The Combined Relationship of Temperature and Molybdenum Concentration to Nitrogen Fixation By *Anabaena Cylindrica*

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Abstract. The joint effects of growth temperature, incubation temperature, and molybdenum concentration on the nitrogen fixation rate of *Anabaena cylindrica* were determined using the acetylene-reduction technique. The nitrogen-fixation response to increased molybdenum concentration varied among three growth temperatures (15°, 23°, and 30° C). The pattern of rate change was similar within a growth temperature but increased overall in magnitude with the three incubation temperatures (also 15°, 23°, and 30° C). The maximum rate of nitrogen fixation occurred at 30°C regardless of previous growth temperature. The minimum molybdenum concentration necessary to yield substantial acetylene reduction varied with growth temperature: at 15°C, 15 μg l^{-1} was effective; at 23°C, less than 5 μg l^{-1} was effective; and at 30°C, 50 μg l^{-1} was effective. At all three growth temperatures, increases in molybdenum concentration above the minimum effective concentration produced increases in acetylene reduction. However, at higher molybdenum concentrations inhibition of nitrogen fixation occurred.

Introduction

Nitrogen fixation by blue-green algae is a major source of nitrogen to many aquatic ecosystems. Knowledge of factors regulating this process is essential to any consideration of eutrophication. "Blue-green algae are becoming more conspicuous in this age of increasing environmental pollution. In nutrient enriched water, blooms of planktonic blue-green algae are more frequent, denser, and longer lasting. Similarly, thermal pollution from the coolant of power plants has, as one of its most striking effects, the promotion of blue-green algal growth" [6]. Interactions of nutrient dynamics with temperature have not been well documented. That temperature affects the state of matter in water is well established, as is the effect of temperature on various physiological processes. The question arises, to what extent do temperature and an ambient nutrient concentration interact to determine the rate of a metabolic process? In this study, we sought

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to answer this question for molybdenum concentration and the process of nitrogen fixation. When this interaction is known, we may be better able to answer the practical question "can thermal enrichment indirectly produce aquatic ecosystem nitrogen fertilization through the promotion of nitrogen fixation by the heat-associated blue-green algae?"

Molybdenum is a requirement for nitrogen fixation by blue-green algae [3]. Weathering provides the primary natural sources of molybdenum at concentrations posing no detrimental effects. Artificial sources are primarily from mine drainage [19] and produce a range of concentrations which may affect the state of blue-green algae in natural systems. Extensive industrial use of molybdenum poses another potential source of artificial input into natural systems.

Our objectives were to: (1) determine the effects of temperature on the rate of nitrogen fixation, (2) determine the effects of different molybdenum concentrations on the rate of nitrogen fixation, (3) determine if a synergistic nitrogen fixation rate response exists between temperature and molybdenum concentrations, and (4) determine if a predictable pattern may be observed with respect to the two dependent variables.

The indirect measurement of nitrogen fixation by acetylene reduction is widely used. Nitrogen-fixing organisms, in addition to reducing nitrogen gas to ammonia, are capable of reducing acetylene to ethylene. Numerous investigators have quantified studies using the acetylene reduction technique and have concluded there is a direct relationship to nitrogen fixation [13, 20, 23]. We thus consider ethylene production as analogous to nitrogen fixation.

Our choice of the blue-green alga, *Anabaena cylindrica*, was based on the fact that members of this genus are often common components of nuisance algal blooms and are well documented as capable of nitrogen-fixation.

Methods

Culture Methods A bacteria-free culture of *Anabaena cylindrica* was obtained from the culture collection, Department of Plant Sciences, Indiana University. The culture medium of Allen and Arnon [1], without combined nitrogen, was chosen because of its capacity to promote rapid growth and its extensive micronutrient content. It was prepared, without molybdenum, using double (glass) distilled water. The Dithiol photometric extraction technique was used to determine molybdenum concentration [5]. Molybdenum from a stock solution was added to separate batches of media to establish final concentrations of 150, 100, 50, 15, and 5 $\mu g\ l^{-1}$. Accuracy of the final concentrations was confirmed by the Dithiol technique. Because the oxide of molybdenum is the most abundant natural form in aquatic systems and its wide use in previous studies, MoO_2 was used in the culture medium.

