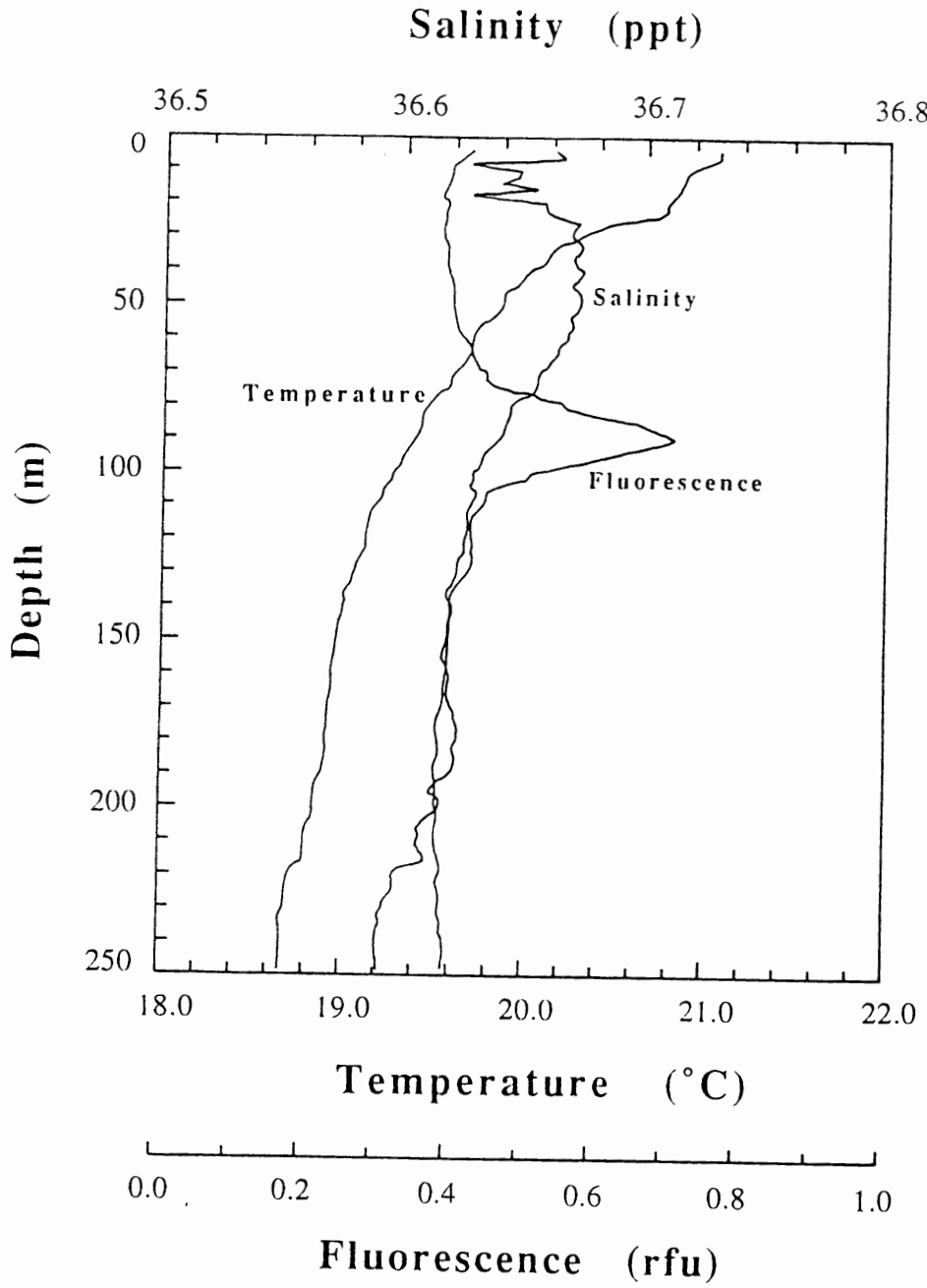


Figure 23. Vertical profile of nitrate + nitrite at BATS station in the Sargasso Sea in April and May 1991.

