AN EVALUATION OF THE ACETYLENE REDUCTION ASSAY USING *ANABAENA FLOS-AQUAE*

By

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ASSAY USING ANABAENA FLOS-AQUAE

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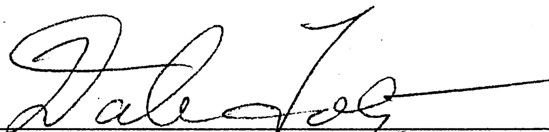

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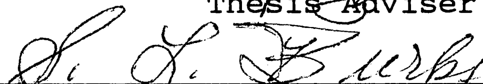

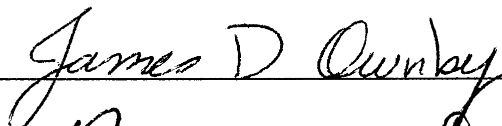

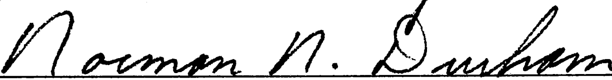

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NOMENCLATURE

Ar	argon
ARA	acetylene reduction activity
BNF	biological nitrogen fixation
C_2H_2	acetylene
CO_2	carbon dioxide
DDD	double distilled deionized
H_2	dihydrogen
H_2O	water
HCl	hydrochloric acid
ml	milliliter
N	Normal
OD	optical density as the (log 1/% transmittance)
SD	standard deviation
uE	microeinsteins

CHAPTER I

INTRODUCTION

Biological nitrogen fixation (BNF) has been studied extensively in the field and laboratory. Assay methods for BNF include the use of ^{15}N, H_2 evolution, the reduction of azide, cyanide, methylisocyanide and acetylene (Burris, 1972). Of these methods, the reduction of acetylene to ethylene is one of the easiest and most sensitive (Klucas, 1969). It has its basis in the fact that nitrogenase, the enzyme complex in the cell that reduces nitrogen to ammonium, also reduces acetylene to ethylene. It should be noted that the acetylene reduction assay serves as an indirect measure of BNF. The exact relationship between nitrogen fixation and acetylene reduction activity (ARA) may vary (Mayne, 1984).

The acetylene reduction assay has been applied in terrestrial and aquatic environments to better understand the BNF process on the community and molecular level using many taxa (Granhall and Lundgren, 1971; Wyatt and Silvey, 1969; Lyne and Stewart, 1973; Reed et al., 1980; Tesfai and Mallik, 1986). The acetylene reduction assay has been used to determine the effect of toxicants on BNF by nitrogen fixing cyanobacteria. Brookes et al. (1986), Horne and

Goldman (1974) and Wurtsbaugh and Apperson (1978), DaSilva et al. (1975) and Lundvist (1970) and Bastian (1981), used the acetylene reduction assay in this context with cyanobacteria in terrestrial communities, aquatic communities, and individual species, respectively. However, many such reports fail to deal with the experimental error of the assay.

This research sought to clarify causes of variability in the acetylene reduction assay. A review of papers using the acetylene reduction assay reveals much variation in protocol (Burris, 1972; Fay, 1980; Hardy et al., 1972; Klucas, 1969; Stewart et al., 1968). For example, some authors preflushed the headspace with a mixture of $Ar:O_2:CO_2$ while others do not.

Possible sources of error can be due to the mechanical procedures in the acetylene reduction assay or to the physiological condition of the culture. Some of these sources of error that could affect the outcome of the acetylene reduction assay were examined here. The objective of this research was to evaluate the ruggedness of mechanical aspects of the acetylene reduction assay protocol.

The method used to evaluate the ruggedness was a modification of the first phase of laboratory evaluation of the test protocol suggested by McKenzie and Olsson (1984). The experimental design used in ruggedness testing is a fractional replication of a factorial experiment. Fractional replication allows examination of the main effects the fac-

tors may have on the test result using a reduced number of experiments (Cochran and Cox, 1957). In the case of ruggedness testing, a 1/16 fractional factorial uses eight separate experiments to examine the main effects of seven different factors. A full factorial experiment would require 2^7 or 128 separate experiments to examine the effect of seven factors on a test result. An assumption necessary to the use of this design is that interaction between factors is negligible. The factors most likely to interact in this research are the factors involving measurement of volume. These were media volume, C_2H_2 volume and volume of boiling water injected. These may have interacted to affect gas pressure of ethylene within each experimental bottle. For the purposes of statistical analysis, I assumed that such interactions, if they occurred, had little effect. Ruggedness testing examines the effect of inducing small technical errors in protocol to learn their effects on the final test result. Analysis of ruggedness testing can identify items in the protocol where strict compliance to protocol is necessary. Identification of these steps can also suggest areas where quality assurance measures should be taken.

The ruggedness testing of McKenzie and Olsson (1984) was slightly modified in this research. For example, some induced technical errors in the protocol were actually deletions from the protocol. This modification allowed possible simplification of protocol and identification of

essential protocol steps. Another alteration of the method used was to test the ruggedness of the assay using <u>Anabaena flos-aquae</u> exposed and not exposed to a known toxicant, cadmium. This enabled the ruggedness of the acetylene reduction assay to be examined as a toxicity test and also simply as a measure of nitrogen fixation.

CHAPTER II

METHODS AND MATERIALS

Culture Conditions

Stock Cultures

Stock cultures were obtained from the Culture Collection at the University of Texas, Austin. Stock agar cultures of *Anabaena flos-aquae* (UTEX 1444) were kept at constant temperature and irradiance of 15°C and 70 uEinsteins/m^2/sec. Growth media used here and below was Allen's blue-green media (modified) (James, 1979).

Inoculum Cultures

A subculture was transferred axenically from the stock culture to 5-10 ml liquid media in a test tube. Cultures were incubated in continuous light (65 ± 5 uEinsteins/m^2/sec) and constant temperature (25 ± 1°C) for 3-5 days until green.

Batch Cultures

A nitrogen-free batch culture was started by pouring the 3-5 ml inoculum into 4000 ml autoclaved media minus $NaNO_3$. It was aerated with a mixture of 2% CO_2 in air prefiltered through a 0.20 um Millipore filter. The culture

was agitated with a stirring bar at a continuous irradiance of 65$\pm$5 uEinsteins/m^2/sec and constant temperature of 25$\pm$1°C.

Monitoring Batch Cultures

After 7 days of growth, the batch culture was examined to ensure it was axenic by withdrawing a sample and streaking a loop of this sample onto autoclaved Tryptic Soy Agar in a petri dish. Growth along the inoculum line after 2-4 weeks indicated a non-axenic culture.

Cell and optical density were monitored daily in triplicate throughout batch culture growth. Optical density was measured as percent transmission using a Bausch and Lomb Spectronic 20 colorimeter. The average of these values was converted into ($\log_2$(log(1/%Transmission))+10 for mean and standard deviation calculations (n=3). Cell density was determined by counting number of cells in a 20 x 20, 1 mm^2 grid of a hemocyometer and dividing the result by the volume, 1 x 10^{-4} ml. Eight counts were averaged for each sample.

Both cell and optical density were monitored until greatest acetylene reduction activity (ARA) was attained. Ruggedness tests were performed at this point (maximum ARA).

Samples Used to Test Ruggedness of the Acetylene Reduction Assay

Three types of samples were used in every ruggedness test; blank, control and experimental, all in triplicate and all in 70 ml Wheaton "400" serum bottles. Final volume was 50.0 ml. Blank samples contained 50.0 ml double distilled deionized (DDD) water plus 2.0 ml 4N HCl. Blank samples accounted for trace amounts of ethylene in the acetylene used and any abiotic production of ethylene. Control samples contained 50.0 ml of batch culture media at maximum ARA plus 2.0 ml 4N HCl. Control samples accounted for any ethylene production after termination of the assay.

There were two types of experimental samples. Experimental samples containing 50.0 ml batch culture media at maximum ARA not exposed to toxicant will be called ENTOX samples. ARA of ENTOX samples was measured in two hour incubations. Experimental samples containing 50.0 ml of media exposed to 2.084×10^{-4} moles Cd/l will be called ETOX samples. ETOX samples were exposed to cadmium for 96 hours. Then, the ARA was measured using a two hour incubation. Exposure to cadmium involved splitting the culture into two approximately equal volumes upon reaching maximum ARA. I then added 0.3 ml concentrated $CdCl_2$ to the ETOX volume and diluted the resulting solution by 5% with N-free media. Control volumes were also diluted 5% with N-free media. All cultures were incubated during testing at a

continuous irradiance of 65±5 uEinsteins/m^2/sec and constant temperature of 25±1°C.

Two types of ruggedness tests were performed. In three cases ruggedness was tested using cells that had reached maximum ARA (using ENTOX samples). Henceforth, these tests will be called non-poisoned ruggedness tests. Two tests were also performed on cells that had reached maximum ARA and were subsequently poisoned with cadmium as described above (using ETOX samples). Henceforth, these tests will be called poisoned ruggedness tests.

Acetylene Reduction Assay Protocol

The protocol used to determine ARA is a modification of the methods described by Hardy et al. (1973) and Turner and Gibson (1978). It is described below in some detail. The modifications of the protocol (induced technical errors) used to test the ruggedness of this protocol follow the protocol description.

The headspace of sample bottles containing 50.0 ml media was purged for 1.5 min. with a Ar/CO_2/O_2 gas mixture, the samples stoppered, 2.0 ml of gas withdrawn and 2.0 ml of commercial grade C_2H_2, which had been filtered through double distilled deionized water, was injected into the bottle. These were then shaken for 1.5 min. by hand to mix the gas phase with the media. The bottles incubated on a shaker table with 62±2 uEinsteins/m_2/sec irradiance and 25±1°C for two hours. Injection of 2.0 ml of 4N HCl was

used to terminate ARA in experimental samples. Then each sample bottle was sealed with silicon sealant for storage until ethylene quantification could take place using a Tracor 560 Gas Chromatograph (GC). All sample bottles of each ruggedness test were analyzed at the same time. GC conditions for all analyses were given in Appendix A.