Erlenmyer flasks (500 ml) were used as batch culture vessels. One-hundred milliliters of freshly prepared media (pH 7.8) was placed in each vessel which was then stoppered with cotton around an aeration tube and autoclaved. An algal inoculum was placed in a vessel with

molybdenum-free medium and grown for 7 days to deplete cellularly stored molybdenum. Subcultures were made into vessels containing 0, 5, 15, 50, 100, and 150 $\mu g\ l^{-1}$ added molybdenum. Cultures with no added molybdenum were analytically confirmed to contain less molybdenum than is detectable by the Dithiol technique ($1 \mu g\ l^{-1}$). Growth of each subculture was measured photometrically at 670 nm. The growth period for the final subcultures was determined by choosing a common optical density in the log phase. A 10-day growth period was established for cultures at 15°C, 6 days at 23°C, and 5 days at 30°C. The final subcultures were made by inoculating a 1 ml algal suspension into the respective molybdenum concentration from each of the six vessels of the first subculture. Replicate cultures were grown at 15°, 23°, and 30°C for each molybdenum concentration.

Growth conditions of each vessel were matched as closely as possible at a light intensity of 450 f.c. A 13-hour light-11 dark cycle was used. Each vessel was constantly agitated at 88 oscillations min^{-1} [10]. Air was passed over the medium in each vessel from a constant dispersion apparatus to maintain saturation conditions similar to surface waters of most lakes and reservoirs.

Acetylene Reduction. Forty-milliliter glass serum bottles, fitted with rubber serum stoppers, were used for the acetylene reduction experiments. A 5-ml algal suspension from each culture vessel was added to each serum bottle containing 15 ml of fresh sterile medium of the same molybdenum concentration. Samples were taken from each replicate growth flask and incubated in triplicate at 15°, 23°, and 30°C. Serum bottles containing 20 ml of sterile medium provided controls. The atmosphere of each serum bottle, after stoppering, was evacuated at 15 psi and filled with argon. The evacuation-argon filling process was repeated three times to assure complete nitrogen removal.

Each serum bottle was placed in a constant temperature bath at the incubation temperature and maintained for 30 min to eliminate any lag in the nitrogenase system resulting from exposure to the argon environment [18]. Two-milliliters of argon were removed from each incubation bottle by syringe and 2 ml of acetylene injected to establish a 10% acetylene environment. Each serum bottle was transferred to the incubation chamber and shaken for 30 min under constant light intensity. Acetylene reduction was terminated by injection of 0.5 ml of 50% trichloroacetic acid (TCA). TCA injection into the controls monitered possible abiotic production of ethylene. The gas phase of each vial was analyzed using a Beckman GC 65 Gas Chromatograph with flame ionization detector.

After gas phase analysis the dry weight of the alga was determined. Aliquots from each culture were evaporated and dried at 105°C for 24 hr. Dry weight of algal cells was determined to the nearest milligram and used to express nitrogen fixation as micromoles ethylene per milligram dry weight per minute. When necessary for comparative purposes, a relationship of 0.5 mg protein/1.0 mg dry weight was assumed.

Data Analysis Replicate values of incubation flasks were pooled and a split plot factorial analysis of variance and F test were used to determine significant interactions among treatments (molybdenum concentration, growth temperatures, and incubation temperatures). Significant interactions among treatments were further analyzed to determine simple main effects of each treatment comprising the significant interactions. The F test was used to determine if significant differences within treatments existed and an overall trend analysis of significant treatments was carried out using orthogonal polynomials [17]. All statistical analyses were performed at the $P<0.05$ level.

Results and Discussion

Nitrogen fixation measured as ethylene production significantly varied with molybdenum concentration, growth temperature, and incubation temperature. As a result, the data were anlayzed for simple main effects by maintaining two factors constant while varying the third.