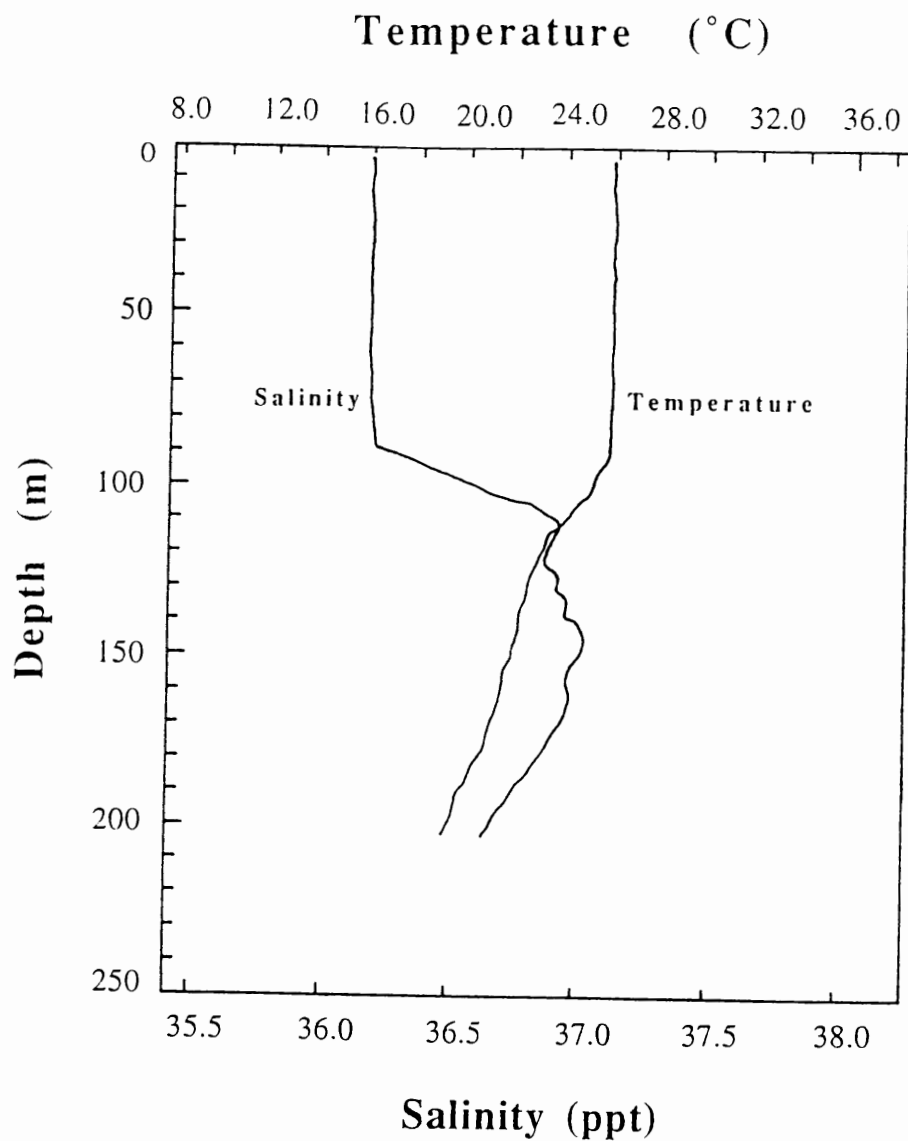




**Figure 24.** Vertical profile of salinity, temperature and fluorescence (rfu = relative fluorescence units) at BATS station in the Sargasso Sea on 30 April, 1991.



**Figure 24.** Vertical profile of salinity, temperature and fluorescence (rfu = relative fluorescence units) at BATS station in the Sargasso Sea on 30 April, 1991.



**Figure 26.** Vertical profile of salinity and temperature in the Caribbean Sea on 4 February, 1991.

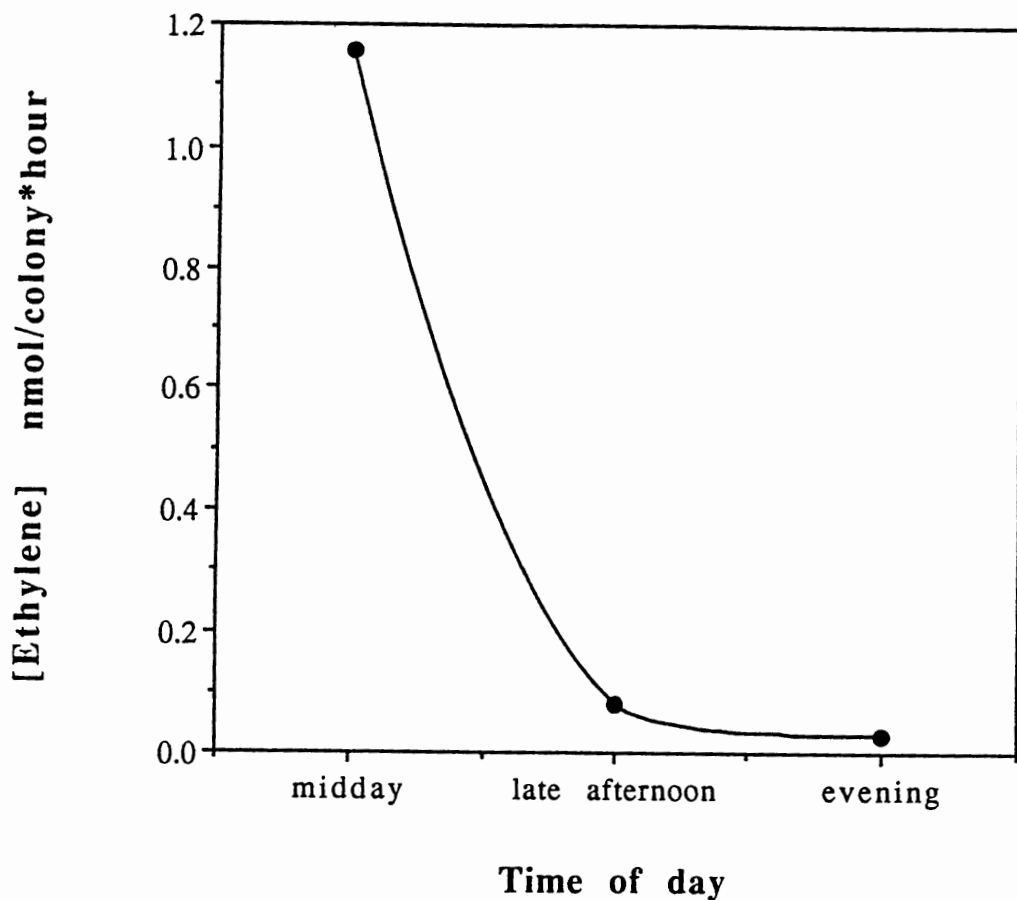


Figure 27. Acetylene reduction measured as ethylene production rate (average per colony \* hour) as a function of time of the day.

