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Hydrogen Oxidation Soil Bioassay Using the Single Laboratory Method

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Before a biological process can be used as an operational bioassay, the quality of data produced by the method must be evaluated. The most vigorous, and therefore expensive, form of evaluation is the collaborative test. While there is no question that such testing is appropriate, economic constraints dictate that some consistent approach be employed to select viable bioassays for testing. Single laboratory testing has been proposed as a method to establish the data quality that could be achieved within a single laboratory. This data can then be used as an indication of the kind of results which could be expected from a collaborative test. Recently, guideline methods were published by EPA (McKenzie and Olson, 1984) which stated that a method should be evaluated based upon its capability for ruggedness (identifying procedural variables that must be carefully controlled), evaluation of systematic error (bias), and identification of method preceision and accuracy.

Because of a potential usefulness to regulatory agencies, the Hydrogen Oxidation Soil Bioassay was selected as a candidate for single laboratory testing. Rogers and McFarland (1982) previously demonstrated that this bioassay was a potential method for monitoring the effect of available soil borne hazardous wastes. The method responded in a predictable manner to various concentrations of monmixtures of toxic compounds (cadmium, silver, mercury, fluoride, monuron, methanearsonic acid, dalapon, 2, 4-D, ozone, NO_2, and tricresyl phosphate) as well as complex environmental samples. While numerous assays were performed on soil amended with these compounds and complex mixtures, sufficient performance data to qualify the method for collaborative testing was lacking. The purpose of this paper is to present the results obtained from the single laboratory evaluation of the Hydrogen Oxidation Soil Bioassay.

MATERIALS AND METHODS

Only a brief overview of Hydrogen Oxidation Soil Bioassay will be presented to provide the reader with the essentials of the method. A more detailed discussion of the method can be found elsewhere (Rogers, 1984).

The soil used for the bioassay was a Calico series fine sandy loam (Aquic Xerofluvent). After collection, it was air dried to a constant weight, sieved to pass a 0.60-mm screen, and stored in plastic bags at room temperature. Chemicals whose toxicity was evaluated by the method were all reagent grade and included mercuric nitrate [$Hg(NO_3)_2$], mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_3$), cadmium nitrate [$Cd(NO_3)_2$], cadmium chloride ($CdCl_2$), and sodium pentachloraphenate (PCP). All testing was done with aqueous solutions of these compounds.

The bioassay was initiated by adding 10 ml of water carrying the toxic compounds or distilled water, if a control, to 100 g of the air dried soil residing in a 1-L, round-bottom flask. The treated soil was then incubated at 25 C in the dark for 16 h. All testing was set up in triplicate.

To evaluate the H_2 oxidizing activity of the treated soil, the following procedure was used. Each flask was flushed with air and then sealed with rubber stoppers. Immediately after that, 5 ml of nitrogen (N_2) containing 0.5 µCi of HT was injected through the stopper. After charging, the flasks were incubated an additional 2 h. The HT oxidizing reaction was stopped at the precise time (2 h) by flushing the flasks with air and the quantity of HT oxidized was determined by recovering the reaction product, HTO, from the soil by distillation.

An index analogous with the LC50 was used to compare the relative effect that compounds have on the oxidation of hydrogen. Because the hydrogen oxidation test measures the rate of a chemical reaction rather than lethality, the term $P_2$50 was used to identify that concentration of a compound that reduces the reaction rate (P_2) by 50 percent. The $P_2$50 term was obtained from a linear regression line generated from a plot of the ratio (as a percent) of a P_2 Control (P_2c) and at least three P_2 treatment (P_2t) values versus the log concentration of the treatment. The calculated concentration that resulted in a P_2t value that was 50 percent of the P_2c was designated as the treatment $P_2$50 value.

RESULTS AND DISCUSSION

A preliminary study was conducted in order to select two chemicals which would be used for the single laboratory evaluation. The test was conducted in such a manner that chemical concentration ranges and sample matrix were similar to what might be encountered when using a hazardous waste sample. Other work had indicated that the bioassay was sensitive to the effects of the heavy metals Hg, Ag, and Cd and some organics (Rogers and McFarlane, 1982). These metals as well as PCP apparently act as enzyme inhibitors (Williamson and Johnson, 1982; Ito and Ohnishi, 1982).

Initially, the nitrate (NO_3) forms of Hg, Ag, and Cd were tested. It was determined that Hg and Ag had $P_2$50 values of 26 ppm and 76 ppm while the one for Cd was 133 ppm. In addition, the chloride (Cl) compounds of Hg and Cd (AgCl was not used because it is

not soluble in H_2O at the working concentrations) were evaluated. The $P_2$50 value for $HgCl_2$ (36 ppm) was found to be comparable with that of $Hg(NO_3)_2$ (26 ppm) while that of $CdCl_2$ (707 ppm) was six times greater than Cd $(NO_3)_2$ (133 ppm).

