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Evaluation of Measurement Errors in Toxicity Tests for Nitrogen Fixation

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Biological nitrogen fixation (BNF) has been studied extensively in the field and laboratory using the acetylene reduction technique because it is rapid and sensitive (Klucas 1969 and Burris 1972). The technique has its basis in the fact that nitrogenase, the enzyme complex in the cell that reduces nitrogen to ammonium, also reduces acetylene to ethylene. However, it is an indirect measure of BNF, because there is no exact relationship between nitrogen fixation and acetylene reduction activity (ARA) (Mayne 1984). It has been used to study BNF on the ecological and organismic level (Granhall and Lundgren 1971; Reed et al. 1980; Tesfai and Mallik 1986). It has also been used to determine the effect of toxicants on BNF by nitrogen fixing cyanobacteria (Brookes et al. 1986; Horne and Goldman 1974; Wurtsbaugh and Apperson 1978; DaSilva et al. 1975; Lundkvist 1970; Bastian and Toetz 1985). In toxicity testing the experimental error of ARA is often large and attributed to either physiological state of the cells or measurement error (Bastian and Toetz 1985). There is a need, then, to determine which of these two sources of error is important. This research sought to determine sources of error in procedural aspects of the acetylene reduction assay.

The method used below to evaluate procedural aspects of the assay is called ruggedness testing (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. Ruggedness testing can identify items in the protocol where strict compliance to protocol is necessary. We tested the ruggedness of the ARA assay as a toxicity test and as a measure of nitrogen fixation. The experimental design used in ruggedness testing is a fractional replication of factorial experiment, which allows examination of the main effect factors may have on a test result using a reduced number of experiments (Cochran and Cox 1957). A full factorial experiment would require 128 separate experiments to examine the effect of seven factors on a test result. In the full experiment one factor would be manipulated at a time, for all possible combinations of the others. In ruggedness testing, a 1/16 fraction factorial uses eight separate experiments to examine the main effects of seven different factors.

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errors based on experience. In addition, we included two induced technical errors, which were anticipated to have a predictable response. Since N_2 is a competitive inhibitor of ARA, purging the system with argon would eliminate any competition with N_2. This gas purge step has been found to be unnecessary as long as the partial pressure of injected ethylene is above 0.2 atm. (Hardy et al. 1973). Thus, the induced technical error not to purge the head space would not be an error at all as one could anticipate that these samples would have the same ARA as unpurged samples.

Table 1. The steps, protocol and induced technical errors used to test ruggedness.

Step	Protocol	Induced Technical Error
Gas purge	Purge	No purge
C_2H_2 (commerical grade) inoculation	Filtered with deionized water	Unfiltered
Volume of media used	50 mL	47 mL
C_2H_2 injection volume	2.0 mL	1.8 mL
Shaker table	Used	Not used
ARA incubation period	2.0 hr	2.2 hr
Volume of boiling H_2O injected prior to C_2H_4 analysis	5.0 mL	4.7 mL

Table 2. Experimental design of each ruggedness test. All experiments were in triplicate.

Experiment	Volume Used mL	Media Purge	Filter C_2H_2	C_2H_2 Volume Injected mL	Shaker Table Use	Incubation Time hr	Water Volume Injected mL
1	50.0	Yes	Yes	2.0	Yes	2.0	5.0
2	50.0	Yes	No	2.0	No	2.2	4.7
3	50.0	No	Yes	1.8	Yes	2.2	4.7
4	50.0	No	No	1.8	No	2.0	4.0
5	47.0	Yes	Yes	1.8	No	2.0	4.7
6	47.0	Yes	No	1.8	Yes	2.2	5.0
7	47.0	No	Yes	2.0	No	2.2	5.0
8	47.0	No	No	2.0	Yes	2.0	4.7

Tank ethylene often contains contaminants such as acetone which could reduce ARA (Hardy et al. 1973). Thus, samples receiving

METHODS AND MATERIALS

Stock cultures of Anabaena flos-aquae (UTEX 1444) were obtained from the Culture Collection at the University of Texas, Austin. A subculture was transferred axenically from the stock culture to 5-10 mL liquid media. The growth media used was Allen's blue-green media (James 1979). Cultures were incubated in continuous light (65 ± 5 Einsteins/m^2/sec) and constant temperature ($25\pm1^{o}C$) for 3-5 days until green. This irradiance and temperature was also used in the ruggedness tests.