TABLE II

AVERAGE ACETYLENE REDUCTION RATES (nmol Ethylene/colony\*hour) FOR *TRICHODESMIUM* IN A SHORT TERM INCUBATION (LESS THAN 5 HOURS), WITH IRON ADDITIONS. FeCl<sub>3</sub> and FeCl<sub>3</sub>+EDTA = 1.8\*10<sup>-8</sup> M Fe, Dust = 4.5\*10<sup>-6</sup> M Fe. STANDARD DEVIATION (SD<sub>n-1</sub>) BETWEEN REPLICATE LINEAR INTEGRATIONS ARE GIVEN IN PARANTHESIS. ND = No Data. \*\*\* = AVERAGE OF 3, \*\* = AVERAGE OF 2 AND \* = AVERAGE OF 1 REPLICATE LINEAR REGRESSION(S).

Date	Control	FeCl <sub>3</sub>	Dust	FeCl <sub>3</sub> +EDTA
April 16th, 1990 (0.254)	0.315*** (0.177)	0.586*** (0.113)	0.352***	ND
February 5th, 1991	0.297*	0.444** (0.404)	0.189** (0.006)	ND
February 6th, 1991	0.648** (0.082)	0.271** (0.006)	0.272** (0.057)	ND
February 7th, 1991	1.663*** (0.081)	1.227*** (0.224)	ND	ND
January 20th, 1992	0.439** (0.119)	0.530** (0.013)	0.259** (0.059)	0.374** (0.196)
January 22nd, 1992	0.161** (0.021)	0.228** (0.070)	0.076** (0.059)	0.065** (0.049)
January 23rd, 1992	0.042*** (0.024)	0.071*** (0.033)	0.058*** (0.033)	0.026*** (0.024)
August 30th, 1992	0.358** (0.080)	0.309** (0.058)	0.191** (0.001)	ND

TABLE III

AVERAGE ACETYLENE REDUCTION RATES (nmol Ethylene/colony\*hour) FOR *TRICHODESMIUM* IN A LONG TERM INCUBATION (5-20 HOURS), WITH IRON AND EDTA ADDITIONS. APRIL 1990 AND FEBRUARY 1991; FeCl<sub>3</sub> and FeCl<sub>3</sub>+EDTA = 1.8\*10<sup>-8</sup> M Fe, Dust = 4.5\*10<sup>-6</sup> M Fe. JANUARY 29th, 1992; FeCl<sub>3</sub> = 1.8\*10<sup>-7</sup> M Fe, Dust = 9.0\*10<sup>-6</sup> M Fe, FeCl<sub>3</sub> + EDTA = 1.8\*10<sup>-7</sup> M Fe. JANUARY 31st, 1992; FeCl<sub>3</sub> = 1.8\*10<sup>-7</sup> M Fe, Dust = 9.0\*10<sup>-6</sup> M Fe, EDTA = 1.0\*10<sup>-6</sup> M EDTA. STANDARD DEVIATION (SD<sub>n-1</sub>) BETWEEN REPLICATE LINEAR INTEGRATIONS ARE GIVEN IN PARANTHESIS. ND = No Data. \*\*\* = AVERAGE OF 3, \*\* = AVERAGE OF 2 AND \* = 1 REPLICATE LINEAR REGRESSION(S).

Date	Control	FeCl <sub>3</sub>	Dust	FeCl <sub>3</sub> +EDTA	EDTA
April 16th, 1990	0.011*** (0.015)	0.030*** (0.035)	0.012*** (0.014)	ND	ND
February 7th, 1991	0.810*** (0.329)	0.537*** (0.113)	0.725*** (0.349)	ND	ND
January 29th, 1992	0.160** (0.069)	0.192** (0.000)	0.138** (0.165)	0.016** (0.008)	ND
January 31st, 1992	0.190** (0.122)	0.089** (0.049)	0.062** (0.054)	ND	0.151** (0.148)

FeCl<sub>3</sub> ( $1.8 \times 10^{-8}$  M) but not a significantly higher rate. A short-term incubation (iron added directly to vial) experiment and a longer term (5 1/2 hours) showed similar results. This experiment was repeated in February 1991 and the opposite result was obtained. Iron in the form of FeCl<sub>3</sub> had lower average acetylene reduction rates in both short term (iron added directly to vials) and long-term (9 hrs) incubation experiments.

