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Project Summary

Single Laboratory Evaluation of the Hydrogen Oxidation Soil Bioassay

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The Hydrogen Oxidation Soil Bioassay was single laboratory tested as a potential method for monitoring hazardous wastes and hazardous waste sites. The bioassay is based on the rate of hydrogen consumption by soil microorganisms. Oxidation of hydrogen to water is inhibited when various pollutants are present in the soil and the rate of this reaction can be used as an indication of potential hazard to the soil ecosystem.

The single laboratory evaluation included ruggedness testing, a determination of method sensitivity and precision, and tests to determine the limits of reliable measurement. Since there was no "true value" or "true response" to a reference material, the method's capability for bias (systematic error) was not determined. Aqueous solutions of mercuric chloride were used as sample material during the evaluation. Some preliminary tests were also conducted using both organic compounds and actual hazardous waste samples.

The bioassay was found to be "rugged" in the sense that modest procedural variations did not produce an altered test result. The method's capability for precision, expressed as a CV of 7.8 percent, was determined by conducting 10 separate bioassays using the same concentration of mercuric chloride. Within a mercury concentration range of 10 ppm to 150 ppm, the technique was capable of distinguishing between concentration differences of 25 ppm. The limits of reliable measurement were established at 10 ppm and 750 ppm mercury when mercuric chloride solutions are used as sample material. The complete Hydrogen Oxidation Soil Bioassay protocol, the results of chemical analyses (i.e., gas chromatography/mass spectrometry, atomic absorption, and inductively coupled argon plasma spectroscopy) conducted on actual samples that were used during the single laboratory test, and the preliminary bioassay responses to different types of sample material are included in the project report.

Before this bioassay can be considered for collaborative testing, it will be necessary to conduct portions of the single laboratory test again using sample material that more realistically simulates either a hazardous waste site leachate, or an analytical fraction of actual hazardous waste material. However, results from this evaluation suggest that this terrestrial monitoring technique should ultimately be a candidate method for collaborative testing and should be of subsequent benefit to a hazardous waste monitoring network.

This Project Summary was developed by EPA's Environmental Monitoring Systems Laboratory, Las Vegas, NV, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Single laboratory testing is used to establish the data quality that can be achieved within a single laboratory. It also provides a basis for deciding whether

or not a given method merits collaborative testing. The previously prepared guidelines for testing biological methods (EPA-600/S4-83-056) have been followed during this evaluation. This approach calls for an identification of procedural variables that must be carefully controlled (ruggedness testing), determination of method sensitivity and precision, and identification of the limits of reliable measurement. In addition, if the response of the evaluated method to a standard reference material is known (true response), method bias should also be determined.

The Hydrogen Oxidation Soil Bioassay is based on the bio-oxidation of hydrogen (H_2) to water (H_2O) hy the hydrogenase enzyme. This enzyme is of microbiological origin (their ability to oxidize H_2 is documented). The microbial forms are assumed to be ubiquitous in soil. If tritium (designated as either $^3H_2$, T_2, or HT; HT is used in this Summary) is introduced, it is oxidized in proportion to its abundance (Figure 1). The product (HTO) from this reaction can be used to determine the rate of H_2 oxidation in a given soil.

With experimentation, it has been found that HT oxidation rates in soil amended with toxic compounds decreased linearly with the log of the compound concentration. This has been shown to occur with toxic liquid, solid, and gaseous compounds.

During the evaluation it was assumed, that if the technique was being routinely used, sample material (e.g., actual hazardous waste material, leachate, analytical fractions of hazardous waste material, water samples that potentially contain waste site chemicals, etc.) would be sent to the assay laboratory. Soil from a hazardous waste site area could potentially be used as test material, but the method has not been evaluated for this type of application. During the single laboratory test, the method protocol was strictly followed. A copy of the complete Hydrogen Oxidation Soil Bioassay protocol is included in the project report.