Quantification of the ethylene present in each sample bottle required increasing headspace pressure by the injection of 5.0 ml boiling water into the serum bottle. Then all water was shaken from the stopper. Immediately prior to GC injection the syringe was thoroughly purged with headspace gas. The volume injected into the GC was 1.00 ml.

Standard curves for ethylene were made by injecting at least three volumes of ethylene standard gas (Matheson Gas Products) in triplicate to encompass the maximum and minimum responses elicited from sample injection. Linear regression of data yielded r-values no less than 0.95. Final calculated values are in moles of ethylene produced per heterocyst per hour and moles of ethylene produced per milliliter media per hour.

Induced Technical Errors in Protocol to Test Ruggedness

The first step in ruggedness testing is to identify the items in protocol where technical error is most likely to occur. The magnitudes of induced technical errors are

listed in the right hand column of Table I. Differences from the protocol were directed towards the most likely bias based on experience to date. For example, protocol in Table I calls for 2.0 ml acetylene injection into the headspace of sample bottles. Experience indicates that the negative pressure in the syringe before penetration of the septa sometimes forces the plunger into the barrel and the actual volume taken is less than 2.0 ml. The induced error was to inject 1.8 ml acetylene instead of 2.0 ml.

Although seven protocol steps were manipulated, testing of these induced technical errors involved eight experiments, each incorporating a different combination of the seven induced technical errors. An experiment is defined as following the previously stated protocol or a variation as stated in each row (Table II). Table II shows all completed experiments for each ruggedness test. Experiment 1 involved no induced differences from protocol.

Cadmium Analysis

Quantification of total cadmium for the samples in Tests 4 and 5 was performed for all samples on May 13, 1988 on a Perkin-Elmer 5000 Atomic Absorption Spectrometer using the graphite furnace technique. Instrument conditions and sample preparation were performed as prescribed by the EPA (1979). The final cadmium concentrations in the range finding experiment was not measured directly.

TABLE I

THE STEPS, PROTOCOL AND INDUCED TECHNICAL ERROR USED TO TEST RUGGEDNESS

Step	Protocol	Induced Technical Error
Volume of media used	50 ml	47 ml
Gas purge time (Velocity of gas just below breaking media tension)	1.5 min	No purge
C_2H_2 (commercial grade) inoculation	Filtered with pure water	Used unfiltered C_2H_2
C_2H_2 injection volume	2.0 ml	1.8 ml
ARA incubation conditions	Shaker table used	Did not use a shaker table
ARA incubation period	2.0 hr	2 hr 10 min
Volume of boiling H_2O injected prior to C_2H_4 quantification	5.0 ml	4.7 ml

TABLE II

EXPERIMENTAL DESIGN TO TEST THE RUGGEDNESS OF THE ACETYLENE REDUCTION ASSAY PROTOCOL

Exp. #	Volume Used	Media Purge Time	Filter C_2H_2	C_2H_2 Volume Injected	Shaker Table Use	Incubation Time	Water Volume Injected
1	50.0 ml	1.5 min	Yes	2.0 ml	Yes	2.0 hr	5.0 ml
2	50.0 ml	1.5 min	No	2.0 ml	No	2 hr 10 min	4.7 ml
3	50.0 ml	0.0 min	Yes	1.8 ml	Yes	2 hr 10 min	4.7 ml
4	50.0 ml	0.0 min	No	1.8 ml	No	2.0 hr	4.0 ml
5	47.0 ml	1.5 min	Yes	1.8 ml	No	2.0 hr	4.7 ml
6	47.0 ml	1.5 min	No	1.8 ml	Yes	2 hr 10 min	5.0 ml
7	47.0 ml	0.0 min	Yes	2.0 ml	No	2 hr 10 min	5.0 ml
8	47.0 ml	0.0 min	No	2.0 ml	Yes	2.0 hr	4.7 ml

CHAPTER III

RESULTS

The larger mean value of either sample blanks (n=3) or controls (n=3) was subtracted from the experimental values for each respective experiment as an initial step in calculations. Lack of detection of ethylene in any of the samples upon injection was interpreted as zero ARA.

Establishing Maximum ARA

To establish nitrogenase activity over batch culture growth, acetylene reduction activity was monitored every other day after cell density reached approximately 1×10^6 cells/ml. The temporal increment in growth typical of a batch culture was plotted using optical density measurements from Appendix B (Figure 1). Ethylene production per ml of the same culture showed an appreciable increase in ethylene production at 220 hours age (Figure 2). The time when ethylene production per ml media was maximum was chosen as the earliest time that ruggedness testing should begin (at an optical density of 6.13 $Log_2(OD)+10$, cell density of 2×10^6 cells/ml and approximately 220 hours of

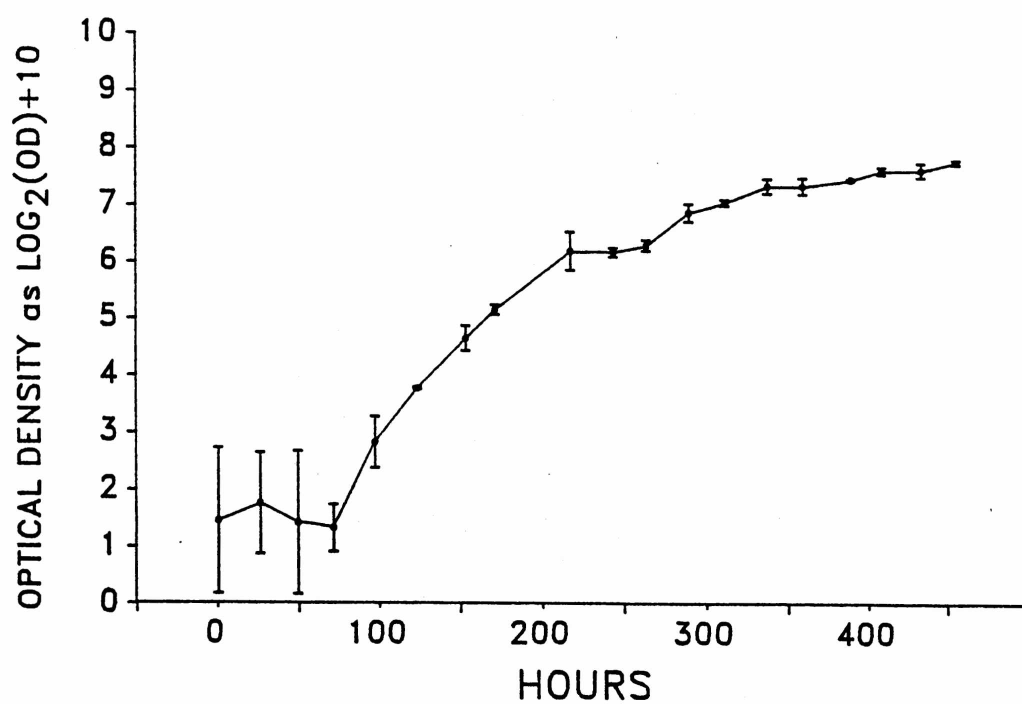

Figure 1. Growth Curve ($\log_2$(OD)+10 vs. Time) from Appendix B of Culture Used to Establish Time of Maximum ARA. Error Bars Represent Two Standard Deviations