In January 1992, experiments with iron additions were repeated with the addition of FeCl<sub>3</sub> ( $1.8 \times 10^{-8}$ M), dust (10 mg in 200 ml FSW) and with the addition of FeEDTA ( $10^{-8}$  M). Colonies were incubated in the iron solutions for 3 1/2 hours and yielded slightly higher average AR rates with the FeCl<sub>3</sub>, but, the rates were not significantly higher than in the no-iron control. Experiments were repeated for three consecutive days in which iron was added directly to assay vials at 1300 hours. On two of those days FeCl<sub>3</sub> had a slightly higher average AR rate but the difference was insignificant when compared to control samples. Concentrations of FeCl<sub>3</sub> and FeEDTA were increased to  $10^{-7}$  M and dust was increased to 20 mg in 200 ml FSW in an overnight incubation (approximately 20 hr) on Jan. 29, 1992, and resulted in no stimulation of AR rates. This experiment was repeated with colonies collected on a blue water dive on Jan. 30, 1992, but with the addition of EDTA only ( $10^{-6}$  M) instead of FeEDTA. Iron and dust solutions yielded slightly lower average AR rates and the EDTA solution had rates similar to that of the control.

An additional experiment was conducted in August 1992 with the original conditions of  $\text{FeCl}_3$  ( $1.8 \times 10^{-8}$  M) and dust (10 mg in 200 ml FSW). Colonies were incubated for three hours in the morning before acetylene was added. Colonies incubated in dust had the lowest average AR rates and  $\text{FeCl}_3$  did not produce a stimulatory effect.



## DISCUSSION

### Protein and Iron Content

Results from this study show intercolonial variation in protein content that ranged from 9.7-26  $\mu\text{g}$  per colony. Using the same method, Hutchins (1989) reported a range of 5.1-20.4  $\mu\text{g}$  protein per colony and Rueter (1988b) reported a range of 4.8-7.9  $\mu\text{g}$  protein per colony for *Trichodesmium* from the Caribbean Sea. As seen in Table I, growth rates and nitrogen fixation rates vary in historical literature. Part of the discrepancy in reported turnover times for cellular nitrogen in *Trichodesmium* is a result of variable amounts of nitrogen per colony. Carpenter (1973) used a value of 1.5  $\mu\text{g}$  N per colony and Carpenter et al. (1987) used a value 0.2  $\mu\text{g}$  N per colony. The protein nitrogen per colony from this thesis ranged from 1.1 to 4.3  $\mu\text{g}$  per colony based on the assumption that 14% of protein is nitrogen (Dayhoff et al. 1965). Elemental analysis of particulate organic nitrogen content in *Trichodesmium* colonies have shown that this estimate is accurate (Elardo, unpub. data). This demonstrates the importance of determining biomass of the population studied and to avoid adopting average estimates from other sources.

Acid leachable iron content was higher in April 1990 than February 1991 (Figure 7 and Figure 8). During the diel in April 1990 cellular iron was lower in the dark hours of the day and increased in the daylight (Figure 7). During this month, iron may have been more

available in the daylight due to the process of photoreduction which produces ferrous iron (Rich and Morel 1990, Wells and Mayer 1991). An interesting question that arises is why would *Trichodesmium* lose iron or release iron if iron as a micronutrient is limiting? One possibility is that this particular population was iron replete and reached a capacity to store excess iron.

The lower iron values during February 1991 coupled with the relatively higher protein content produced high N/Fe ratios (Figure 10). The N/Fe data show that *Trichodesmium* have a higher limit of N/Fe ratio than previously thought (Hutchins 1988). Rueter (1989) estimated from data in the Caribbean Sea and near Barbados that *Trichodesmium* would have N/Fe ratios of 1000. His measurements ranged from 60-140 pmol per colony. The lowest measurements in pmol obtained in this thesis during February 1990 ranged from 81-371 pmol per colony with N/Fe ratios near 3000. Rueter and Unsworth (1991) found a N/Fe ratio of 2700 for cultures of marine *Synechococcus* grown in  $10^{-8}$  M Fe. They concluded that rapidly growing populations of marine phytoplankton could be expected to have N/Fe ratios of 3000 to 1500 in order to grow fast enough to avoid predation and sinking. Nitrogen doubling times for *Trichodesmium* have been estimated to be from 105 days (Carpenter and McCarthy 1975) to 1.5 days (Carpenter et al. 1987) which indicate that at times, growth can be very rapid. Such a magnitude of variation in growth rates could produce a large variation in N/Fe ratios.

Clearly, cellular iron concentrations are highly variable in *Trichodesmium* colonies. The highest average concentration of acid leachable cellular iron during April 1990 was 817 pmol per colony (Figure 7). *Trichodesmium* colonies most likely obtain these high levels from a source outside of the oceanic environment. Data from the N. Atlantic ocean show that particulate and dissolved iron reached approximately 1.1 nmol in the upper 50 meters during April 1989 (Powell et al. 1994). Dry and wet iron deposition to Bermuda had a seasonal peak between April and November in the years 1989-1990 (AEROCE data, T. Church, unpub. data). The highest peak was in September and in 1989 reached 50,000 pmole/cm<sup>2</sup>. Rueter et al. (1992) showed evidence of *Trichodesmium* colonies adhering to dust particles and proposed this mechanism as the major source of iron acquisition. The seasonal dust input may explain the high levels of cellular iron obtained during April 1990.