Procedure

The soil used for the bioassay is a Calico series fine sandy loam (Aquic Xerofluvent) from southeastern Nevada. Chemicals used as sample material were all reagent grade and included mercuric nitrate [$Hg(NO_3)_2$], cadmium chloride ($HgCl_2$), silver nitrate ($AgNO_3$), cadmium nitrate [$Cd(NO_3)_2$], mercuric chloride ($CdCl_2$), pyrocatechol, m-chlorophenol, and p-chlorophenol. Samples

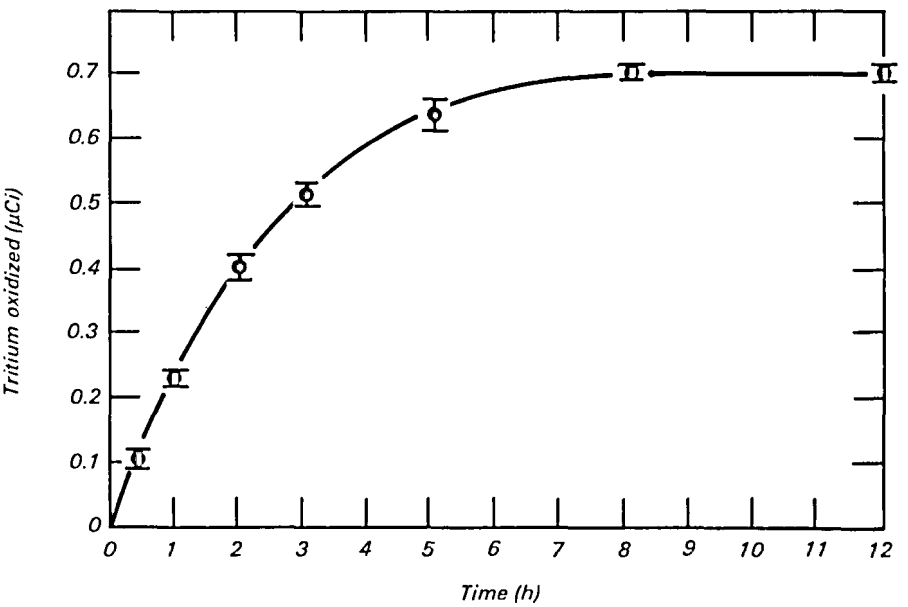

Figure 1. HT oxidation in soil with time.

which had been collected from an actual hazardous waste site were also used during a preliminary evaluation (Table 1). Testing was initiated by adding 10 ml of water carrying the toxic compounds, 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 hours. The assay must be conducted in triplicate (triplicate flasks for each treatment and control) so that a mean and coefficient of variation (CV) can be determined.

Following the incubation period, each flask was flushed with air for 10 s (20L/min) and then sealed with a rubber stopper. 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 returned to the environmental chamber (25°) for an additional 2 hours. The HT oxidizing reaction was stopped at the precise time (2 hours) by flushing the flasks with air. Since the consumption of the H_2/HT is rapid and begins immediately, care was used to charge the flasks sequentially

*Table 1. Major Components of Hazardous Waste Site Material Used During the Method Evaluation**

Semivolatile Organics (μg/L)	*Volatile Organics (μg/L)*
phenol	*trichloroethylene*
2-nitrophenol	*benzene*
benzoic acid	*toluene*
pentachlorophenol	*methylene chloride*
alpha-BHC	
beta-BHC	
gamma-BHC	
delta-BHC	*Inorganics (mg/L)*
fluoranthene	
dibenzofuran	*aluminum*
trimethylnaphthalene	*copper*
benzo(c)fluoranthene	*iron*
benzo(a)pyrene	*sodium*
di-n-butylphthalate	*nickel*
2-6-dinitrotoluene	*selenium*
1,2-benzene dicarboxylic acid	*zinc*

**Hazardous waste sample analysis conducted by Acurex Corporation, Mountain View, California 94039*

with the same order being followed when the reactions were stopped. To determine the amount of HT oxidized, the reaction product, HTO was first recovered from the soil by distillation (Figure 2). Then the quantity of HTO in the distillate was determined by liquid scintillation analysis. An outline for the entire method is shown in Figure 3.

Treatment effects caused by each test compound were determined by calculating a ratio of zero-time reaction rates for treatments and controls. Reaction rates were calculated from the exponential growth model

$$Y = P_1 [1-\exp(-P_2 t)] + E$$

where:

Y = tritium content

P_1 = the asymptotic tritium content

P_2 = the reaction rate parameter

t = time in hours

E = the error function (assumed to be Gaussian).