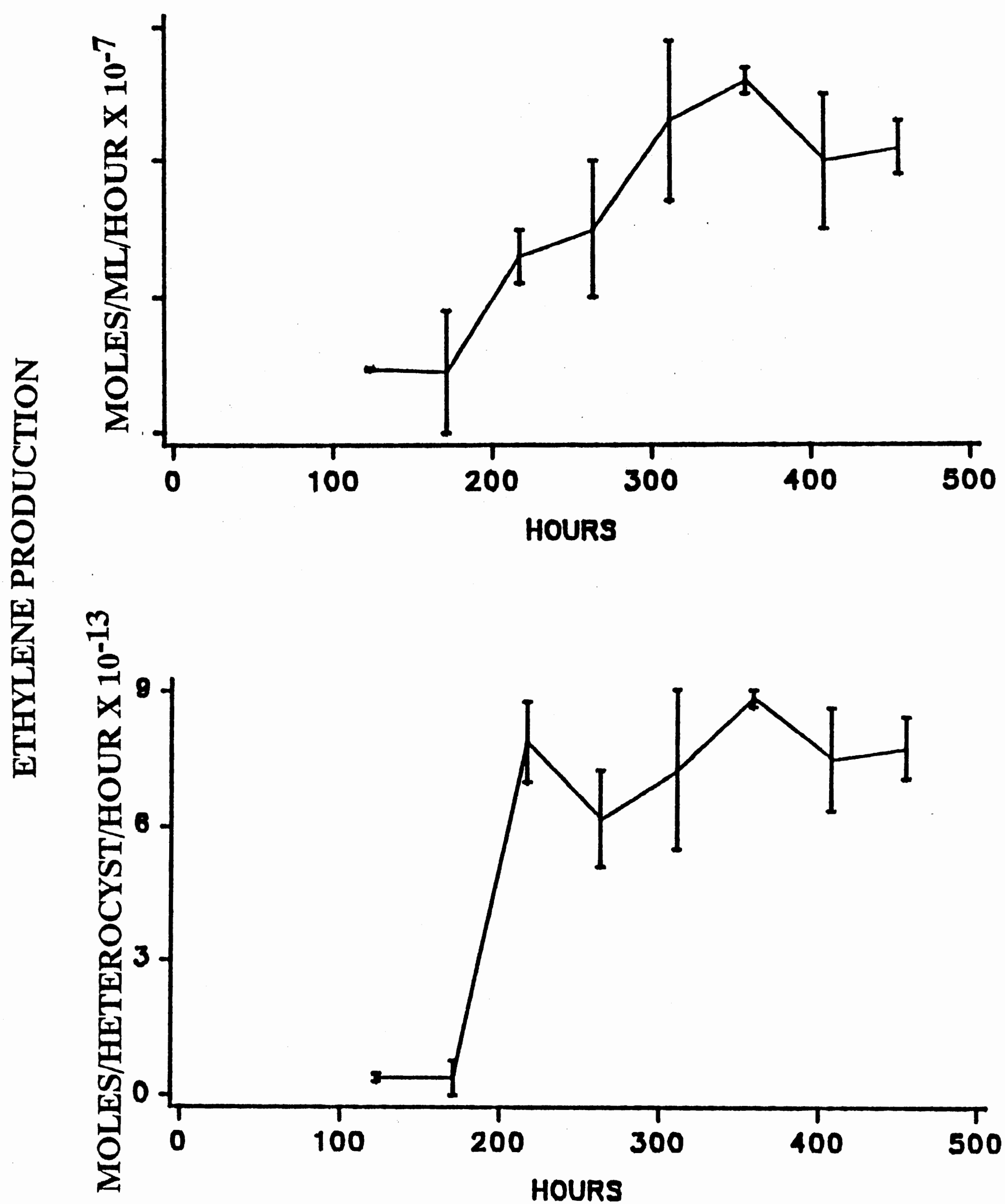

Figure 2. ARA Over Culture Growth to Maximum ARA for Non-poisoned Ruggedness Testing. Error Bars Represent Two Standard Deviations

culture age). Ruggedness tests were done on cultures aged 220 hours or more.

Analyzing Ruggedness Data

The analysis of data generated by ruggedness tests can suggest areas of the protocol needing strict compliance and also demonstrate the overall worth of the ARA protocol as a toxicity test. In order to determine the importance of an induced technical error the average of the results for the experiments with no induced error were compared to the average of the results for the experiments with that induced error. McKenzie and Olsson (1984) suggest that considerable information might be obtained from such comparisons in a single test (statistical methods and confidence interval not specified). For example, the means (A and a) in Table III may be compared in any number of ways. However, McKenzie and Olsson stress that information can be obtained by repeating the test and examining differences using an approximation of a t-test. It is maintained that this gives another indication that an induced technical error is affecting the test result.

I followed their suggested method of testing for differences. The procedure used to evaluate the importance of a technical error in media volume is shown in Table III. The mean of the test results from Experiments 1 through 4 (no induced error in media volume) was compared to the mean of the test results from Experiments 5 through 8 (induced

TABLE III

RUGGEDNESS RESULTS FOR MEDIA VOLUME
FROM TEST 1

Exp. #	Media Volume	Mean ARA as Moles C_2H_4/ml media/hr (n=3)	Average	Two Times Standard Deviation
1	A(50.0 ml)	2.47×10^{-8}		
2	A(50.0 ml)	5.97×10^{-8}		
3	A(50.0 ml)	1.91×10^{-8}	3.685×10^{-8}	3.714×10^{-8}
4	A(50.0 ml)	4.29×10^{-8}		
5	a(47.0 ml)	3.28×10^{-8}		
6	a(47.0 ml)	4.45×10^{-8}		
7	a(47.0 ml)	2.70×10^{-8}	3.408×10^{-8}	1.482×10^{-8}
8	a(47.0 ml)	3.20×10^{-8}		

Difference between means = 0.278×10^{-8}

Conclusion: The difference (0.278×10^{-9}) is less than 2 X either standard deviation therefore this induced technical error does not significantly affect the ARA results.

error in media volume). Since the difference between means was no greater than two times the standard deviation for either mean, I concluded that the induced error of 3.0 ml media volume did not affect the test. The procedure was the same to evaluate the six other induced errors, four experiments with the induced error were compared to the four without the induced error. If the same induced technical error shows an effect in repeated tests, it is almost certain to be an important item in a test protocol meriting the attention of the analyst.

Statistical analysis to determine ruggedness was performed for values of ARA expressed as moles ethylene produced per ml media per hour and moles ethylene produced per heterocyst per hour.

Non-poisoned Ruggedness

Batch culture growth of non-poisoned cells for ruggedness Tests 1, 2 and 3 can be found in Appendix C. ARA values are in Tables XXVIII through XXXII (Appendix F). Cultures for Tests 1 and 3 were axenic, whereas the culture for Test 2 was non-axenic. Statistical analyses for Test 1 are found in Tables IV and V. Both analyses show no significant difference of ARA values due to any of the seven induced technical errors in protocol. Analysis of ruggedness for Tests 2 and 3 also show no significance for any of the induced technical errors as shown in Tables VI-VII and VIII-IX, respectively.

TABLE IV

ARA VALUES PER HETEROCYST AND SIGNIFICANCE OF RUGGEDNESS TEST PERFORMED ON NOVEMBER 30, 1987 USING ENTOX CULTURE STARTED ON NOVEMBER 20, 1987. TEST 1.

Protocol Step	Acetylene Reduction Activity (moles C_2H_4/Heterocyst/Hour) x 10^{-13}				
	No Technical Error $X \pm SD$	Technical Error $X \pm SD$	Difference of Means	2 X Largest SD	Significant
Media Volume Used	12.40±6.24	11.45±2.53	0.95	12.48	No
Purge Use Gas	13.61±5.12	10.25±3.50	3.36	10.24	No
C_2H_2 Filtration	8.71±1.89	15.16±3.82	6.46	7.63	No
C_2H_2 Injection Volume	12.05±5.46	11.81±4.01	0.24	10.92	No
Shaker Table Use	10.14±3.69	13.73±4.88	3.59	9.74	No
Incubation Period	11.23±2.66	12.63±6.13	1.40	12.25	No
Volume of H_2O Injected	11.78±3.60	11.64±5.94	0.14	11.87	No

Note: An induced technical error was significant if the difference between technical error mean and no technical error mean was greater than twice the largest standard deviation.

TABLE V

ARA VALUES PER ML MEDIA AND SIGNIFICANCE OF RUGGEDNESS TEST PERFORMED ON NOVEMBER 30, 1987 USING ENTOX CULTURE STARTED ON NOVEMBER 20, 1987. TEST 1.

Protocol Step	Acetylene Reduction Activity (moles C_2H_4/ml Media/Hour) x 10^{-8}				
	No Technical Error $X \pm SD$	Technical Error $X \pm SD$	Difference of Means	2 X Largest SD	Significant
Media Volume Used	14.18 ± 7.13	13.08 ± 2.88	1.10	14.26	No
Purge Gas Use	15.54 ± 5.85	11.71 ± 4.00	3.83	11.70	No
C_2H_2 Filtration	9.94 ± 2.16	17.32 ± 4.36	7.38	8.72	No
C_2H_2 Injection Volume	13.76 ± 6.24	13.49 ± 4.58	0.27	12.48	No
Shaker Table Use	11.07 ± 4.22	15.58 ± 5.57	4.61	11.14	No
Incubation Period	12.83 ± 3.04	14.43 ± 7.00	1.60	14.00	No
Volume of H_2O Injected	13.45 ± 4.12	13.30 ± 6.78	0.14	13.56	No

Note: An induced technical error was significant if the difference between technical error mean and no technical error mean was greater than twice the largest standard deviation.

TABLE VI

ARA VALUES PER HETEROCYST AND SIGNIFICANCE OF RUGGEDNESS TEST PERFORMED ON DECEMBER 12, 1987 USING ENTOX CULTURE STARTED ON NOVEMBER 20, 1987. TEST 2.

Protocol Step	Acetylene Reduction Activity (moles C_2H_4/Heterocyst/Hour) x 10^{-13}				
	No Technical Error X±SD	Technical Error X±SD	Difference of Means	2 X Largest SD	Significant
Media Volume Used	9.95±2.92	9.18±1.68	0.76	5.84	No
Purge Gas Use	10.55±2.29	8.58±1.98	1.97	4.58	No
C_2H_2 Filtration	8.21±1.07	10.93±2.38	2.72	4.76	No
C_2H_2 Injection Volume	9.24±2.70	9.89±2.04	0.65	5.40	No
Shaker Table Use	8.49±1.93	10.64±2.23	2.15	4.46	No
Incubation Period	9.17±1.78	9.96±2.86	0.79	5.72	No
Volume of H_2O Injected	9.68±1.91	9.42±2.81	0.26	5.62	No

Note: An induced technical error was significant if the difference between technical error mean and no technical error mean was greater than twice the largest standard deviation.

TABLE VII

ARA VALUES PER ML MEDIA AND SIGNIFICANCE OF RUGGEDNESS TEST PERFORMED ON DECEMBER 17, 1987 USING ENTOX CULTURE STARTED ON DECEMBER 7, 1987. TEST 2.

Protocol Step	Acetylene Reduction Activity (moles C_2H_4/ml Media/Hour) x 10^{-8}				
	No Technical Error X±SD	Technical Error X±SD	Difference of Means	2 X Largest SD	Significant
Media Volume Used	11.41±3.29	10.45±2.79	0.92	6.58	No
Purge Gas Use	12.65±2.75	9.24±1.98	3.41	5.50	No
C_2H_2 Filtration	9.66±2.82	12.24±2.61	2.58	5.64	No
C_2H_2 Injection Volume	10.55±3.79	11.35±2.11	0.80	7.58	No
Shaker Table Use	9.81±1.25	12.06±3.78	2.25	7.56	No
Incubation Period	9.95±2.20	11.94±3.40	1.99	6.80	No
Volume of H_2O Injected	11.14±1.91	10.76±3.93	3.82	7.86	No

Note: An induced technical error was significant if the difference between technical error mean and no technical error mean was greater than twice the largest standard deviation.