Nitrogen fixation by *Anabaena cylindrica* has been reported to range from 1.0 to 8 n*M* ethylene mg protein^{-1} min^{-1} [18, 21, 22]. Our maximum rate was 7.9 n*M* ethylene mg protein^{-1} min^{-1}, and the minimum rate was 0.04 n*M*. The minimum rate was measured in media without added molybdenum. The occurrence of any nitrogen fixation in the absence of added molybdenum indicates that either the organism is capable of using submicrogram quantities, or substituting alternate cations, such as vanadium [3].

Temperature Response. The effect of temperature on metabolic processes is well established and follows a general pattern of increasing metabolic rate with increasing temperature. Our data followed this general pattern. At a constant growth temperature, cultures of *Anabaena cylindrica* increased ethylene production with increasing incubation temperature for most molybdenum concentrations (Table 1).

The nitrogen-fixation temperature coefficients (Q_{10}) for the temperature span of 15°-30°C ranged from 1.03 to 1.86 (Table 2). These coefficients were generally higher at greater molybdenum concentrations. This relationship be-

Table 1. *Mean (± s) Ethylene Production (n*M *mg^{-1} min^{-1}) by* Anabaena cylindrica *as a Function of Growth Temperature (*n*=6), and the Molybdenum Concentration Present throughout Growth and Incubation*

Growth temp.	Molybdenum (μg^{-1})	Incubation Temp. 15°C	23°C	30°C
15°C	0	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.02
	5	0.09 ± 0.07	0.09 ± 0.09	0.15 ± 0.12
	15	1.21 ± 0.36	1.32 ± 0.47	1.60 ± 0.51
	50	1.49 ± 0.20	2.02 ± 0.20	2.47 ± 0.25
	100	1.36 ± 0.48	1.25 ± 0.51	1.41 ± 0.57
	150	0.51 ± 0.24	0.79 ± 0.22	0.84 ± 0.14
23°C	0	0.64 ± 0.22	0.97 ± 0.19	0.96 ± 0.71
	5	2.12 ± 0.33	2.44 ± 0.26	3.41 ± 0.38
	15	2.31 ± 0.56	2.87 ± 0.57	3.94 ± 0.75
	50	1.96 ± 0.17	3.02 ± 0.07	3.64 ± 0.23
	100	0.94 ± 0.17	1.37 ± 0.15	1.76 ± 0.40
	150	1.04 ± 0.14	1.21 ± 0.12	2.07 ± 0.71
30°C	0	0.27 ± 0.02	0.40 ± 0.04	0.43 ± 0.06
	5	0.27 ± 0.09	0.41 ± 0.13	0.58 ± 0.21
	15	0.22 ± 0.05	0.34 ± 0.05	0.42 ± 0.07
	50	1.38 ± 0.35	2.41 ± 0.58	3.29 ± 0.83
	100	1.79 ± 0.66	1.86 ± 0.70	3.84 ± 0.86
	150	1.45 ± 0.43	2.50 ± 0.61	3.65 ± 0.39

Table 2. *Van't Hoff Temperature Coefficients (Q_{10}) for Nitrogen-Fixation Rate by* Anabaena cylindrica *at Three Growth Temperatures*

Molybdenum conc. (μg^{-1})	Growth Temp. 15°C	23°C	30°C
0	1.21	1.32	1.37
5	1.38	1.38	1.69
15	1.20	1.43	1.54
50	1.40	1.51	1.79
100	1.03	1.53	1.67
150	1.40	1.59	1.86

tween molybdenum concentrations and the magnitude of Q_{10} suggests that molybdenum affects the response of the nitrogenase system to short-term temperature change.

Cultures grown at 30°C had the greatest temperature coefficient values. This was expected since 30°C is the upper limit for growth for *Anabaena cylindrica*. In preliminary work, we found that cultures at 35°C had limited growth. Similar results were obtained by Fogg and Than Tun [10], also with *Anabaena cylindrica*. It is reasoned that cultures grown at 23°C and incubated at 30°C may show suppression, whereas cultures grown at 30°C and incubated at 30°C would not.