By entering the asymptotic HT content (the amount injected) and the amount of HTO recovered at the time sampled, the rate of hydrogen oxidation (P_2) can be calculated. The rate of hydrogen oxidation in treated soil (P_{2t} or P_2 treatment), divided by the rate of hydrogen oxidation in untreated soil (P_{2c} or P_2 control), is then tabulated for each treatment concentration. These data are then graphically plotted.

Results

Preliminary test results confirmed that the assay was responsive to aqueous solutions of Hg, Ag, and Cd, to metal combinations, to phenolic compounds, and to actual hazardous waste site material (i.e., metals added to waste site material to provide a more realistic sample matrix). Aqueous solutions of mercuric chloride were used as sample material during the single laboratory evaluation because Hg appeared to be more toxic than Ag and Cd, and because $HgCl_2$ is more soluble than $Hg(NO_3)_2$. An aqueous solution of mercuric chloride might also be used as a much simplified example of a hazardous waste site leachate. Qualitative and quantitative analyses were conducted on all mercuric chloride sample material to confirm the chemical composition and to ensure lack of sample contamination.

The first phase of the single laboratory evaluation was to determine if minor departures from the method protocol would result in an altered bioassay result. A method's ability to produce an unaltered test result when subjected to minor procedural variations is an indication of method ruggedness. If the results are altered by small procedural variations, it is important to emphasize in the protocol that a specific step must be strictly followed or, in some cases, to provide more detail on any quality control steps associated with the critical procedure.

The prescribed method procedure and the corresponding procedural variations used during the ruggedness evaluation are summarized in Table 2. The seven protocol directed procedures (A-G) were chosen because they are the ones which, in our judgment, could inadvertently be altered as indicated by the variations a-g.

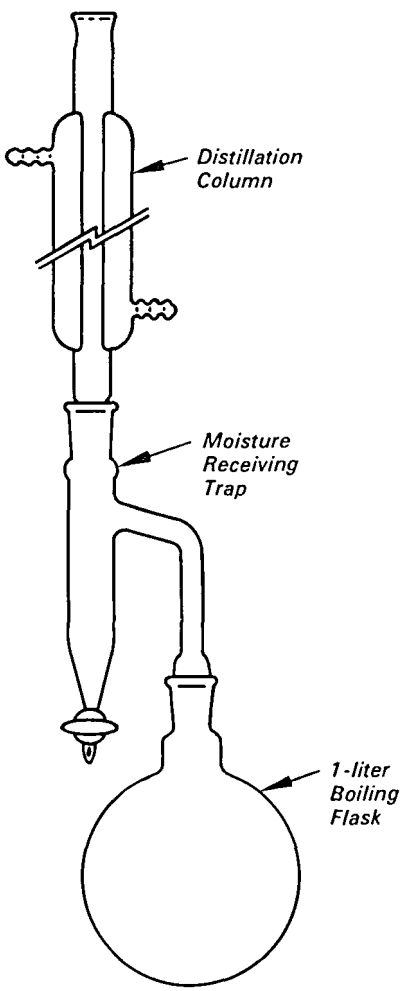

Figure 2. Distilling apparatus for the collection of tritiated water from soil exposed to tritium during the Hydrogen Oxidation Soil Bioassay.