TABLE VIII

ARA VALUES PER HETEROCYST AND SIGNIFICANCE OF RUGGEDNESS TEST PERFORMED ON FEBRUARY 7, 1988 USING ENTOX CULTURE STARTED ON JANUARY 28, 1988. TEST 3.

Protocol Step	Acetylene Reduction Activity (moles C_2H_4/Heterocyst/Hour) x 10^{-13}				
	No Technical Error X±SD	Technical Error X±SD	Difference of Means	2 X Largest SD	Significant
Media Volume Used	27.22±18.13	31.67±5.58	4.45	36.26	No
Purge Gas Use	28.72±15.49	30.17±11.49	1.45	30.96	No
C_2H_2 Filtration	19.87±9.73	39.02±5.85	18.15	19.46	No
C_2H_2 Injection Volume	30.01±16.57	28.88±9.88	1.13	37.14	No
Shaker Table Use	23.70±14.30	35.19±8.98	11.49	28.60	No
Incubation Period	29.06±14.64	29.84±12.59	0.78	19.28	No
Volume of H_2O Injected	26.38±13.15	32.51±13.23	6.13	26.46	No

Note: An induced technical error was significant if the difference between technical error mean and no technical error mean was greater than twice the largest standard deviation.

TABLE IX

ARA VALUES PER ML MEDIA AND SIGNIFICANCE OF RUGGEDNESS TEST PERFORMED ON FEBRUARY 7, 1988 USING ENTOX CULTURE STARTED ON JANUARY 28, 1988. TEST 3.

Protocol Step	Acetylene Reduction Activity (moles C_2H_4/ml Media/Hour) x 10^{-8}				
	No Technical Error X±SD	Technical Error X±SD	Difference of Means	2 X Largest SD	Significant
Media Volume Used	43.25±28.61	49.90±8.46	6.65	57.21	No
Purge Gas Use	45.15±24.93	48.00±17.10	2.85	49.86	No
C_2H_2 Filtration	31.70±15.62	61.45±9.24	29.75	31.24	No
C_2H_2 Injection Volume	46.95±26.50	46.20±14.70	0.75	53.00	No
Shaker Table Use	37.50±22.40	55.70±14.10	18.15	44.80	No
Incubation Period	45.50±23.50	47.70±19.10	2.20	47.00	No
Volume of H_2O Injected	41.40±21.30	51.80±19.90	10.40	42.60	No

Note: An induced technical error was significant if the difference between technical error mean and no technical error mean was greater than twice the largest standard deviation.

Range Finding to Establish Dosage for ETOX Cultures

A batch culture was grown to maximum ARA then exposed to three concentrations of $CdCl_2$ (0, 2.084 and 208.4 umoles Cd/l). Each culture was monitored at 24 hour intervals to determine what concentration and length of exposure to cadmium was necessary to produce a marked reduction of ARA. Data on growth and ARA of this culture up to and after poisoning are given in Appendix E. This culture was not axenic. Only after 72 hours was the optical density of the 208.4 umoles Cd/l culture significantly less than that of either control or the 2.084 umoles Cd/l culture (Figure 3).

Examination of ARA values as moles ethylene produced per ml media per hour and moles ethylene produced per heterocyst per hour showed similar results (Figures 4 and 5, respectively). No significant difference from control was found for the 2.084 umoles Cd/l culture throughout the experiment. Comparison of the 208.4 umoles Cd/l culture to control shows a significant difference at 72 and 96 hours of exposure for both measures of ARA. For purposes of ensuring substantial ARA inhibition from control, I concluded that poisoned ruggedness testing should proceed after exposing ETOX cultures to 208.4 umoles Cd/l for 96 hours.

Poisoned Cultures

Batch culture growth data up to and after poisoning for ruggedness Tests 4 and 5 are given in Appendix D. ARA values for Tests 4 and 5 are listed in Appendix F. Test 4

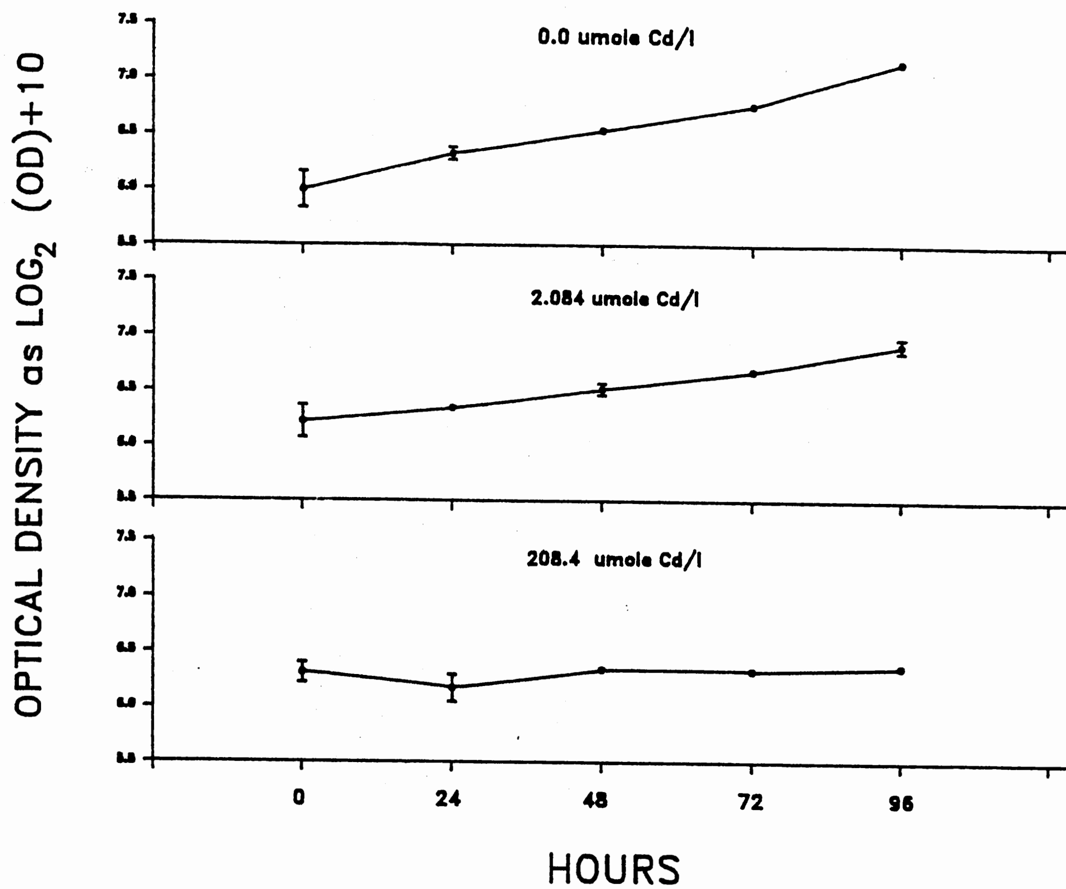

Figure 3. Optical Density Measurements of Range Finding Experiment After Exposure to 0.0, 2.084 and 208.4 umoles Cd/1. Error Bars Represent Two Standard Deviations

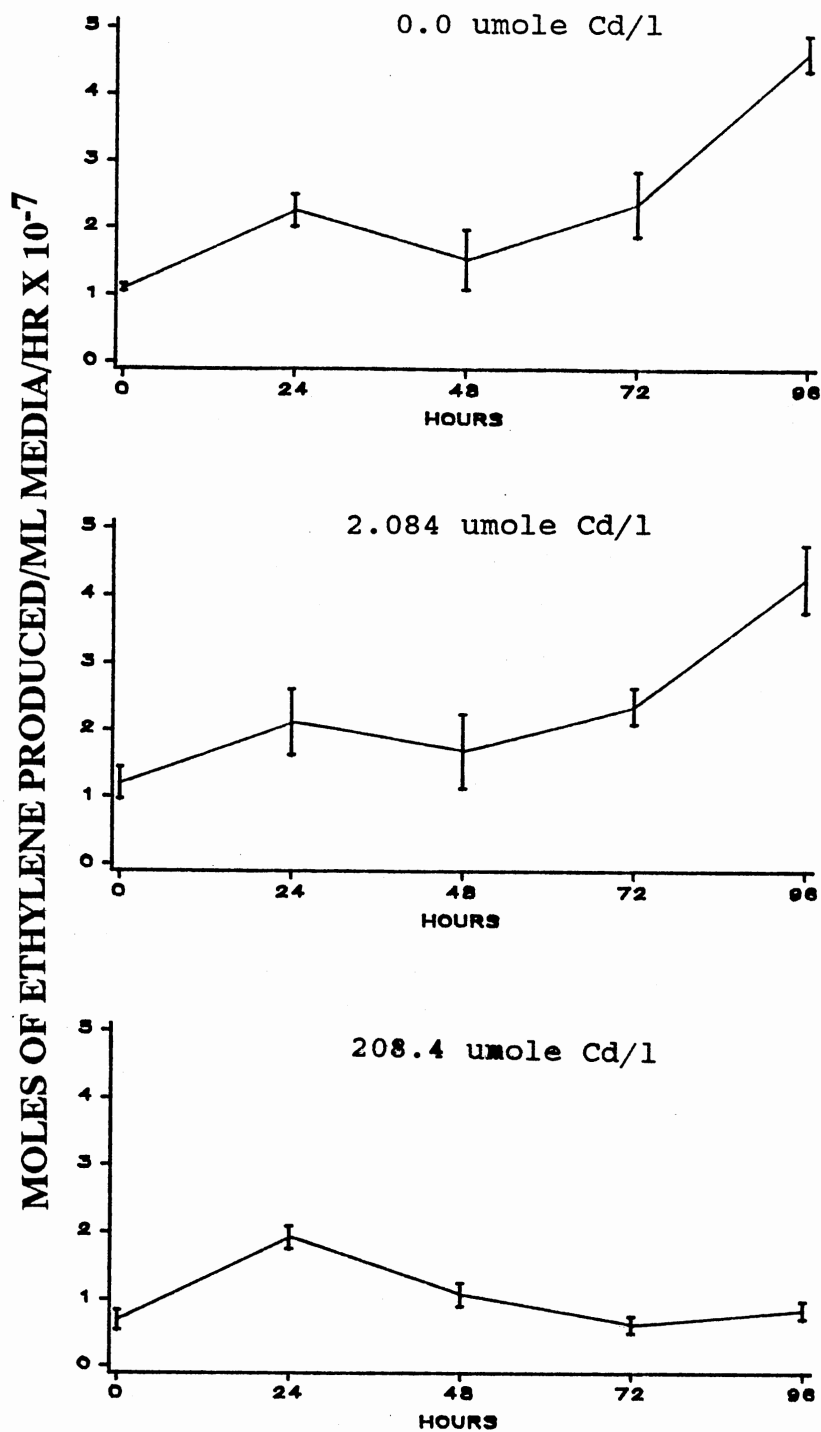

Figure 4. ARA (per ml Media) Over Time of Range Finding Experiment After Exposure to 0.0, 2.084 and 208.4 umoles Cd/l. Error Bars Represent T