Temperature coefficients for nitrogen fixation reportedly range from 2 to 6. Burk, as cited by Fogg and Than Tun [10], reported a Q_{10} of approximately 2 in *Azotobacter*. Goering and Neess [11] and later Horne and Fogg [15] reported *in situ* values of approximately 3. In the Antarctic, Fogg and Stewart [9] found Q_{10} values as high as 6 for *Nostoc* during the short yearly growth period. The highest Q_{10} values for *in situ* nitrogen fixation may be a result of an enhancement of the nitrogenase system normally supressed in *in situ* systems that respond prolifically to the favorable conditions that develop during early bloom situations in temperate lakes or the short growing period in the Antarctic. Laboratory cultures are under more steady-state conditions and the nitrogenase system may be more stable to short-term temperature fluctuations. Consequently, lower Q_{10} values may be expected under laboratory conditions.

Molybdenum Concentration Response. The nitrogen fixation response to molybdenum concentration among growth temperatures generally followed a pattern of initial limitation at low molybdenum concentrations, followed by a region of maximum ethylene production, and finally a region of declining ethylene production at higher molybdenum concentrations. These results are typical of sys-

tems where a region of optimum concentration is bounded by regions of limitation and inhibition (Table 1).

Molybdenum as a cofactor for the nitrogenase complex is essential to the fixation of nitrogen. Our results indicate that the critical or minimum molybdenum concentration for maximum ethylene production varies between 0 and 50 μg l^{-1}, depending on growth temperature (Fig. 1-3). It is probable that almost no nitrogen fixation occurs when molybdenum is absent. Eyster [7] indicated that the critical minimum concentration of molybdenum for nitrogen fixation in *Nostoc* was 10 μg l^{-1}. Wolfe [24] indicated that healthy growth of *Anabaena cylindrica* occurred at 200 μg l^{-1} Mo with N_2 as the sole nitrogen source. This inference that concentrations of 200 μg l^{-1} are required for nitrogen fixation is in disagreement with our results. Our data agree with Eyster in that the critical minimum concentration for nitrogen fixation is below 50 μg l^{-1}. The comparison of molybdenum requirements for different species of nitrogen-fixing blue-green algae assumes that common systems and components are utilized by each species. The nitrogenase enzyme complex has been observed as a common component of nitrogen-fixing species of blue-green algae, and molybdenum is a common cofactor of this nitrogenase system.

At each growth temperature, cultures had a decline in nitrogen fixation at higher molybdenum concentrations. Fogg [8] reported that the concentrations of molybdenum supporting nitrogen fixation are inhibitory to phosphatase activity in *Anabaena cylindrica*. The decreases observed in ethylene production at higher molybdenum concentrations may thus be the result of either direct inhibition of the nitrogenase complex by excessive molybdenum or indirect inhibition of the nitrogenase complex resulting from the direct inhibition of an interrelated system such as phosphatase.

Molybdenum and Temperature Interactions. The nitrogen-fixing responses of *Anabaena cylindrica* to varying molybdenum concentrations (0-150 μg l^{-1}) and incubation temperatures (15°, 23°, and 30°C) for each growth temperature (also 15°, 23°, and 30°C) are described in Figs. 1-3. The overall response patterns among cultures were very similar. However, close comparison among the three growth temperatures reveals some substantial differences. The critical minimum molybdenum concentration varied with growth temperature. In cultures grown at 15°C, ethylene production increased significantly between molybdenum concentrations of 5 and 15 μg l^{-1} and optimum rates extended over a range of 15 to 50 μg l^{-1}. Cultures grown at 23°C had substantial ethylene production at submicrogram Mo concentrations, with optimum rates extending over a range of 5 to 50 μg l^{-1} Mo. At the 30°C growth temperature, ethylene production achived maximum rates between 100 and 150 μg l^{-1} and the optimum range extended from 50 to 150 μg l^{-1}. Trends observed were consistent within growth temperatures and suggest that these data are indicative of the real patterns. Individual differences in the optimum molybdenum ranges were insignificant.