A nitrogen-free batch culture was started by preparing 4,000 ml of Allen's media without nitrogen ($NaNO_3$), autoclaving the media and adding 3-5 ml of algal inoculum. It was aerated with a mixture of 2% CO_2 in air prefiltered through a 0.20μm pore size Millipore filter. The culture was agitated with a stirring bar at the irradiance and constant temperature reported above.

After 7 days of growth, batch cultures were examined to determine if they were axenic by withdrawing a sample and streaking a loop of this sample onto autoclaved Tryptic Soy Agar in a petri dish. Cell and optical density were monitored daily throughout batch culture growth.

The protocol used to determine ARA was a modification of the methods described by Hardy et al. (1973). The headspace of sample bottles containing 50.0 mL media was purged for 1.5 min with $Ar/CO_2/O_2$ gas mixture (79%, 0.04%, 21%), 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 were incubated for two hours on a shaker table under the irradiance and temperature described above. ARA was terminated with 2.0 mL 4N HCl. Ethylene quantification was accomplished using a Tracor 560 Gas Chromatograph. The headspace pressure in each bottle was increased by injection of 5.0 ml boiling water. The syringe was thoroughly purged with headspace gas prior to injection (volume 1.0 mL).

Blanks and controls were used in triplicate, respectively, in each ruggedness test. Blank samples contained 50.0 mL double distilled deionized (DDD) water plus 2.0 mL 4N HCl, and accounted for trace amounts of ethylene in the acetylene. Control samples contained 50.0 mL of batch culture media at maximum ARA plus 2.0 ml 4N HCl.

Two types of ruggedness tests were performed. Ruggedness was tested using cells that had reached maximum ARA but not exposed to toxicants (tests 1-3). Two ruggedness tests were performed on cells that had reached maximum ARA and were subsequently poisoned with Cd as described below (tests 4 and 5).

We identified five steps in the above protocol where technical error was most likely to occur (Table 1). Differences from the protocol are called induced technical errors, and are probable

unfiltered ethylene would have a different ARA than samples receiving filtered ethylene. In this case known error was deliberately introduced. The design of a single ruggedness test is shown in Table 2. The first experiment always followed the protocol exactly.

We determined the time of maximum ARA in a batch culture as follows. Every other day ARA was monitored after cell density reached approximately 1×10^6 cells/mL. There was no appreciable increase in ethylene production per heterocyst after 220 hr at cell densities of 2×10^6/mL. Thus, ruggedness tests were accomplished using other batch cultures after 220 hours of growth.

A batch culture was grown to maximum ARA and then exposed to three concentrations of $CdCl_2$ (0, 2.1 and 210μM Cd/1), as a way to determine the concentration of Cd toxic to ARA. Each culture was then monitored every 24 hours to determine what concentration and length of exposure to cadmium was necessary to produce a marked reduction of ARA. There was no significant difference between the control ARA and ARA of the 2.084μM Cd/1 culture. But ARA of the 208.4μM Cd/1 culture was significantly different from the control at 72 and 96 hours of exposure. We concluded that poisoned ruggedness testing should proceed after exposing cultures to about 200μM Cd/1 for 96 hr. Actual exposures in tests 5 and 6 were 239.4 and 208.4μM Cd/1, respectively. Cadmium concentrations were determined on a Perkin-Elmer 5000 Atomic Absorption Spectrometer using the graphite furnace technique (EPA 1979). Standard curves for ethylene were made by injecting ethylene standard gas (Matheson Gas Products Co.) to encompass the maximum and minimum responses elicited from sample injection ($r > 0.95$). All tests except 2 and 5 were axenic.

RESULTS AND DISCUSSION

Ethylene was not detected in all samples. Thus, to avoid zero values a value corresponding to 50% of the detection limit was subtracted from each experimental value, and these differences were used to calculate a mean and a standard deviation for each experiment in a test. Statistical analysis to determine ruggedness was performed for values of ARA expressed as ethylene production per mL media per hr and ethylene production per heterocyst per hr. Since the outcome was the same for both, only the results for the latter are given below.