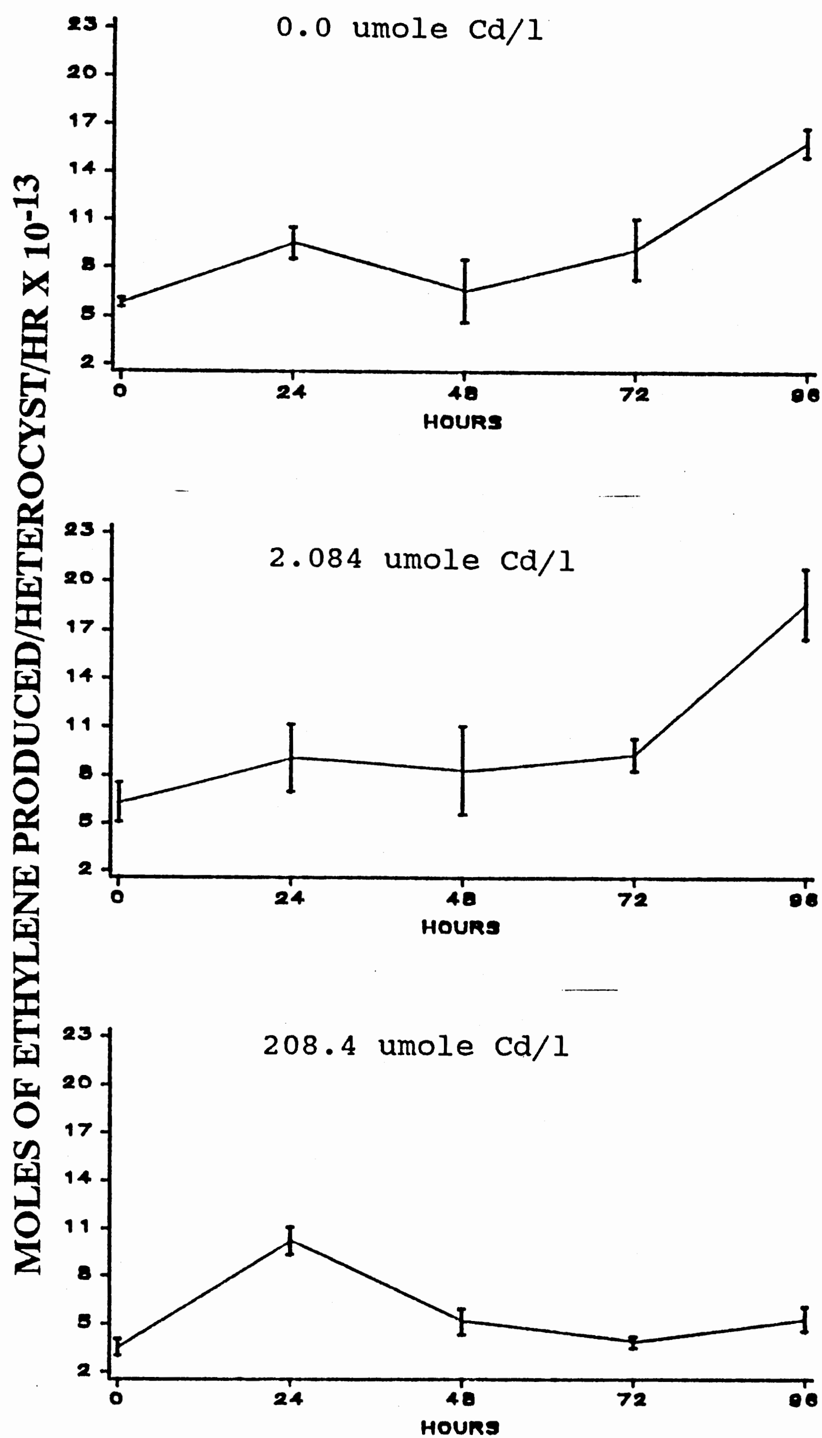

Figure 5. ARA (per Heterocyst) Over Time of Range Finding Experiment After Exposure to 0.0, 2.084 and 208.4 umoles Cd/l. Error Bars Represent T

was an axenic culture whereas Test 5 was a non-axenic culture. Test 4 exposure was 239.7 umoles Cd/l for the poisoned cells and 0.4168 umoles Cd/l for the control. Test 5 poisoned cell exposure was 208.4 moles Cd/l with a control exposure of 1.667 umoles Cd/l. The detection limit for the analysis was 0.2084 umoles Cd/l.

The results of Test 4 (Tables X and XI) show no significant difference of ARA between any of the seven protocol steps. The results of Test 5 (Tables XII and XIII) are similar.

TABLE X

ARA VALUES PER HETEROCYST AND SIGNIFICANCE OF RUGGEDNESS TEST PERFORMED ON NOVEMBER 11, 1987 USING ENTOX CULTURE STARTED ON OCTOBER 30, 1987. TEST 4.

Protocol Step	Acetylene Reduction Activity (moles C_2H_4/Heterocyst/Hour) x 10^{-13}				
	No Technical Error X±SD	Technical Error X±SD	Difference of Means	2 X Largest SD	Significant
Media Volume Used	9.60±2.16	7.90±1.46	1.80	4.32	No
Purge Gas Use	8.59±2.82	8.80±0.84	0.21	5.74	No
C_2H_2 Filtration	8.66±2.822	8.74±1.03	0.08	5.64	No
C_2H_2 Injection Volume	9.18±2.22	8.22±1.86	0.96	4.44	No
Shaker Table Use	9.57±1.92	7.83±1.82	1.74	3.84	No
Incubation Period	9.17±2.85	8.22±0.54	0.95	5.70	No
Volume of H_2O Injected	9.85±1.89	7.54±1.37	2.31	3.78	No

Note: An induced technical error was significant if the difference between technical error mean and no technical error mean was greater than twice the largest standard deviation.

TABLE XI

ARA VALUES PER ML MEDIA AND SIGNIFICANCE OF RUGGEDNESS TEST PERFORMED ON NOVEMBER 11, 1987 USING ENTOX CULTURE STARTED ON OCTOBER 30, 1987. TEST 4.

Protocol Step	Acetylene Reduction Activity (moles C_2H_4/ml Media/Hour) x 10^{-8}				
	No Technical Error X±SD	Technical Error X±SD	Difference of Means	2 X Largest SD	Significant
Media Volume Used	3.25±0.79	2.75±0.52	0.50	1.58	No
Purge Gas Use	2.89±0.973	3.11±0.30	0.22	1.94	No
C_2H_2 Filtration	3.00±0.90	3.00±0.51	0.00	1.80	No
C_2H_2 Injection Volume	3.10±0.78	2.90±0.66	0.20	1.56	No
Shaker Table Use	3.32±0.57	2.68±0.680	0.64	1.36	No
Incubation Period	3.18±0.92	2.82±0.35	0.36	1.84	No
Volume of H_2O Injected	3.42±0.57	2.57±0.51	0.85	1.14	No

Note: An induced technical error was significant if the difference between technical error mean and no technical error mean was greater than twice the largest standard deviation.

TABLE XII

ARA VALUES PER HETEROCYST AND SIGNIFICANCE OF RUGGEDNESS TEST PERFORMED ON FEBRUARY 29, 1988 USING ENTOX CULTURE STARTED ON FEBRUARY 14, 1988. TEST 5.

Protocol Step	Acetylene Reduction Activity (moles C_2H_4/Heterocyst/Hour) x 10^{-14}				
	No Technical Error X±SD	Technical Error X±SD	Difference of Means	2 X Largest SD	Significant
Media Volume Used	16.35±8.47	6.40±12.79	9.95	25.58	No
Purge Gas Use	6.02±6.95	16.73±13.29	10.71	26.58	No
C_2H_2 Filtration	10.18±13.73	12.56±10.45	2.38	27.46	No
C_2H_2 Injection Volume	12.41±10.46	10.33±13.75	2.08	27.50	No
Shaker Table Use	16.58±13.36	6.17±7.12	10.41	26.52	No
Incubation Period	12.39±10.46	10.36±13.76	2.03	27.52	No
Volume of H_2O Injected	5.99±6.92	16.75±13.27	10.76	26.54	No

Note: An induced technical error was significant if the difference between technical error mean and no technical error mean was greater than twice the largest standard deviation.

TABLE XIII

ARA VALUES PER ML MEDIA AND SIGNIFICANCE OF RUGGEDNESS TEST PERFORMED ON FEBRUARY 29, 1988 USING ENTOX CULTURE STARTED ON FEBRUARY 14, 1988. TEST 5.