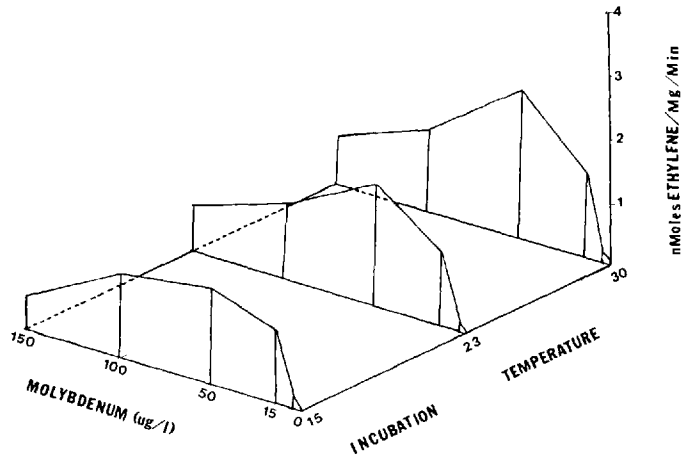

Fig. 1. Nitrogen-fixation response as ethylene production, by *Anabaena cylindrica* grown at 15°C to different incubation temperatures and molybdenum concentrations.

The initial decline in ethylene production observed at higher molybdenum concentrations occurred at different molybdenum concentrations for different growth temperatures. Cultures grown at 15°C exhibited a gradual decline from 50 to 150 μg l^{-1}. Cultures grown at 23°C produced a decline in ethylene production after 50 μg l^{-1}. With further study, we determined that the decline in ethylene production for cultures grown at 30°C occurred after 150 μg l^{-1} for cultures incubated at both 23° and 30°C, whereas it occurred between 100 and 150 μg l^{-1} for cultures incubated at 15°C.

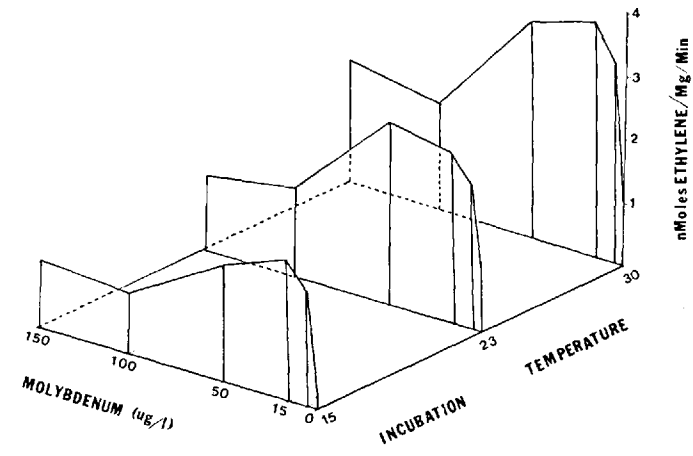

Fig. 2. Nitrogen-fixation response as ethylene production, by *Anabaena cylindrica* grown at 23°C to different incubation temperatures and molybdenum concentrations.

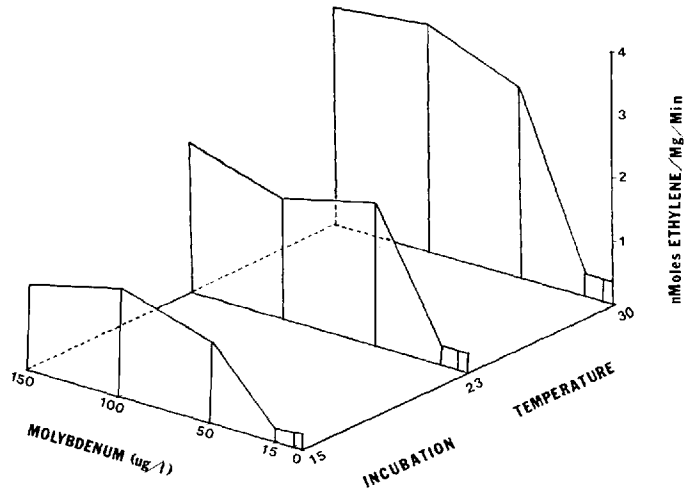

Fig. 3. Nitrogen-fixation response as ethylene production, by *Anabaena cylindrica* grown at 30°C to different incubation temperatures and molybdenum concentrations.