### SDS-PAGE

Changes in proteins subjected to SDS-PAGE were observed in samples of *Trichodesmium* (Figures 12, 13, 14). The unknown protein(s) observed had an apparent molecular weight near 26 kDa and 25 kDa. The proteins changed on diel samples and also with iron and dust additions. Iron and dust additions caused changes in the proteins that resulted in the two forms being present at once. Because the protein bands responded to iron additions, it was thought that they may be involved in iron storage as a ferritin-like protein. Phytoferritin, the iron complexing protein found in plants,

has 24 identical subunits with molecular weights 20 to 28 kDa (Briat et al. 1988). Bacterioferritin isolated from the cyanobacterium *Synechocystis* P.C.C. 6803 has a subunit molecular weight of 19 kDa (Laulhere et al. 1992). The unknown proteins had a close proximity to the molecular weight of bacterioferritin but did not stain with iron specific stains. Prussian blue was not sensitive enough and did not stain any protein on the gel. Bathophenanthroline is more sensitive but did not react with *Trichodesmium* proteins. The proteins may not have been concentrated enough for the reaction and when restained with coomassie blue showed faint bands that indicated this. However, equine ferritin did react at approximately 3  $\mu$ g. Equine ferritin was used as a standard because all ferritins whether animal or plant share structural properties (Theil 1987).

Phycoerythrin was ruled out as the identity of the unknown proteins by Western blot immunoassay (Figure 15). On diel studies, phycoerythrin subunits do not change in molecular weight but remain consistent throughout the day and night (Figure 15). However, because of low protein on the gels, the role of an iron storage protein can not be ruled out.

### Immunoassays for Dinitrogenase Reductase

Changes in the iron protein of nitrogenase (dinitrogenase reductase) was observed in diel samples from the Caribbean Sea and in samples incubated in iron and dust. The changes in molecular weight of the subunits of dinitrogenase reductase are referred to here as a "modified" form although the molecular basis for the

change in molecular weight is not known. Diel samples in the Caribbean Sea showed a diel shift from the active unmodified form in the daytime to a modified form at night (Figures 17, 18). Both of the identical subunits of dinitrogenase reductase became modified at night. An interesting observation in the diel studies for this population was that dinitrogenase reductase was present early in the morning at 0400 hours in the modified form (Figure 17). The population of *Trichodesmium* in this study appear to undergo reversible modification of dinitrogenase reductase without degradation of the enzyme. In an earlier report Capone et al. (1990) concluded that modification of dinitrogenase reductase in *Trichodesmium* is a product of *de novo* synthesis and degradation. Du and Gallon (1993) found this process also in *Gleotheca*. Zehr et al. (1993) concluded that in natural populations of *Trichodesmium* a diel cycle of synthesis and degradation accompanies the modification of the Fe protein. However, in cultures of *Trichodesmium*, Ohki et al. (1992) found the Fe protein present over a 24 hour period. In studies with *Anabaena*, Ernst et al. (1990a) found that reversible modification is more significant than *de novo* synthesis. It is proposed in this thesis, at least for the Caribbean population studied in this thesis, that reversible modification occurs in *Trichodesmium*. It is much more efficient to cap the unused enzyme and protect it from oxygen by "modifying" when not in use and simply lop off the modifier and have a ready made active enzyme.

Caribbean Sea populations of *Trichodesmium* incubated with dust, and in most cases soluble iron, resulted in the expression of

both forms of the subunits of the iron protein even after nitrogen fixation ceases and the control (no Fe) is in the inactive modified form (Figures 17, 18). This means that either half of the colonies have modified iron protein or the colonies have only one of the identical subunits modified. Under the influence of oxygen only, *Anabaena* converts both subunits to the modified form but, in the presence of ammonium chloride, modified and non-modified forms are found in equal amounts (Reich and Boger 1989). Possibly, nutrients may cause a different response in the modification of dinitrogenase reductase. Since oxygen irreversibly destroys nitrogenase, modification of dinitrogenase reductase is believed to be an oxygen protection mechanism. It may be possible that this mechanism works in a different way when exposed to different nutrient regimes. This may represent an ancient adaptation that evolved when the presence of oxygen became a problem for diazotrophs and now this system is used as a regulating system for nitrogen fixation secondarily. An example similar to this would be the peptides metallothionein in animals and phytochelatin in plants that function as intercellular chelators that originally sequestered trace metals and maintained metal homeostasis for nutritional purposes but with the increase of heavy metals in the environment due to pollution are also used to detoxify (Grill et al. 1987).

*Trichodesmium* colonies from the Sargasso Sea however did not show a diel change in subunits of the iron protein of nitrogenase (Figure 19). Instead, both forms of the iron protein in a 1:1 distribution were present at all times in the puff form colonies. Tuft

form colonies did not show a strong signal for nitrogenase (Figures 21, 22). The dominate colony form in the Sargasso Sea in Spring was the puff forms. During summer however, tufts become the dominate form. It appears that the two colonial morphologies of *Trichodesmium* have different physiologies. This is quite interesting since all of the Caribbean colonies used in experiments were tuft forms.