PCP was found to have a $P_2$50 of 177 ppm. These results are consistent with data which showed that the amount of PCP required to decrease nitrogenase activity by 50% had a range of between 49 and 660 ppm depending upon soil conditions (Tam and Trevors, 1981). This wide range is apparently due to the unavailability of the chemical after its addition to soil. For example, even with extreme extraction procedures (low pH, methylene chloride, sonication) only 80% of applied PCP could be recovered immediately after application from the soil used in the above study.

As a result of this preliminary work, aqueous solutions of $HgCl_2$ and PCP were selected for use as sample materials for the single laboratory test. Mercury was selected both because it appeared to be more toxic than Ag and Cd, and because $HgCl_2$ is more soluble than $Hg(NO_3)_2$. PCP was used as a representative toxic organic which could be associated with hazardous waste.

After selection of representative toxic chemicals, the single laboratory test was initiated. This testing was intended to supply data on ruggedness, precision, accuracy, sensitivity, and reliability of measurement. The results of these evaluations follow.

A method's ability to produce unchanged results while being subjected to minor procedural variations is an indication of its "ruggedness" (Youden, 1969). Procedural methods and variations used for the ruggedness test are summarized in Table 1. The seven protocol directed methods (A-G) were chosen because they are the ones which, in our judgement, could inadvertently be altered and are indicated by a-g. The protocol directed method and its corresponding variation were then arranged into a series of eight trails. Each trail consisted of a single analysis of a single concentration of the test compound, 50 ppm Hg or 100 ppm PCP, and a combination of procedural variations. Final test results from the eight trails are indicated as s-z.

The effect of each individual method or variable was obtained by calculating the mean oxidation value for all the treatments which contained that factor. For example, the factor A was part of the trail which produces results for analyses s, t, u, and v. Therefore, the mean for A can be calculated from the summation of s-v [A = (s + t + u + v)/4]. Means for the other factors (A-G and a-g) were calculated the same way. After tabulation of the means, the differences between the method and variation were computed, e.g., A-a, etc. If one or two variables were having an effect on test results, their respective differences would be substantially larger than the group of differences associated with the other variables.

Table 1. Variations in the Hydrogen Oxidation Soil Bioassay Used to Determine "Ruggedness"

Item	Protocol Directed	Variation
1. Time a flask is purged with air.	A. Purge time 10 s	a. Purge time 6 s
2. Time soil is pre-incubated with test compound.	B. Preincubation time 16 h	b. Preincubation time 20 h
3. Time soil is incubated with HT.	C. Incubation time 120 min	c. Incubation time 135 min
4. Water, containing test compound, applied to soil.	D. Amount water 10 ml	d. Amount water 11 ml
5. Frequency of mixing soil.	E. Frequency of mixing 2 beats/s	e. Frequency of of mixing 1 beat/s
6. HTO derived from distillation.	F. Quantity of HTO 8 ml	f. Quantity of HTO 7.9 ml
7. HTO distilled from soil.	G. Amount of HTO distillate 15 ml	g. Amount of HTO distillate 17 ml

Results of this test are in Table 2. It is apparent that some differences can result from procedural changes. The differences, however, were not consistent between the two toxic compounds. This was most noticeable with factors D(PCP) and G(Hg). Why these factors did not produce similar results with each test is not known, but these data would suggest that the variations resulted from undetermined sources.

Table 2. Effect of procedural variations for "ruggedness" testing with Hg and PCP

	% Change due to Procedural Variation	
Factor	Hg	PCP
A	7.9	2.4
B	7.9	2.4
C	9.0	11.4
D	1.4	15.8
E	12.3	2.4
F	12.3	2.4
G	15.5	1.1

In general, it appears that the modest procedural variations evaluated had little effect on the test results. This indicates that the test is "rugged" in the sense that variations in the method of a magnitude that could be made by a qualified laboratory following a written method protocol would not be expected to significantly alter test outcomes.

Method precision was determined by conducting 10 separate assays using the same concentration of the same test material. Each of the separate determinations represented a valid test response as directed by the method protocol. Testing was conducted on alternate days and used $HgCl_2$ (75 ppm Hg) and PCP (100 ppm). The average test response for $HgCl_2$ was 61.6% (±4.8%) HT oxidation and for PCP 63.6% (±3.4%). These data showed that the method has good precision, but that some variability is present.

Because the Hydrogen Oxidation Bioassay had not been widely used neither a standard reference material nor a known method repsonse to the materials used for testing were available. Therefore, under single laboratory test guidelines the question of accuracy was approached by conducting 20 independent assays of the two test compounds. For this evaluation, 100 ppm of both Hg and PCP were used. The responses to these concentrations were 59.4% (±5.4%) for Hg and 64.4% (±2.2%) for PCP. Future use of the test under similar conditions can use these data as reference points for method response.