Regardless of incubation temperature, the maximum ethylene production by *Anabaena cylindrica* occurred in cultures grown at 23°C and molybdenum concentrations of less than 100 μg l^{-1}. At 100 and 150 μg l^{-1} Mo, the maximum rates occurred at the highest growth temperature.

The fact that the critical minimum molybdenum concentration required to produce maximum ethylene production was generally lower at lower growth temperatures indicates a temperature-dependent relationship between nitrogen fixation and efficiency of molybdenum usage. Goldman's [12] primary production data for Castle Lake, California may also be interpreted on this basis. Although exact water temperatures were not reported, he observed increased rates of photosynthesis on addition of 100 μg l^{-1} Mo to cultures from Castle Lake in June, whereas, in October, 50 μg l^{-1} Mo yielded the highest photosynthetic rates. Early the following summer 5 μg l^{-1} Mo was more effective than higher concentrations. One may conclude that a number of different factors affected primary production in Castle Lake in response to molybdenum, i.e., different species utilizing molybdenum at various times of the year, variation in ambient molybdenum concentration, light intensity affecting photosynthesis, rainfall and turbidity, and nitrogen content; each may account for the variation observed. However a notable change in lake temperature would also be apparent at these times and may possible be correlated with molybdenum usage. Becacas [2] also observed differential rates of primary production on molybdenum addition at various times of the year in Lago Maggiore, Italy; a 12% increase in May and a 6% increase in July.

Enhanced nitrogen fixation at varying temperatures and molybdenum concentrations may be the result of a greater availability of the cation to the organisms by increased uptake efficiences. Laboratory studies [14] indicate the blue-green alga *Plectonema boryanum* concentrates different elements more efficiently at different temperatures. Cobalt uptake increased until 25°C then decreased through 40°C, magnesium uptake increased until 35°C and then decreased. Other cations (iron, strontium, zinc, and cesium) increased in concentrations up to 40°C.

Consequently, it appears that our temperature-molybdenum interaction data may be explained either as a result of increased availability of molybdenum through more efficient uptake, or an increased efficiency of utilization as indicated previously, or both.

Quantitatively, cultures grown at 23°C had the greatest ethylene production for most of the molybdenum concentrations used (Fig. 4). Each of the cultures was grown at its respective growth temperatures for two generations, thus acclimation was assumed to have occurred. The data for 23°C indicates that either the nitrogenase system is capable of utilizing the available molybdenum more effectively at that temperature, or an acclimated uptake system is making the cation more readily available, or both. A comparison of the range of maximum ethylene production in cultures grown at 23° and 30°C, indicated a broader range of maximum ethylene production at 30°C, although the maximum was slightly less. This accounts for the greater quantitative rates found at 100 and 150 μg l^{-1} Mo at 30°C. It is interesting to note that increasing temperature has not raised the maximum rate but did shift the range of molybdenum for cultures grown at 30°C to higher concentrations.

Patterns Involving Temperature-Molybdenum Effects. Similarities in response patterns occurred among cultures grown at constant temperatures and incubated at different temperatures (Figs. 1-3). At low concentrations of molybdenum, ethylene production was also low. Increasing the molybdenum concentration enhanced ethylene production to a maximum after which declines occurred. In acclimated systems, these data may be useful in determining the nitrogen-fixation response to brief variations in temperature and molybdenum concentrations.

For a given molybdenum concentration, responses of cultures grown at different temperatures but incubated at the same temperature were very similar (Fig. 4). In general, an apparent optimum growth temperature of 23°C consistently yielded the maximum rate of ethylene production at all molybdenum concentrations below 100 μg l^{-1}. The patterns at the two highest molybdenum concentrations were similar to each other and basically the inverse of those patterns at lower concentrations.