#### Hydrographical Influence on Dinitrogenase Reductase in *Trichodesmium* in the Sargasso and Caribbean Seas

The Caribbean Sea is a permanently stratified oligotrophic ocean as shown in a CTD profile from February 1991 (Figure 26). The Sargasso Sea undergoes strong seasonal patterns of production, biomass and sinking flux (Menzel and Ryther 1960, Deuser 1986, Lohrenz et al. 1992, Michaels et al. 1994). The spring is characterized by a phytoplankton bloom that is a result of convective mixing in the winter which injects nutrients from deeper water to the surface layers. In April 1991, nitrate and nitrite were present in the euphotic zone as a result of recent mixing (Figure 23). This source of inorganic nitrogen may have affected the iron protein of *Trichodesmium* resulting in the expression of both forms of the subunits during the day instead of only the active, unmodified form. Goering et al. (1966) showed by using  $^{15}\text{N}$  uptake *Trichodesmium* in the Atlantic Ocean to fix nitrogen and take up nitrate simultaneously. However, Carpenter and McCarthy (1975) found no significant uptake in  $^{15}\text{N}$ -nitrate. In the Carpenter and McCarthy (1975) study,

*Trichodesmium* from the oligotrophic Sargasso Sea were incubated in nitrate for only 2 hours in seawater from the most oligotrophic station. The Goering et al. (1966) study assayed colonies that were near the Amazon River plume and were influenced by nitrate from the river. Studies with nitrate reductase in phytoplankton have shown that the enzyme is induced by nitrate and nitrate reductase is present only if water contained nitrate (Eppley et al. 1969).

*Trichodesmium* from the Sargasso Sea in April 1991 in this study could have been induced because of the presence of nitrate in the water column. As the nitrate decreased because of uptake by phytoplankton, *Trichodesmium* switched to nitrogen fixation as a source of nitrogen. Figure 22 shows that at this time changes occurred in the iron protein. Studies in the regulation of nitrogen fixation in cultures of *Trichodesmium* found that inorganic nitrogen repressed nitrogenase activity and only the single modified (inactive) form of the iron protein appeared in Western blots (Ohki et al. 1991). However, the cultures had both forms of the enzyme when actively fixing nitrogen. *Anabaena* was observed to have both forms of the iron protein in the presence of ammonia (Reich and Boger 1989, Ernst et al. 1990b) and Smith et al. (1987) showed that when both forms of the iron protein were present in *Anabaena*, it did not always result in a decrease in acetylene reduction.

The temperature of the Sargasso Sea surface water in April and early May 1991 was 21°C which is approaching the thermal minimum for physiologically active colonies. Mainly puff forms were found and tuft forms were rare. Actively growing populations rarely



occur in water colder than 20°C (Carpenter 1983b). Maximum photosynthetic activity occurs at 30°C with a sharp decline below 20°C and above 35°C (Carpenter and Capone 1983).

#### Acetylene Reduction Assay and Iron Additions

Acetylene reduction (AR) rates with iron additions showed no significant stimulatory effect (Tables II, III). *Trichodesmium* colonies that showed slightly higher average rates in April 1990 also had high cellular iron. If these colonies were pre-exposed to iron *in situ*, perhaps they have synthesized specific iron uptake porter proteins and are able to take up iron in a relatively short time. Colonies incubated in iron in February 1991 had slightly lower average AR rates and lower cellular iron. These colonies may not be able to take up iron in short term incubations. However, this is only speculative since the changes in AR rates with iron additions are not significantly different from control rates. Interestingly, the highest overall AR rates were during February 1991 when cellular iron was low.

Similar results for nitrogen fixation rates for *Trichodesmium* at  $10^{-8}$  M Fe were presented by Rueter (1988). Although his study showed no stimulation in AR rates with  $10^{-8}$  M Fe, he showed that stimulation of nitrogen fixation occurred at  $10^{-6}$  M Fe after incubation in iron for two days. Rueter et al. (1990) demonstrated stimulation of nitrogen fixation in cultures of *Trichodesmium* incubated in iron from  $10^{-9}$ ,  $10^{-8}$  and  $10^{-7}$  M Fe after 15 days. Nitrogen fixation was stimulated in the cultures after several days

following an increase in chlorophyll-*a*. The result being light-dependent nitrogen fixation would be stimulated only after the enhancement of photosynthetic pigments. Rueter et al. (1990) considered the possibility that *Trichodesmium* may respond to varying amounts of iron in the environment with small physiological changes. In this way *Trichodesmium* is able to optimize iron usage.

The longest period of time colonies were incubated in iron for acetylene reduction in this thesis was 20 hours. Colonies were incubated in January 1992 in FeCl<sub>3</sub> ( $1.8 \times 10^{-7}$ ), dust (20 mg in 200 ml FSW) and FeEDTA ( $10^{-7}$  M) (TABLE III). The results did not show any stimulation of nitrogen fixation. This experiment was repeated with blue water dive collected colonies with the exception of EDTA ( $10^{-6}$ ) replacing FeEDTA but with similar results (TABLE III). This may not be long enough to get a physiological response of increased nitrogen fixation rates with iron additions. However, Paerl et al. (1994) demonstrated stimulation of nitrogen fixation with  $1 \mu\text{M}$  Fe as FeEDTA in "short term" (24-48 hours) and "long term" (5-11 days) incubations. Ambient dissolved iron concentrations in the N. Atlantic Ocean are approximately between 0.2 and 0.6 nM in the euphotic zone (Powell et al. 1994). Natural populations of *Trichodesmium* colonies are never exposed to iron concentrations as high as  $1 \mu\text{M}$ . Perhaps high iron concentrations stimulate other iron uptake systems that are normally secondary or induce synthesis of iron uptake proteins. Only the dust concentrations in this thesis were  $\mu\text{M}$  ( $4.5 \mu\text{M}$ ). Stimulation of nitrogen fixation did not occur and in most cases, average AR rates were slightly lower than control (no Fe).