The importance of an induced technical error was determined by comparing the average of the results for the four experiments with no induced error to the average of the four experiments with that induced error using a t-test (Table 3). As an example, mean ARA was 12.41 ± 6.24 and 11.45 ± 2.53 M 10^{-13} per heterocyst per hr respectively, for media volumes of 50 and 47 mL in test 1. The difference was not significant (Table 3). Other tests of significance were made in a similar way.

The induced technical errors of media volume, acetylene injection

volume, incubation time, shaking and water injection volume had no effect on ARA. In these respects the assay is "rugged" and small deviations in each will have no effect on test results. When acetylene was filtered through water, ARA was significantly lower than when it was used directly from the tank in tests 1 and 3, and marginally so in test 2 (Table 4). No such diffference was observed in poisoned tests. No other induced technical error caused a significant difference in ARA.

Table 3. Ethylene production per heterocyst, t-tests and significance (test 1, unpoisoned). *Significant at 0.05% level.

Ethylene Production (M C_2H_4/heterocyst/hr) x 10^{-13}

	No Technical Error X±SD	Technical Error X±SD	Difference between Means	P	t
Media Volume Used	12.41±6.24	11.45±2.53	0.96	0.7871	0.3
Purge Gas Use C_2H_2	13.61±5.12	10.25±3.50	3.35	0.3212	1.1
Filtration C_2H_2	8.70±1.89	15.16±3.82	6.46	0.0230	3.0*
Injection Volume	12.05±5.46	11.81±4.01	0.23	0.9521	0.0
Shaker Table Use	10.13±3.69	13.73±4.87	3.59	0.2843	1.2
Incubation period	11.23±2.67	12.63±6.13	1.40	0.6896	0.4
Volume of H_2O Injected	11.71±3.61	12.09±5.74	0.32	0.9330	0.0

Table 4. Ethylene production per heterocyst, t-tests and significance by test for the experiment involving acetylene filtration. *Significant at 0.05% level.

Acetylene Reduction Activity (M C_2H_4/heterocyst/hr) x 10^{-13}

Test	No Technical Error X±SD	Technical Error X±SD	Difference between Means	P	t
1	8.70±1.89	15.16±0.382	6.46	0.0230	3.0*
2	3.50±1.25	6.66±2.35	3.15	0.0556	2.4
3	17.88±9.75	36.86±6.40	18.97	0.0170	3.3*
4	1.77±2.05	1.59±0.66	0.19	0.8704	0.2
5	1.45±2.87	0.92±0.98	0.83	0.7401	0.3

The mean values of ARA for a given experiment were very different between tests. For example, the ARA value for no technical error

for media volume was 12.41, 5.67 and 25.46 M ethylene per heterocyst per hr x 10^{-13} in tests 1-3, respectively. The ARA for media volume (no technical error) was 2.42 and 180 M per heterocyst per hr x 10^{-13} in poisoned tests 5 and 6, respectively. Thus, ARA values were consistent within tests, but not between them.

The test results did not reveal a consistent procedural error. Thus, if the same protocol for testing is followed again there is little evidence to anticipate that procedural errors in the protocol are important sources of variability. Rather, the problem of variability seems to be in variability in the physiological state of algae in different batches. Batch cultures of Anabaena seem to exhibit considerable physiological differences. Apparently, we were not able to reproduce the exact physiological state in all cultures, even though theoretically it should be so. Future research needs to address this problem.

An assumption necessary to the use of the ruggedness design is that interaction between factors is negligible. The factors most likely to interact in this research are the factors involving measurement of media volume, ethylene volume and volume of boiling water injected. These may have interacted to affect gas pressure of ethylene within each experimental bottle. For purposes of statistical analysis, we assumed that such interactions, if they occurred, had little effect.

Filtration of tank acetylene through water probably removed only acetone and not other contaminants such as H_2S (Hardy et al. 1973). However, our initial assumption was that this induced technical error in the protocol would show an effect. This assumptiom was verified at least in the unpoisoned tests. In addition, gas purge did not affect ARA as was anticipated. Thus, the induced technical errors programed into the ruggedness tests had the effects anticipated.

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