IMPACTS OF TOXICANTS ON BIOGEOCHEMICAL CYCLING

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PREFACE

The following report contains data and accompanying text from a project supported by the University Center for Water Research (UCWR), Oklahoma State University. This report is not intended to be a citable reference, because data and interpretations are preliminary and later may be updated or altered. If an individual wishes to cite data or interpretations contained in this report, the senior author should be contacted for updated information, and the material should be cited as a personal communiction.

Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the UCWR.

ABSTRACT

The objective of this research was to determine the ruggedness of the acetylene reduction assay (ARA) as a measure of nitrogen fixation. The ARA involves a multistep procedure. Each step has potential for error, so the variance can be quite high. This research identified that the following steps contributed no error to the final result of either poisoned or non poisoned cultures of Anabaena flos-aquae: Volume of media, gas purge time, filtration of acetylene vs. non filtration, injection volume of acetylene, incubation conditions (shaking vs non shaking; length of incubation) and volume of headspace of the incubation vessel. Variance in ARA from test to test seems to be due to physiology of cultures, not errors in measurement.

PROBLEM ADDRESSED

Biological nitrogen fixation (BNF) is an important step in the global nitrogen (N) cycle. One assay method for BNF is the reduction of acetylene to ethylene. It has its basis in the fact that the same enzyme complex reducing nitrogen to ammonium in the cell also reduces acetylene to ethylene.

The acetylene reduction assay has been applied in terresterial and aquatic environments to understand the BNF process. The acetylene reduction assay has been used also to determine the effect of toxicants on BNF by nitrogen fixing cyanobacteria (Brookes, 1986, Horne and Goldman, 1974, and Wurtsbaugh and Apperson, 1978, DaSilva et al, 1975, Lundvist, 1970 and Bastian and Toetz, 1985. However, many such reports fail to deal with the variability of the assay.

This research sought to clarify sources of variability in the acetylene reduction assay. Sources of variation are the mechanical procedures in the acetylene reduction assay and the condition of the culture.

OBJECTIVE OF STUDY

The objective of this research was to test the ruggedness of the mechanical aspect 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). 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.

METHODOLOGY

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 under irradiance of 15°C and 70 uE/m^2/sec. Allen's media used here and below were prepared according to James (1979).

Inoculum cultures. A subculture was transfered axenically from the stock culture to 5-10ml liquid media in a test tube. Cultures were incubated in continuous light (65±5 uE/m^2/sec) and constant temperature(25±°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 continuously agitated with a stirring bar at a continuous irradiance of 65±5 uE/m^2/sec and constant temperature of 25±1°C.

Monitoring Batch Cultures. After 7 days of growth, the batch cultrue 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 days 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 Baush and Lomb spectronic 20 colorimeter. The average of these values was converted into the $\log_2$(1 % Transmission) + 10 for mean and standard deviation calculations (n=3). Cell density was determined by counting number

of cells in a hemocyometer and dividing the result by volume, 1 x 10^{-4}ml. Eight counts were averaged for each sample.

Both cell and optical density were monitored until the culture reached the level of greatest acetylene reduction activity (ARA). At this point(maximum ARA) the ruggedness tests were performed.

The Ruggedness of the Acetylene Reduction Assay

Samples. 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 x 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, upon reaching maximum ARA, into two approximately equal volumes. Then added 0.3 ml concentrated $CdCl_2$ was added to the ETOX volume the resulting solution was diluted 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 $uE/M^2/sec$ and constant temperature of 25±1°C.

Two types of ruggednesss tests were performed. In three cases a ruggedness test was performed on cells that had reached maximum ARA (using ENTOX samples). Henceforth, these tests will be called non-poisoned ruggedness tests. Two tests were performed on cells that reached maximum ARA and subsequently poisoned with cadium as described above (using EXTOX 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 (1973) and Turner (1978). It is described below in some detail. I then describe the modifications of the protocol (induced technical errors) used to test the ruggedness of this protocol.

Protocol. 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 C_2H_2, which had been filtered through double distilled deionized water, was injected into the bottle. These were then to be 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 $uE/m_2/sec$ 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.

Quantification of the ethylene present in each sample bottle reequired the injection of 50.0 ml boiling water into the serum bottle, tapping all water out of the stopper and thoroughly purging the syringe with bottle gas immediately prior to gas chromatograph injection. 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 headspace 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 errors is most likely to occur. The magnitudes of induced technical errors are listed in the left hand column of Table 1. Differences from the protocol were directed towards the most likely bias based on experience to date. For example, protocol in Table 1 calls for 2.0 ml air removal from 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 remove 1.8 ml air 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 2). Table 2 shows all completed experiments for each ruggedness test. Experiment 1 involved no induced differences from protocol.

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 nitorgenase activity over batch culture growth, acetylene reduction activity was monitored every other day after cell density reached approximately 1 $x10^6$ cells/ml. Examination of ARA per ml media over culture growth showed an appreciable increase in ethylene production at 220 hours. Ethylene production per heterocyst exhibited relative constant ARA values over batch culture growth. The point where the largest increase of ethylene production occurred (as ethylene per ml media per hour) was interpreted as when ruggedness testing should begin (maximum ARA at optical density measurement of 10.200 $Log_2(OD)+10$, cell density of 2 x 10^6cells/ml and approximately 220 hours of culture age). It is at this point where cell density is high enough for appreciable and relatively constant ARA. Thus, 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 thus 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 mean of the test results from

experiments 5 through 8 (induced 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 technical error of 3.0 ml media volume did not affect the test. This criteria was used throughout the analysis of data.