Another problem with the incubations in this thesis are bottle effects. *Trichodesmium* colonies that were incubated overnight in no Fe were compared to colonies freshly collected the next morning. Incubated controls had lower AR rates indicating effects from being contained in the flasks. Lack of stimulation of nitrogen fixation with iron additions could be due to the fact that colonies could not be kept in good enough condition long enough to observe a change in nitrogenase activity.

Possibly what is more significant in these experiments are the shorter term incubations where iron was added directly to the acetylene reduction vial or incubated 1-2 hours (TABLE II). Since colonies can have protein-N to iron ratios approaching 3000 and the colonies can actively fix nitrogen on low cellular iron, this develops an hypothesis that the colonies may not be iron-limited. Perhaps, iron additions added to colonies may not be needed during the nitrogen fixation phase of that day. The extra iron might be taken up without an instantaneous change in physiology but with changes in proteins. Evidence for this is seen in the Western blots for the iron protein of nitrogenase (Figure 18). Repeatedly in the Caribbean Sea populations, colonies incubated in iron or dust had both forms of the iron protein when the control did not. This may be a precursor to higher nitrogenase activity in the future. Iron additions also induced changes in unknown proteins with SDS-PAGE (Figure 12) which is similar to changes in the iron protein.

## Conclusion

*Trichodesmium* protein and iron content can vary over diel time scales and within populations and appears to be related to the growth phase of that population. Higher protein-N to iron ratios exist for *Trichodesmium* than previously thought indicating that this organism is adapted to low cellular iron levels. This is supported by a lack of stimulation of nitrogen fixation rates with near ambient iron concentrations. Changes in the iron protein of nitrogenase and an unknown protein(s) were observed with iron additions that may be a more subtle response to iron nutrition. Diel studies of the changes in the iron protein of nitrogenase offer evidence that natural populations of *Trichodesmium* in the Caribbean Sea have reversible modification of the iron protein. *Trichodesmium* colonies in the cooler Sargasso Sea in spring do not show a diel change in subunits of the iron protein of nitrogenase when nitrate is present in the water column. These environmental conditions alter the colony morphology and physiology of *Trichodesmium* colonies in this seasonal environment.

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## APPENDIX I

0.5 Ionic strenght buffer

(Tris-HCL buffer)

0.1 M NaCl, 0.01 M MgCl<sub>2</sub>, 0.01 M Ca Cl<sub>2</sub>, 50 ml. of 1 M HCl, titrated to pH 7.5 with 1 M Tris

Attempt 1

Thirty to fifty colonies were placed into 50 µl of 0.05 I-buffer and 44 colonies were placed on a 0.45 µm Millipore filter (HA) attached to a 12 cc syringe fitted with a Millipore Swinex-13 filter rig clamped vertically on a ring stand (Figure ). Gentle suction was employed to rinse off the sample on the filter with 100 ul of 0.05 I-buffer to reduce saltwater effects on matrix of gel. The sample was then scraped off the filter with a pipette tip and placed in 50 µl of 0.05 I-buffer. SDS sample buffer (0.0625 M tris-OH, 2% SDS, 10% glycerin, 0.001 M EDTA, and 0.01% bromophenyl blue) was made fresh from 2 X stock and prepared immediately before use by adding 0.8 ml of phenylmethlysulfonyl fluoride (PMSF) stock solution (3 mg PMSF/0.5 ml ethanol made immediately prior to use and kept on ice) and 50 mM dithiothreitol. Immediately following the preparation of sample buffer, 50 µl of prepared sample buffer was added to all samples. Samples were ground with a pipette tip and boiled for 1.5 minutes to denature the protein and then centrifuged at 6,000 g for 2 minutes. The samples were stored at -4 °C and run on a 2 14% SDS gels at 200 V for 30 minutes. All samples showed smearing but the



sample placed on the Millipore filter prior to buffer addition showed less smearing. High molecular weight bands were detected.

### Attempt 2:

Colonies to SDS buffer ratio was 1:1 (40 colonies to 40 ul SDS sample buffer) and 1:2 (20 colonies to 40 ul SDS sample buffer). One sample from each condition was flash frozen in liquid nitrogen for 2 min. prior to solubilization. The loading volumes were similar to the first attempt, 10 and 15 ul, on a 14% gel run at 200 V for 30 min ( 100 V per gel) but there was no improvement observed. In an attempt to isolate the reason for the poor resolution, samples were run that were prepared using a different SDS sample buffer (Zehr 1990). There was no appreciable improvement in resolution in these gels.

The gels were smearing in the low molecular weight range (including the molecular weight standard) and it was thought that this might be due to heat. The previous gels were run at 100 V per gel (200 v per two gels) so the voltage was decreased to 90 V. The samples in this run were diluted 1:4 (20 colonies:80 ul buffer) as this combination gave good results. There was still no appreciable improvement.