In the context of a single laboratory test, a method's sensitivity is defined as its capability to respond to small changes in the concentration of test compound. The ability of the method to distinguish between changes in sample concentrations of Hg ($HgCl_2$) and PCP was tested by selecting one concentration of each compound greater than a reference concentration and one lower. Ten independent analyses for each of the concentrations were then conducted. If the method could distinguish between the reference and the extreme concentrations, a new concentration equal distance from the reference and extreme concentrations were tested. This was repeated until the difference between the reference and a treatment was 25 ppm for Hg and 50 ppm for PCP. The reference concentration for Hg was 75 ppm with 10 and 150 ppm being the extremes and 50 and 100 ppm serving as midpoints between the reference concentration and the extremes. For PCP, the reference concentration was 200 ppm, with 1, 100, 150, 250, 300, 400 ppm being the bracketing concentrations. The testing with Hg allowed for an upper and lower detection of 75 ppm (65 ppm on the lowest concentration) and 25 ppm, and for PCP, these concentrations were 200, 100, and 50 ppm. The ranges were expanded for PCP because background data on how it affected the method was not available.

Test results for the different concentrations of sample material are given in Table 3. Statistical comparison between the reference concentration and those used as brackets were made (ANOV). Differences significant at the 5% level for Hg were found for concentrations which differed by 75 and 25 ppm from the reference

concentration while for PCP, the range was 200, 100, and 50 ppm. These data indicate that with Hg concentrations ranging between 10 to 150 ppm, increments of at least 25 ppm should produce effects which will be statistically distinguishable. For PCP, a range between 1 and 250 ppm should distinguish 50 ppm intervals. The upper limit on PCP concentration was dictated by the lack of significance between 250 and 300 ppm PCP.

Table 3. Method sensitivity of the hydrogen oxidation soil bioassay using aqueous solutions of Hg, $HgCl_2$, and PCP

Chemical	µg/g Soil	Average Response (% of Control)
Hg	10	80.3[a]* ± 5.1
	50	67.9[b] ± 7.5
	75	61.6[c] ± 4.8
	100	56.2[d] ± 4.4
	150	38.3[e] ± 2.2
PCP	1	97.7[a] ± 3.6
	100	63.6[b] ± 3.4
	150	56.7[c] ± 3.5
	200	52.0[d] ± 5.0
	250	47.6[e] ± 4.3
	300	45.1[e] ± 3.9
	400	39.9[f] ± 5.9

* Mean values within each chemical treatment followed by the same letter are not significantly different at the 5% level as determined by ANOV.

The tests designed to establish the limits of reliable measurement determined the concentration range of test compound for which the method was capable of providing useful data. More specifically, the single laboratory test was used to verify that the method's capabilities for sensitivity and precision did not deteriorate at the upper and lower extremes of the detection range.

While upper and lower detection limits for the compounds used in the Hydrogen Oxidation Bioassay were not available from the literature, data from the preliminary testing showed that the test compounds produced extremely different effects when applied in concentrations ranging between 10 and 1,000 ppm. Therefore, an effort was made to collect data on the reliability (i.e., precision and sensitivity) of the method at both of these extremes as well as for intermediate concentrations of the two test compounds.

Concentrations of Hg (as $HgCl_2$) used for this evaluation were 10, 100, 500, 750, and 1000 ppm. For PCP, the range was the same except the 500 ppm concentration was not used. Mean test results for each sample concentration were compared using ANOV as the indicator of differences in the test results (Table 6). These data indicate that the method was sensitive (at the 5% level) to incremental increases of Hg up to 750 ppm and PCP to 1000 ppm.

Results for Hg between 750 and 1000 ppm were not statistically distinguishable. While the method's capability for precision with Hg deteriorated from 500 to 1000 ppm, it might still be able to provide useful data (in terms of precision) at elevated concentrations of toxic compounds. Precision for PCP was acceptable over the range of concentrations tested though it did decrease at 750 ppm and above.

Table 4. Limits of reliable measurement for the hydrogen oxidation soil bioassay using aqueous solutions of Hg($HgCl_2$) and PCP

Chemical	µg/g Soil	Average Response (% of Control)
Hg	10	80.3^{a*} ± 5.1
	100	56.2^{b} ± 4.4
	500	11.5^{c} ± 4.2
	750	2.6^{d} ± 0.7
	1000	1.2^{d} ± 0.4
PCP	10	86.2^{a} ± 4.3
	100	65.3^{b} ± 3.4
	750	27.4^{c} ± 3.4
	1000	17.7^{d} ± 2.2

* Mean values within each chemical treatment by the same letter are not significantly different at the 5% level as determined by ANOV.

Based on the above test, the limits of reliable measurement for the Hydrogen Oxidation Soil Bioassay are therefore presented as 10 and 750 ppm of Hg when aqueous solutions of $HgCl_2$ are used and 10 and 1000 ppm for aqueous solutions of PCP.

In conclusion, by following the single laboratory test protocol there is much better understanding of the performance of the Hydrogen Oxidation Soil Bioassay. The assay was determined to be rugged in the sense that slight procedural variations had little effect on test outcomes and at the same time, the method's sensitivity and precision were very good within its limits of reliable measurements. Based on these findings, the assay could be recommended as a candidate for collaborative testing.

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