Non-poisoned Ruggedness. Batch Cultures for Tests 1 and 3 were axenic, whereas the culture for Test 2 was non-axenic. 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. Results of analysis for test one are found in Tables 4 and 5. Both analyses show no significant change of ARA values due to any of the seven induced technical errors in protocol. Analysis of ruggedness for test 2 and 3 showed that none of the induced technical errors caused differences in ARA. Tabular data supporting these results and those described below for tests 4 and 5 are omitted here for brevity.

Range Finding to Establish ETOX Cultures. A batch culture was grown to maximum ARA then exposed to three concentrations of $CdCl_2$ (0, 2.084×10^{-6} and 2.084×10^{-4} moles/l). Each culture was monitored at 24 hour intervals to determine what concentration and length of exposure to cadmium. Comparison of growth between exposure levels did not show significant difference until 72 hours. At this point optical density of the 2.084×10^{-4} moles Cd/l culture was significantly less than that of either control or the 2.084×10^{-6} moles Cd/l exposure.

Examination of ARA values as moles ethylene produced per ml media per hour and moles ethylene produced per heterocyst per hour showed similar results. No significant difference from control was found for the 2.084×10^{-6} moles Cd/l culture. Comparision of the 2.084×10^{-4} moles Cd/l to control shows a significant difference at 72 and 96 hours of exposure.

For purposes of insuring substantial ARA inhibition from control, we concluded that poisoned ruggedness testing should proceed after exposing cultures to 2.084×10^{-4} moles Cd/l (1000 ug/l) for 96 hours. ETOX (poisoned) samples recieved this treatment.

Poisoned Cultures. Exposure concentrations as deterimined by atomic absorption were 2.397×10^{-6} moles Cd/liter and 2.084×10^{-6} moles Cd/liter for Tests 4 and 5, respectively. Statistical analysis was performed to determine the ruggedness of the poisoned acetylene reduction assay expressed in moles ethylene produced per ml media per hour and in moles ethylene produced per heterocyst per hour. Results of Test 4 show no significant change of ARA for any of the seven chosen protocol steps. The results of Test 5 were are similar.

DISCUSSION

The Effect of Ruggedness Testing on ARA

Each ruggedness analysis suggests the 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". Use of a purge gas, filtered commercial grade acetylene and a shaker table during incubation could possibly be omitted from the protocol to yield an economical assay for use in the field with minimal amount of equipment.

Further investigation should be done with each item separate to determine the degree each can effect 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 are 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.

Analysis of ARA values between cultures would not be statistically valid due to confouding effects of different contamination and toxicant concentrations with each culture grown. Further tests using replicate test conditions would enable separation of within test variation into components of culture contamination, toxicity, physiological state, and measurement error. It is important to state which aspects of the acetylene reduction assay protocol are being used, modified, or deleted in published results. This will reveal areas of variance for comparison between reports.

Conclusions

The acetylene reduction assay has been shown to be "rugged" using poisoned and non-poisoned aquatic nitrogen fixing cyananobacteria. Small variances of media volume, volume of acetylene injected, incubation time, and volume of water injected in the assay protocol did not effect the ARA. Use of a purge gas, filtered acetylene, and a shaker table are items of the protocol that could be eliminated from the protocol depending on the restrictions of the researcher.

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Table 1. The chosen steps, protocol and induced technical error used to test the ruggedness of the acetylene reduction assay.

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 2: Experimental design to test the ruggedness of the acetylene reduction assay protocol.

Exp. #	Media Volume Used	Purge Time	Filter C_2H_2	C_2H_2 Volume Injected	Shaker Table use	Incubation Time	Water Volume Injected
1	50.ml	1.5 min	Yes	2.0ml	Yes	2.0hr	5.0ml
2	50.ml	1.5 min	No	2.0ml	No	2hr10min	4.7ml
3	50.ml	0.0 min	Yes	1.8ml	Yes	2hr10min	4.7ml
4	50.ml	0.0 min	No	1.8ml	No	2.0hr	5.0ml
5	47.ml	1.5 min	Yes	1.8ml	No	2.0hr	4.7ml
6	47.ml	1.5 min	No	1.8ml	Yes	2hr10min	5.0ml
7	47.ml	0.0 min	Yes	2.0ml	No	2hr10min	5.0ml
8	47.ml	0.0 min	No	2.0ml	Yes	2.0hr	4.7ml

Table 3. Ruggedness test results for media volume using data from the axenic, ENTOX samples performed on Nov. 30, 1987. Test 1.

Experiment Number	Media Volume	Mean ARA as Moles C_2H_4/ml media/hr (n=3)	Average	Two times Standard Deviation
1	A(50.ml)	2.47×10^{-8}		
2	A(50.ml)	5.97×10^{-8}	3.685×10^{-8}	3.714×10^{-8}
3	A(50.ml)	1.91×10^{-8}		
4	A(50.ml)	4.39×10^{-8}		
5	a(47 ml)	3.28×10^{-8}		
6	a(47 ml)	4.45×10^{-8}	3.408×10^{-8}	1.482×10^{-8}
7	a(47 ml)	2.70×10^{-8}		
8	a(47 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.

Table 4. ARA values per heterocyst and significance of ruggedness test performed on 11/30/87 using ENTOX culture started on 11/20/87. Test 1.

Protocol Step	Acetylene Reduction Activity Value (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 Used	12.40±6.24	11.45±2.53	0.95	12.48	No
Purge Gas Use	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

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 5. ARA values per ml media and significance of ruggedness test performed on 11/30/87 using ENTOX culture started on 11/20/87. Test 1.

Protocol Step	Acetylene Reduction Activity Value (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	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	0.15	13.56	No

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.