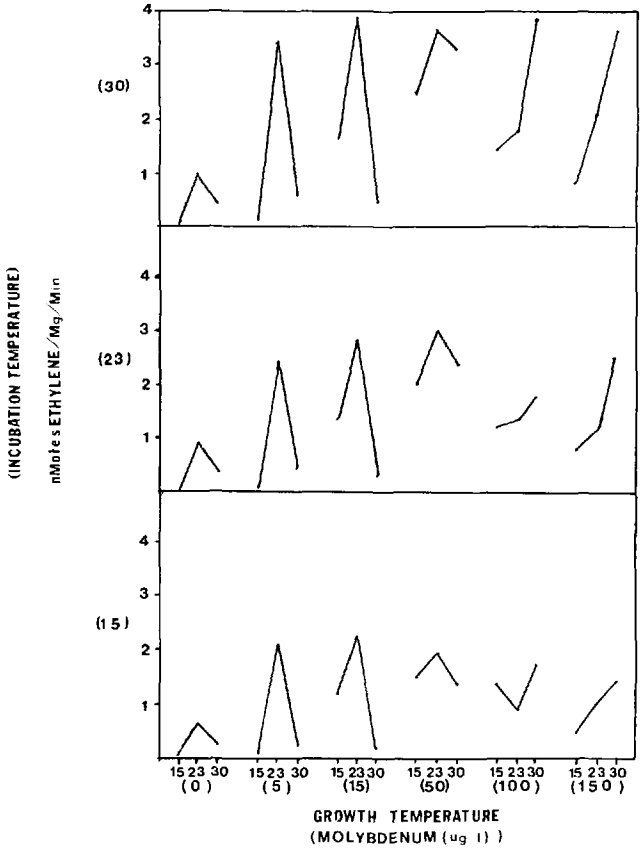

Fig. 4. The effect of culture growth temperature on the rate of nitrogen fixation by *Anabaena cylindrica* relative to molybdenum concentration for each of three incubation temperatures (15°, 23°, and 30°C).

The acute difference in these two patterns is indicative of two separate intracellular control systems. We postulate that some enzyme, isozyme, or similar system that is normally accentuated at the intermediate temperature only is inhibited by high molybdenum concentrations. At these concentrations, a second system, operating on a more fundamental thermodynamic or kinetic principle, becomes dominant at higher temperatures. The postulated regulatory system could be operational on either the membrane-uptake activity or on the nitrogenase activity.

It is possible that an acclimination period longer than two generations is required to override adaptations experienced in the culture collections at Indiana, and greater elucidation of patterns may be achieved by extended acclimation periods. However, cultures grown at 30°C (Fig. 3), when incubated at different

temperatures, did have similar patterns; so it is probable that acclimation had occurred.

Application of these data to systems subjected to thermal inputs suggest that at ambient molybdenum concentrations, nitrogen fixation may be thermally inhibited. Although little data exist on natural molybdenum concentrations, reported values are often less than 1 $\mu g\ l^{-1}$ [4, 16], but as high as 100 $\mu g\ l^{-1}$ [4]. Species acclimated to ambient temperatures are capable of utilizing low molybdenum concentrations more efficiently (Fig. 2). However, thermal inputs may alter responses of nitrogen-fixing species to critical minimum molybdenum concentrations, as in Fig. 3, and limit the occurrence of these species or their efficiency to fix nitrogen. Alternately, enhancement by thermal inputs might occur when ambient concentrations are within inhibitory ranges for acclimated species, or when molybdenum input occurs in conjunction with thermal increases. However, this latter result is unlikely since most ambient concentrations are low.

The extension of these data to the field encounters certain problems: Are metallic substitutes for molybdenum available to the nitrogenase complex that would alter the ethylene production response; are other physical-chemical factors, such as light or other nutrients, limiting in place of, or in addition to, molybdenum; and are there other synergystic responses that directly or indirectly affect nitrogen-fixation systems? Comparison of laboratory data with acetylene reduction field data had yielded good correlation between the two systems for various species of nitrogen-fixing blue-green algae [21, 22]. The response of *Anabaena cylindrica* may also extend to other genera of nitrogen-fixing blue-green algae since the nitrogenase system is characteristic of known nitrogen-fixing species.

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