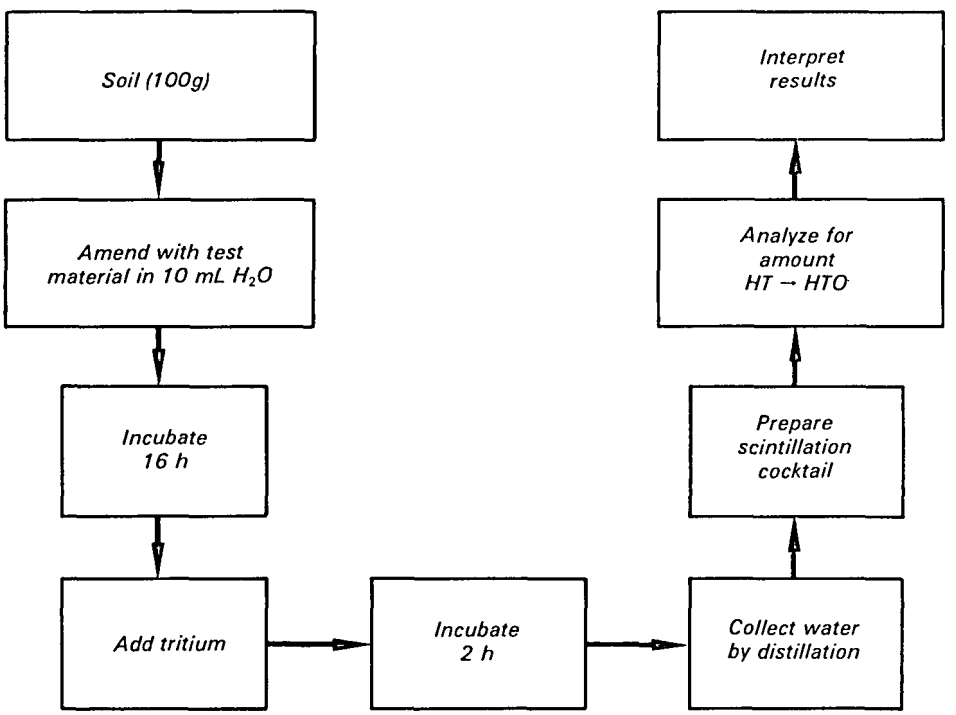

Figure 3. Outline of procedures for the Hydrogen Oxidation Soil Bioassay.

Table 2. Variations in the Hydrogen Oxidation Soil Bioassay Used to Determine "Ruggedness"

Item	Protocol Directed	Variation
1. Length of time a flask is purged with air both before introduction of HT and to flush out remaining HT after incubation.	A. Purge time 10 s	a. Purge time 6 s
2. Length of time soil is pre-incubated with test compound.	B. Preincubation time 16 h	b. Preincubation time 20 h
3. Length of time soil is incubated in presence of HT.	C. Incubation time 120 min	c. Incubation time 135 min
4. Amount of water, containing test compound, applied to soil.	D. Amount water 10 mL	d. Amount water 11 mL
5. Frequency of mixing soil after application of water.	E. Frequency of mixing 2 beats/s	e. Frequency of mixing 1 beat/s
6. Quantity of HTO derived from distillation which is mixed with liquid scintillation cocktail.	F. Quantity of HTO 8 mL	f. Quantity of HTO 7.9 mL
7. Amount of HTO distilled from soil.	G. Amount of HTO distillate 15 mL	g. Amount of HTO distillate 17 mL

The protocol directed procedures and the corresponding procedural variations were then arranged into a series of eight trials. Each trial consisted of a single analysis of a single concentration of $HgCl^2$ (50 ppm Hg) and a pre-selected combination of procedural variations.

Basically, the procedural variations had little effect on the assay response and, based on the ruggedness test results, it was not considered necessary to revise the method protocol. The assay is "rugged" in the sense that modest variations in method procedure would not be expected to alter the assay result.

Method precision was determined by conducting 10 separate tests with each of the separate determinations representing a valid test response. Testing was conducted on alternate days and used 75 ppm Hg as sample material. The average response to this treatment was 61.6 percent reduction in HT oxidation with a CV of 7.8 percent.

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 a test compound. The ability of this bioassay procedure to distinguish between changes in Hg concentration was initially tested using one concentration greater than that used for the precision determination and one lower. Ten independent analyses were conducted for each of the new concentrations. If the method can distinguish between the concentration used during the precision determination and the two newly selected concentrations, the concentration interval is reduced and additional concentrations are tested. For this single laboratory test, the process was repeated until the concentration interval had been reduced to 25 ppm.

The concentration used for the precision test was 75 ppm with 10 and 150 ppm being the initially tested extremes and with 50 and 100 ppm as midpoints between the reference concentration and the extremes. It was therefore possible to determine if the bioassay could initially distinguish between Hg concentrations of 75 ppm (65 ppm in the case of the lower concentration) and then between concentration differences of 25 ppm.