Protocol Step	Acetylene Reduction Activity (moles C_2H_4/ml Media/Hour) x 10^{-9}				
	No Technical Error X±SD	Technical Error X±SD	Difference of Means	2 X Largest SD	Significant
Media Volume Used	13.87±8.79	4.94±9.88	8.92	19.77	No
Purge Gas Use	4.70±5.44	13.02±10.34	8.31	20.67	No
C_2H_2 Filtration	7.96±10.73	9.74±8.03	1.78	16.06	No
C_2H_2 Injection Volume	9.62±8.04	8.08±10.75	1.54	21.50	No
Shaker Table Use	12.88±10.37	4.82±6.57	8.06	20.74	No
Incubation Period	8.10±10.76	9.60±8.04	1.15	21.51	No
Volume of H_2O Injected	4.68±5.41	13.02±10.31	8.33	20.61	No

Note: An induced technical error was significant if the difference between technical error mean and no technical error mean was greater than twice the largest standard deviation.

CHAPTER IV

DISCUSSION

The Effect of Ruggedness Testing on ARA

The results of this research show that seven induced technical errors had little effect on the outcome of the test result. The protocol items of media volume, acetylene injection volume, incubation time and water injection volume are "rugged" and small deviations in each will have no effect on test results. Use of a purge gas, filtering commercial grade acetylene and using a shaker table during incubation could possibly be omitted from the protocol to yield an assay for use in the field with minimal amount of equipment. This is now often the case in field work, but the results given here justify these practices.

Further investigation should be done with each item separate to determine the degree each can affect ARA. Although ruggedness testing showed no significant effect of induced errors on ARA, it is important to note that mean and standard deviation between individual experiments differ significantly within each test. This suggests that some sort of effect between experiments occurred, yet these differences were obscured in the statistical analysis employed. This may be due to several factors including

possible interaction between experiments because of the systematic way experiments were executed.

Comparison of ruggedness data between cultures would not be statistically valid due to confounding effects of different bacterial contamination and toxicant concentrations between cultures. Further tests using replicate test conditions would enable separation of within test variation into components of culture contamination, toxicity, physiological state, and error.

An aspect of the acetylene reduction assay that was not examined were physiological factors that could affect ARA. It is probable that physiological factors could account for the relatively high ARA values in Test 3 and the variation of ARA between cultures. Other possible factors that could effect ARA are pH, growth stage, nitrogen content of the media, and amount and type of storage products. Monitoring of these factors over culture growth would allow these additional factors to be incorporated into an analysis of ruggedness.

It is important for workers to state which aspects of the acetylene reduction assay protocol are being used, modified, or deleted when published results. This will allow for comparison between reports.

The Acetylene Reduction Assay as a Toxicity Test

The poisoned ruggedness tests indicated how the acetylene reduction assay would perform as a toxicity test. The closeness of the range of ARA values for Experiments 1 through 8 in Test 4 indicated a "rugged" protocol in which none of the induced errors affected ARA in any way. Test 5 is less informative because no ethylene was produced in several cases. One possible factor for zero values is that the cadmium exposure per cell was higher than that in Test 4, although the molar concentrations were very similar.

Because of the long exposure time and high concentration of cadmium needed to produce a considerable decrease in ARA from control, I do not feel that the acetylene reduction assay is a good candidate for a test of heavy metal toxicity. Another reason for the lack of sensitivity in this test might be EDTA binding Cd^{++} in the media. However, if acetone actually does stimulate ARA during a 2 hour exposure, the acetylene reduction assay might be a good test for lipophilic compounds, even though the response observed here was apparently to increase ARA. Also, Bastian and Toetz (1985) showed a rapid response of ARA to polyaromatic hydrocarbons.

Overall, this assay does not seem to be a good general indicator of toxicity. Perhaps the use of isolated heterocysts would yield an assay more sensitive to heavy metal

toxicity. A drawback to the use of heterocysts is the need to maintain anaerobic conditions.

Conclusions

The acetylene reduction assay has been shown to be "rugged" using poisoned and non-poisoned cultures of *Anabaena flos-aquae*. Small errors of media volume, volume of acetylene injected, incubation time, and volume of water injected in the assay protocol did not affect ARA. Use of a purge gas, filtered acetylene, and a shaker table could be eliminated from the protocol.

The acetylene reduction assay is "rugged" as a toxicity test, but intact filaments of hetercystous *Anabaena flos-aquae* are not apparently sensitive to heavy metals. But the assay may prove to be a sensitive indicator of the toxicity of lipophilic substances to BNF. To this end further investigation is required.

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APPENDICES

APPENDIX A

GAS CHROMATOGRAPH SETTINGS

TABLE XIV

TRACOR 560 GAS CHROMATOGRAPH CONDITIONS
USED FOR ETHYLENE QUANTIFICATION

Supelco 80/100 porapak R column 6' x 1/8"

Component	Temperature
Injection Port	70°C
Oven	65°C
Flame Ionization Detector	118°C

Gas Type	Pressure (psi)	Flow rate
N_2	45	27 cc/min.
H_2	13	70 cc/min.
Air	60	300 cc/min.

APPENDIX B

TIME COURSE DATA USED TO ESTABLISH MAXIMUM ARA OVER CULTURE GROWTH

TABLE XV

TIME COURSE DATA OF CULTURE STARTED ON JULY 11, 1987. STERILITY TEST POSITIVE (NON-AXENIC)

Culture Age (Hours)	Veg/Het Ratio $X\pm SD$	LOG_2(OD)+10 $X\pm SD$	Cell Density (cells/ml) $X\pm SD$	Moles Ethylene Produced/Hour per Heterocyst $X\pm SD$	per ml Media $X\pm SD$
0	--	1.450 ± 1.280	--	--	--
26	10.6 ± 0.3	1.756 ± 0.898	--	--	--
49	9.1 ± 0.9	1.598 ± 1.428	$(2.1\pm3.7)\text{x}10^{4}$	--	--
71	9.2 ± 0.4	1.317 ± 0.420	$(1.0\pm1.4)\text{x}10^{5}$	--	--
97	9.1 ± 1.0	2.828 ± 0.456	$(1.1\pm1.0)\text{x}10^{5}$	--	--
123	10.4 ± 0.4	3.762 ± 0.466	$(1.2\pm0.7)\text{x}10^{6}$	$(4.0\pm1.0)\text{x}10^{-14}$	$(4.7\pm0.1)\text{x}10^{-8}$
153	12.7 ± 2.8	4.657 ± 0.226	$(1.5\pm0.5)\text{x}10^{6}$	--	--
171	12.0 ± 0.7	5.163 ± 0.093	$(1.3\pm0.2)\text{x}10^{6}$	$(4.0\pm4.1)\text{x}1^{-14}$	$(4.5+4.6)\text{x}10^{-8}$
217	15.8 ± 1.1	6.200 ± 0.343	$(2.7\pm1.8)\text{x}10^{6}$	$(7.9\pm0.9)\text{x}10^{-13}$	$(1.3\pm0.2)\text{x}10^{-7}$
243	15.4 ± 2.4	6.180 ± 0.086	$(2.4\pm0.3)\text{x}10^{6}$	--	--
263	14.0 ± 2.2	6.302 ± 0.102	$(3.0\pm1.0)\text{x}10^{6}$	$(7.3\pm2.2)\text{x}10^{-13}$	$(1.5\pm0.5)\text{x}10^{-7}$
289	15.0 ± 1.4	6.883 ± 0.162	$(4.9\pm1.2)\text{x}10^{6}$	--	--
311	15.3 ± 0.3	7.061 ± 0.060	$(4.8\pm2.8)\text{x}10^{6}$	$(7.3\pm1.8)\text{x}10^{-13}$	$(2.3\pm0.6)\text{x}10^{-7}$
337	21.1 ± 2.8	7.351 ± 0.137	$(3.3\pm0.2)\text{x}10^{6}$	--	--
359	17.1 ± 1.7	7.351 ± 0.140	$(4.9\pm1.2)\text{x}10^{6}$	$(8.9\pm0.2)\text{x}10^{-13}$	$(2.6\pm0.1)\text{x}10^{-7}$
389	14.8 ± 0.6	7.467 ± 0.018	$(4.5\pm0.1)\text{x}10^{6}$	--	--
408	19.5 ± 2.2	7.623 ± 0.070	$(4.5\pm0.6)\text{x}10^{6}$	$(8.7\pm2.3)\text{x}10^{-13}$	$(2.0\pm0.5)\text{x}10^{-7}$
433	13.8 ± 2.5	7.633 ± 0.131	$(3.8\pm0.9)\text{x}10^{6}$	--	--
455	16.4 ± 2.2	7.786 ± 0.044	$(4.3\pm0.8)\text{x}10^{6}$	$(7.8\pm0.7)\text{x}10^{-13}$	$(2.1\pm0.2)\text{x}10^{-7}$

APPENDIX C

GROWTH DATA OF ENTOX CULTURES

TABLE XVI

OBSERVATIONS ON HETEROCYST, CELL AND OPTICAL DENSITY, AND pH OF NOVEMBER 20, 1987 ENTOX CULTURE. NEGATIVE STERILITY TEST (AXENIC).

TEST 1. ARA PERFORMED NOVEMBER 30, 1987

Age (Hours)	Het/Veg Ratio X±SD	Cell Density (Cell/ml) X±SD	$Log_2(OD)+10$ X±SD	pH
0	----	----	1.665±1.479	6.35
51	7.44±0.61	$(5.92 \pm 0.82) \times 10^4$	0.781±0.733	6.60
75	8.41±1.75	$(9.43 \pm 5.68) \times 10^4$	1.883±0.692	6.60
99	8.67±1.20	$(3.88 \pm 1.38) \times 10^5$	2.631±0.202	7.00
123	8.18±0.89	$(4.62 \pm 1.37) \times 10^5$	3.918±0.137	7.00
171	12.77±1.07	$(1.02 \pm 0.11) \times 10^6$	4.654±0.154	6.50
195	17.35±2.48	$(1.23 \pm 0.26) \times 10^6$	5.251±0.059	6.75
219	14.55±0.57	$(1.54 \pm 0.39) \times 10^6$	5.512±0.069	6.85
243	15.52±1.63	$(1.77 \pm 0.18) \times 10^6$	5.717±0.075	6.70

TABLE XVII

OBSERVATIONS ON HETEROCYST, CELL AND OPTICAL DENSITY, AND pH OF DECEMBER 7, 1987 ENTOX CULTURE. POSITIVE STERILITY TEST (NON-AXENIC).