Solutions for 12 % SDS PAGE stock solutions:

### Resolving gel

2.94 ml acrylamide 30%, 0.94 ml resolving buffer , 1.11 ml dH<sub>2</sub>O, 75 µl SDS 10%, 0.94 ml bis 2%, 1.5 ml glycerol 50%  
45 µl APS 10%, 4.5 µl TEMED

Stacking gel

0.67 ml acrylamide 30%, 1.0 ml stacking buffer, 1.95 ml dH<sub>2</sub>O, 40 µl SDS 10%, 0.29 ml bis 2%, 33.3 µl APS 10%, 3.33 µl TEMED

Resolving buffer

36.3 g Tris pH 8 with approximately 48 ml 1.0 M HCl to final volume of 100 ml

Stacking buffer

60 g Tris pH 6.8 with approximately 48 ml of 1.0 M HCl to final volume of 100 ml

Protein Solubilization Buffer (SDS sample buffer)

1% SDS, 50 mM Tris, 5% beta-mercaptoethanol, 2 mM PMSF, 1 mM EDTA, 10% glycerol, 0.2 mg./mL. bromophenol blue

Electrode Buffer 5x

7.5 g Tris-OH, 36 g glycine, 2.5 g SDS to final volume of 500 ml

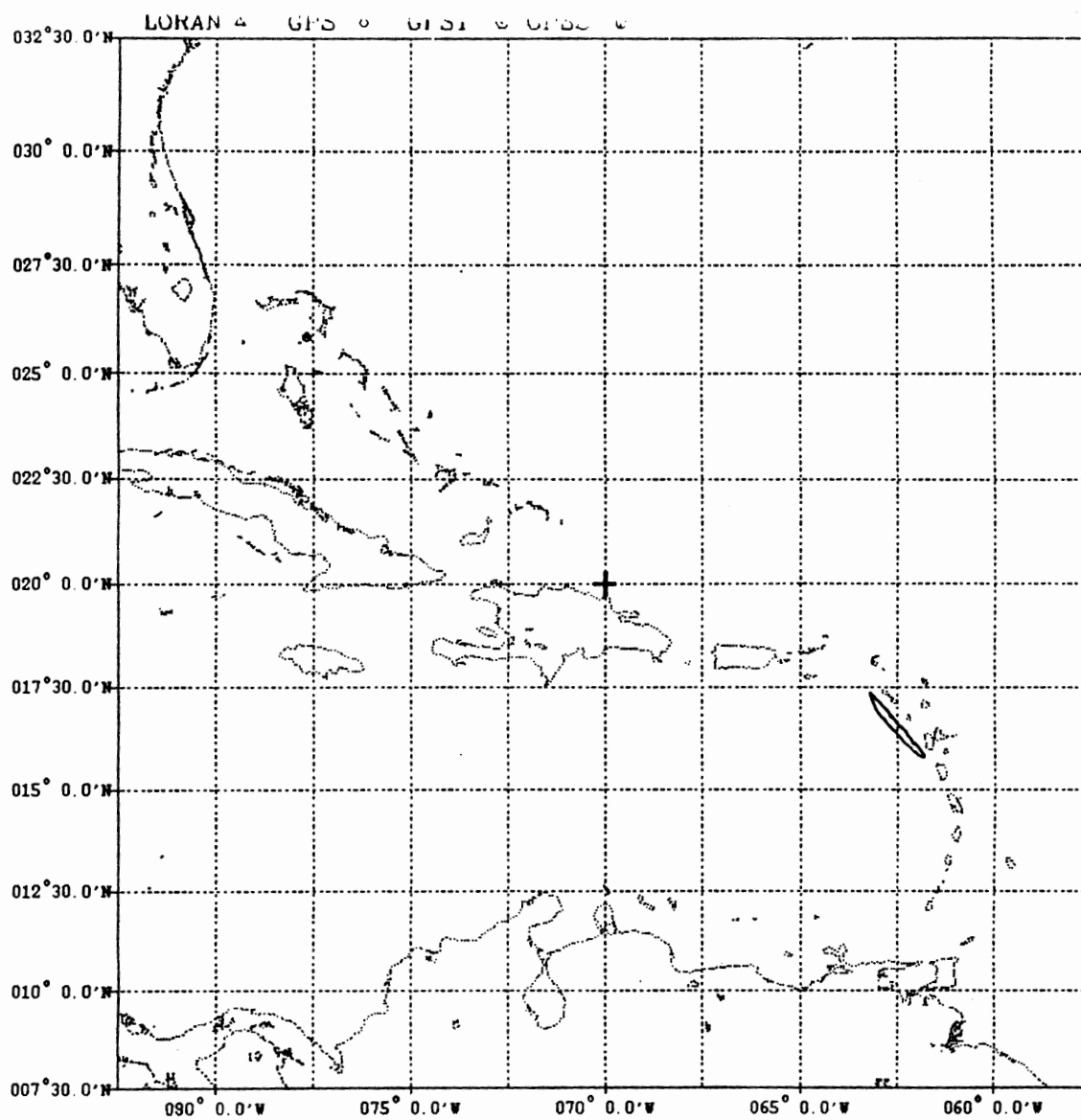
Coomassie Stain

25% isopropyl alcohol, 10% acetic acid and .025% coomassie blue R-250

Destaining Solution

10% acetic acid

## Appendix II



Location of cruise track (inside circle) in the Carriibbean Sea.