Results for the sensitivity determination are included in Table 3. The bioassay was capable of distinguishing between Hg concentration differences of 75 ppm and between differences of 25 ppm (significant at the 5 percent level). Therefore, the method's single laboratory capability for sensitivity has been presented as 25 ppm Hg when aqueous solutions of mercuric chloride are used as sample material.

Tests to establish the limits of reliable measurement should determine the sample concentration range for which the method is capable of providing useful data. In some instances, the single laboratory test may simply verify that the method capabilities for sensitivity, precision, and accuracy (if applicable) do not deteriorate at the upper and lower extremes of the detection range.

Three additional concentrations of Hg were used in addition to those used for the method sensitivity test. These new concentrations were 500 ppm, 750 ppm, and 1,000 ppm. Table 3 presents a compilation of test data obtained from both the additional concentrations and from the concentrations used for the sensitivity determination. The results indicate that the method was sensitive to incremental increases of Hg up to 750 ppm. Results betwen 750 ppm and 1,000 ppm were not statistically distinguishable. As noted previously, test results from the lower concentrations were distinguishable. Between 10 ppm and 150 ppm of Hg, the method's capability for precision can be described as having a CV range of 5.7 to 11.0 percent. Method capability for precision suffered from 500 ppm to 1,000 ppm. The limits of reliable measurement for the Hydrogen Oxidation Soil Bioassay are presented as 10 ppm and 750 ppm Hg when aqueous solutions of mercuric chloride are used as sample material.

Conclusions

The Hydrogen Oxidation Soil Bioassay was single laboratory tested as a potential method for use in hazardous waste monitoring networks. Preliminary test results confirmed that the assay was responsive to aqueous solutions of Hg, Ag, and Cd, to metal combinations, to phenolic compounds, and to actual hazardous waste site material (i.e., metals added to waste site material to

Table 3. Data Used for Determining the Method Sensitivity and the Limits of Reliable Measurement Using Aqueous Solutions of Mercuric Chloride

	Response (% of Control) µg Hg/g Soil							
Determination	10	50	75	100	150	500	750	1000
1	79	73	63	59	38	8	2	1
2	92	68	59	59	41	10	3	2
3	85	68	68	59	38	12	2	2
4	72	62	54	50	41	11	3	1
5	80	80	64	60	38	11	3	1
6	79	68	59	55	35	10	3	1
7	79	73	63	55	38	12	3	1
8	79	68	63	51	35	10	3	1
9	79	68	68	63	38	23	3	1
10	79	51	55	51	41	8	1	1
$\bar{x}$	80.3[a]*	67.9[b]	61.6[c]	56.2[d]	38.3[e]	11.5[f]	2.6[g]	1.2[g]
SD	5.1	7.5	4.8	4.4	2.2	4.2	0.7	0.4
CV	6.4	11.0	7.8	7.8	5.7	36.5	26.9	33.3

**Mean values followed by the same letter are not significantly different at the 5% level.*

provide a more realistic sample matrix). The bioassay was found to be "rugged" in the sense that modest procedural variations did not produce an altered test result. The method's capability for precision, expressed as a CV of 7.8 percent, was determined by conducting 10 separate assays using the same concentration of mercuric chloride. Within a mercury concentration range of 10 ppm to 150 ppm, the technique was capable of distinguishing between concentration differences of 25 ppm. Limits of reliable measurement were established at 10 ppm and 750 ppm of mercury. The single laboratory tested method protocol has, of course, also has been prepared. Before this bioassay can be considered for collaborative testing, it will be necessary to conduct portions of the single laboratory test again using sample material that more realistically simulates either a hazardous waste site leachate, or an analytical fraction of actual hazardous waste material.

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***W. W. Sutton** is the EPA Project Officer (see below).*
The complete report, entitled "Single Laboratory Evaluation of the Hydrogen Oxidation Soil Bioassay," (Order No. PB 84-211 317; Cost: $10.00, subject to change) will be available only from:

National Technical Information Service
5285 Port Royal Road
Springfield, VA 22161
Telephone: 703-487-4650

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