TEST 2. ARA PERFORMED DECEMBER 18, 1987

Age (Hours)	Het/Veg Ratio $X \pm SD$	Cell Density (Cell/ml) $X \pm SD$	$Log_2(OD)+10$ $X \pm SD$	pH
51	8.00 ± 1.05	$(4.96 \pm 1.16) \times 10^4$	1.701 ± 1.150	6.60
75	6.53 ± 1.71	$(2.17 \pm 0.57) \times 10^5$	3.013 ± 0.552	6.85
99	9.26 ± 1.24	$(1.65 \pm 2.64) \times 10^5$	3.084 ± 0.029	6.20
123	8.35 ± 1.25	$(7.62 \pm 5.74) \times 10^5$	4.583 ± 0.785	6.30
147	10.00 ± 1.17	$(8.07 \pm 2.18) \times 10^5$	4.743 ± 0.019	6.60
171	12.69 ± 2.11	$(1.03 \pm 0.16) \times 10^6$	5.061 ± 0.101	6.25
195	13.16 ± 1.18	$(1.53 \pm 0.38) \times 10^6$	5.297 ± 0.048	6.15
218	14.45 ± 1.66	$(1.27 \pm 0.35) \times 10^5$	5.488 ± 0.020	6.25
243	11.92 ± 1.10	$(1.25 \pm 0.14) \times 10^6$	5.642 ± 0.018	6.15
267	15.04 ± 0.98	$(1.80 \pm 0.17) \times 10^6$	5.724 ± 0.066	6.30

TABLE XVIII

OBSERVATIONS ON HETEROCYST, CELL AND OPTICAL DENSITY, AND pH OF JANUARY 28, 1988 ENTOX CULTURE. POSITIVE STERILITY TEST (AXENIC).

TEST 3. ARA PERFORMED FEBRUARY 8, 1988

Age (Hours)	Het/Veg Ratio X±SD	Cell Density (Cell/ml) X±SD	Log_2(OD)+10 X±SD	pH
57	----	$(2.25\pm1.98)X10^4$	1.627+0.244	6.20
103	8.79+0.99	$(7.79\pm2.82)X10^4$	2.747+0.572	6.20
125	10.93+0.80	$(3.37\pm0.54)X10^5$	3.473+0.196	6.10
173	9.51+3.07	$(7.31\pm3.96)X10^5$	3.968+0.100	6.50
226	14.75+3.62	$(1.75\pm0.51)X10^6$	5.259+0.023	6.50
255	13.17+2.96	$(2.08\pm0.13)X10^6$	5.727+0.073	6.55

TABLE XIX

OBSERVATIONS ON HETEROCYST, CELL AND OPTICAL DENSITY, AND pH OF RANGE FINDING CULTURE STARTED OCTOBER 11, 1987. POSITIVE STERILITY TEST (NON-AXENIC).

Age (Hours)	Het/Veg Ratio X±SD	Cell Density (Cell/ml) X±SD	Log_2(OD)+10 X±SD	pH
0	----	----	0.757±0.378	7.15
33	25.53±1.32	(1.04+0.74)x10^{4}	0.780±0.733	6.30
55	11.08±0.02	(6.38+0.35)x10^{4}	1.240±0.563	6.55
78	12.12±3.15	(2.61+1.63)x10^{5}	2.149±0.685	6.35
103	9.93±0.88	(1.64+0.80)x10^{5}	3.292±0.029	6.40
127	9.40±1.32	(3.91+1.26)x10^{5}	4.250±0.033	7.15
175	11.86±1.42	(5.60+1.04)x10^{5}	5.043±0.403	6.65
199	17.75±1.72	(8.45+2.22)x10^{5}	5.299±0.034	6.35
223	16.24±0.29	(1.20+0.31)x10^{6}	5.653±0.0625	6.40
250	12.85±0.14	(1.75+0.50)x10^{6}	6.043±0.047	6.20

APPENDIX D

GROWTH DATA OF RANGE FINDING EXPERIMENT

TABLE XX

OBSERVATIONS OF OPTICAL AND CELL DENSITY OF CONTROL AND EXPOSED CULTURES STARTED OCTOBER 11, 1987 AFTER BEING EXPOSED TO $CdCl_2$. POSITIVE STERILITY TEST (NON-AXENIC)

Total Culture Age (hrs)	Exp. Time (hrs)	0.0 moles Cd/1 (Control)		2.084 umoles Cd/1		208.4 umoles Cd/1	
		Log_2(OD)+10	Cells/ml	Log_2(OD)+10	Cells/ml	Log_2(OD)+10	Cells/ml
250	0	5.998±0.164	$(2.25±0.27)x10^6$	6.172±0.085	$(2.44±0.23)x10^6$	6.310±0.095	$(2.21±0.10)x10^6$
274	24	6.324±0.612	$(2.53±0.02)x10^6$	6.347±0.028	$(2.53±0.08)x10^6$	6.171±0.015	$(2.30±0.04)x10^6$
298	48	6.541±0.352	$(2.59±0.29)x10^6$	6.518±0.054	$(2.66±0.27)x10^6$	6.342±0.103	$(2.34±0.22)x10^6$
322	72	6.770±0.035	$(3.35±0.47)x10^6$	6.680±0.001	$(3.05±0.25)x10^6$	6.329±0.002	$(2.00±0.38)x10^6$
346	96	7.161±0.023	$3.49x10^6$	6.916±0.063	$3.38x10^6$	6.367±0.009	$1.63x10^6$

TABLE XXI

ARA (PER ML MEDIA) OF OCTOBER 11, 1987 CULTURE POISONED OCTOBER 21, 1987 FOR THE RANGE FINDING EXPERIMENT

Culture Age (hrs)	Expo. Time (hrs)	Moles ethylene produced per ml media per hour		
		(Control) 0.0 moles Cd/1	2.084 umoles Cd/1	208.4 umoles Cd/1
250	0	$(1.10\pm0.05)x10^{-7}$	$(1.20\pm0.24)x10^{-7}$	$(0.69\pm0.15)x10^{-7}$
274	24	$(2.26\pm0.24)x10^{-7}$	$(2.12\pm0.49)x10^{-7}$	$(1.92\pm0.17)x10^{-7}$
298	49	$(1.53\pm0.45)x10^{-7}$	$(1.69\pm0.56)x10^{-7}$	$(1.07\pm0.18)x10^{-7}$
322	72	$(2.37\pm0.49)x10^{-7}$	$(2.36\pm0.27)x10^{-7}$	$(0.62\pm0.13)x10^{-7}$
346	96	$(4.65\pm0.27)x10^{-7}$	$(4.26\pm0.50)x10^{-7}$	$(0.84\pm0.13)x10^{-7}$

TABLE XXII

ARA (PER HETEROCYST) OF OCTOBER 11, 1987 CULTURE
POISONED OCTOBER 21, 1987 FOR RANGE
FINDING EXPERIMENT

Culture Age (hrs)	Expo. Time (hrs)	Moles ethylene produced per heterocyst media per hour		
		(Control) 0.0 moles Cd/1	2.084 umoles Cd/1	208.4 umoles Cd/1
250	0	$(5.85 \pm 0.27) \times 10^{-13}$	$(6.29 \pm 1.26) \times 10^{-13}$	$(10.17 \pm 0.90) \times 10^{-13}$
274	24	$(9.54 \pm 0.99) \times 10^{-13}$	$(9.06 \pm 2.10) \times 10^{-13}$	$(10.17 \pm 0.90) \times 10^{-13}$
298	48	$(6.59 \pm 1.95) \times 10^{-13}$	$(8.31 \pm 2.74) \times 10^{-13}$	$(5.13 \pm 0.82) \times 10^{-13}$
322	72	$(9.23 \pm 1.91) \times 10^{-13}$	$(9.32 \pm 0.99) \times 10^{-13}$	$(3.86 \pm 0.34) \times 10^{-13}$
346	96	$(16.03 \pm 0.91) \times 10^{-13}$	$(18.63 \pm 2.17) \times 10^{-13}$	$(5.30 \pm 0.79) \times 10^{-13}$

APPENDIX E

GROWTH DATA OF ENTOX CULTURES

TABLE XXIII

OBSERVATIONS ON HETEROCYST, CELL AND OPTICAL DENSITY, AND pH OF ETOX CULTURE STARTED OCTOBER 30, 1987. ARA PERFORMED NOVEMBER 11, 1987. NEGATIVE STERILITY TEST (AXENIC). TEST 4

Age (Hours)	Het/Veg Ratio $X \pm SD$	Cell Density (Cell/ml) $X+SD$	$Log_2(OD)+10$ $X \pm SD$	pH
0	—	—	$0.787^{+}0.911$	—
78	8.75 ± 0.39	$(1.18 \pm 0.35) x10^5$	2.594 ± 0.523	6.50
102	10.22 ± 0.77	$(2.98 \pm 1.46) x10^5$	3.428 ± 0.048	6.20
126	11.07 ± 1.51	$(1.61 \pm 0.66) x10^6$	4.930 ± 0.125	5.95
150	10.97 ± 1.17	$(1.85 \pm 0.58) x10^6$	5.625 ± 0.036	5.80
174	11.41 ± 0.56	$(2.37 \pm 0.19) x10^6$	5.905 ± 0.040	5.95
198	$CdCl_2$ Exposure Data in Table XXIV		6.181 ± 0.053	6.00

TABLE XXIV

CONTINUATION OF DATA IN TABLE XXIV ON OPTICAL AND FINAL CELL DENSITY OF OCTOBER 30, 1987 CULTURE EXPOSED to 239.7 uMOLES Cd/L AND CONTROL. ARA PERFORMED NOVEMBER 11, 1987. TEST 4

Culture Age (Hour)	Exposure Age (Hour) X±SD	Cultures	
		0.0 umole Cd/l Log_2(OD)+10 X±SD	239.7 umoles Cd/l Log_2(OD)+10
198	0	6.045±0.001	6.009±0.035
222	24	6.084±0.024	6.217±0.053
246	48	6.370±0.019	6.126±0.028
270	72	6.574±0.041	5.712±0.082
294	96	1.721±0.110	5.492±0.063
Final Cell Density (Cells/ml) X±SD		$(2.51\pm.93)\times10^6$	$(5.62\pm1.71)\times10^5$
Veg/Het Ratio X±SD		12.99±0.36	15.92±0.12

TABLE XXV

OBSERVATIONS ON HETEROCYST, CELL AND OPTICAL DENSITY, AND pH OF ETOX CULTURE STARTED FEBRUARY 14, 1988. ARA PERFORMED FEBRUARY 29, 1988. POSITIVE STERILITY TEST (NON-AXENIC) TEST 5.

Age (Hours)	Het/Veg Ratio X±SD	Cell Density (Cell/ml) X±SD	Log_2(OD)+10 X±SD	pH
79	9.32±1.46	$(7.50 \pm 9.30) \times 10^4$	2.807±0.289	6.40
124	12.68±0.51	$(6.82 \pm 1.33) \times 10^5$	3.618±0.082	--
175	11.46±0.97	$(9.90 \pm 5.04) \times 10^5$	4.641±0.124	6.40
200	12.76±0.19	$(9.93 \pm 0.93) \times 10^5$	5.275±0.059	6.45
222	15.21±2.85	$(1.54 \pm 0.15) \times 10^6$	5.599±0.026	6.70
246	13.45±0.53	$(1.11 \pm 0.33) \times 10^6$	5.707±0.102	6.40
268	$CdCl_2$ exposure data on Table XXVI		5.797±0.130	6.25

TABLE XXVI

CONTINUATION OF DATA ON TABLE XXVI ON OPTICAL DENSITY AND FINAL VEGETATIVE/HETEROCYST RATIO OF THE FEBRUARY 14, 1988 CULTURE POISONED FEBRUARY 25, 1988 EXPOSED TO $2.084X10^{-4}$ MOLES Cd/1 AND CONTROL. POSITIVE STERILITY TEST. TEST 5.

Culture Age (hrs)	Exp. Time (hrs)	Control (0.0 moles Cd/1)			208.4 umoles Cd/1		
		Log_2(OD)+10 X±SD	Cells/ml X±SD	pH	Log_2(OD)+10 X±SD	Cells/ml X±SD	pH
268	0	5.690±0.057	$(1.70±0.39)x10^6$	6.45	5.682±0.039	$(1.43±0.14)x10^6$	6.55
292	24	5.968±0.008	$(1.41±0.42)x10^6$	6.65	5.833±0.040	$(1.38±0.21)x10^6$	6.85
316	48	6.154±0.020	$(1.18±0.09)x10^6$	6.55	6.130±0.049	$(1.29±0.37(x10^6$	6.55
330	72	6.357±0.040	$(2.32±0.30)x10^6$	6.80	5.991±0.056	$(1.41±0.22)x10^6$	6.85
354	96	6.617±0.009	$(2.91±0.17)x10^6$	6.65	5.929±0.080	$(1.05±0.21)x10^6$	6.65
Vegetative Cell/ Heterocyst ration X±SD			13.51±0.35			13.43+0.49	

APPENDIX F

ARA DATA FOR ALL RUGGEDNESS TESTS

TABLE XXVII

ARA OF RUGGEDNESS TEST 1 ON NOVEMBER 30, 1987 USING
CULTURE STARTED ON NOVEMBER 20, 1987.
NEGATIVE STERILITY TEST

Exp. #	Moles ethylene produced per hour: per ml x 10^{-8} Replicates I	II	III	X±SD	per Heterocyst x 10^{-12} Replicates I	II	III	X±SD
1	9.40	9.56	9.52	9.50±0.009	0.823	0.837	0.834	0.831±0.008
2	21.22	23.38	24.27	22.95±1.57	1.857	2.047	2.124	2.009±0.138
3	7.81	7.07	7.21	7.37+0.39	0.683	0.619	0.631	0.645±0.034
4	21.70	16.79	12.15	16.88±4.78	1.889	1.470	1.063	1.478±0.418
5	13.21	14.97	9.61	12.60±2.73	1.157	1.310	0.841	1.103±0.239
6	17.85	17.14	16.06	17.12±0.94	1.563	1.527	1.406	1.499±0.082
7	10.28	10.14	10.43	10.28±0.14	0.900	0.888	0.913	0.900±0.013
8	11.21	14.11	11.64	12.32±1.57	0.981	1.235	1.018	1.078±0.137

TABLE XXVIII

ARA OF RUGGEDNESS TEST 2 ON DECEMBER 18, 1987
USING CULTURE STARTED ON DECEMBER 7, 1987.
POSITIVE STERILITY TEST

Exp. #	Moles ethylene produced per hour: per ml x 10^{-8} Replicates I	II	III	X±SD	per Heterocyst x 10^{-13} Replicates I	II	III	X±SD
1	9.29	9.81	9.41	9.50±0.27	7.75	8.18	7.85	7.93±0.23
2	16.35	13.63	17.79	15.96±2.16	11.35	10.33	12.73	11.47±1.20
3	8.10	8.99	8.42	8.50±0.45	9.15	9.73	10.13	9.67±0.50
4	13.61	12.39	15.26	13.78±1.70	11.35	10.33	12.73	11.47±1.20
5	10.96	11.66	12.14	11.59±0.59	9.15	9.73	10.13	9.67±0.50
6	11.81	15.09	13.89	13.63±1.70	9.86	12.58	11.58	11.34±1.38
7	9.31	9.68	10.28	9.76±0.50	7.76	8.07	8.58	8.14±0.41
8	9.52	8.57	9.30	9.13±0.50	7.94	7.15	7.76	7.62±0.42

TABLE XXIX

ARA OF RUGGEDNESS TEST 3 ON FEBRUARY 8, 1988 USING CULTURE STARTED ON JANUARY 28, 1988. NEGATIVE STERILITY TEST

Exp. #	Moles ethylene produced per hour per ml x 10^{-7} Replicates I	II	III	X±SD	per Heterocyst x 10^{-13} Replicates I	II	III	X±SD
1	1.176	1.394	1.357	1.309±0.116	7.448	8.826	8.591	8.289±0.737
2	6.652	7.334	7.724	7.237±0.543	42.119	46.439	48.906	45.845±3.397
3	2.474	2.047	2.834	2.452±0.394	15.667	12.962	17.942	15.524±2.493
4	6.273	6.131	6.185	6.196±0.280	39.721	38.819	39.164	39.234±0.455
5	4.350	4.908	4.581	4.613±0.280	27.545	31.076	29.006	29.209±1.774
6	5.414	5.037	4.491	4.981±0.404	34.282	31.895	28.437	31.538±2.939
7	4.117	4.221	4.204	4.181±0.056	26.070	26.726	26.618	26.472±0.352
8	6.482	6.232	5.976	6.230±0.253	41.039	39.460	37.839	39.446±1.600

TABLE XXX

ARA OF RUGGEDNESS TEST 4 ON NOVEMBER 11, 1987 USING CULTURE STARTED ON OCTOBER 30, 1987. NEGATIVE STERILITY TEST

Exp. #	Moles ethylene produced per hour per ml x 10^{-8} Replicates I	II	III	$\bar{X}\pm SD$	per Heterocyst x 10^{-13} Replicates I	II	III	$\bar{X}\pm SD$
1	6.926	3.012	3.222	4.165±1.819	19.633	8.536	9.133	12.434±6.242
2	2.584	2.343	2.202	2.316±0.283	7.324	6.642	8.581	7.516±0.984
3	2.963	2.925	3.028	2.973±0.052	8.398	8.289	8.581	8.423±0.148
4	3.770	3.256	3.600	3.542±0.262	10.686	9.228	10.203	10.030±0.732
5	0.000	2.908	3.041	1.983±1.719	0.000	8.243	8.620	5.621±4.872
6	3.173	3.135	3.013	3.107±0.084	8.995	8.887	8.540	8.807±0.238
7	2.851	2.878	2.811	2.873±0.020	8.081	8.158	8.194	8.144±0.058
8	3.014	3.003	3.094	3.037±0.050	8.543	8.512	8.770	8.608±0.141

TABLE XXXI

ARA OF RUGGEDNESS TEST 5 ON FEBRUARY 29, 1988 USING CULTURE STARTED ON FEBRUARY 14, 1988. POSITIVE STERILITY TEST.

Exp. #	Moles ethylene produced per hour: per ml x 10^{-9} Replicates I	II	III	X±SD	per Heterocyst x 10^{-13} Replicates I	II	III	X±SD
1	0.000	0.000	27.740	9.133±15.819	0.000	0.000	3.505	1.168±2.023
2	0.000	0.000	29.054	9.685±16.775	0.000	0.000	3.716	1.239±2.146
3	36.668	0.000	31.443	22.704±19.835	4.690	0.000	4.022	2.904±2.537
4	28.084	0.000	0.000	9.603±16.633	3.685	0.000	0.000	1.228±2.127
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	0.000	30.114	29.887	19.670±17.046	0.000	3.852	3.823	2.558±2.215

VITA

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