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Project Summary

Freshwater Assay Using Soil Eluates as Sample Material (Single Laboratory Evaluation)

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The Chlorophyta assay, which uses soil as sample material, has been a useful bioassessment technique for screening hazardous waste site problems. An eluate is prepared from a 125-gram soil sample and then diluted into three separate concentrations prior to being tested using *Selenastrum capricornutum*. The work reported here has attempted to determine the procedure's capability for data quality, to provide a basis for deciding whether the assay merits collaborative testing, and to define more clearly the method's potential for inclusion as part of an operational monitoring network.

The soil used for most of this evaluation was a homogenized clay loam (characterized as being 22% sand, 51% silt, and 27% clay). Samples were chemically analyzed using ICAP, GC/MS, and Kjeldahl nitrogen techniques to confirm that a reasonably homogenized soil had been selected for the evaluation. Soil containing either sodium fluoride or 2,4-dichlorophenol was tested using the algal assay, and several tests were conducted to confirm the dose/response curve using the positive control compound, zinc chloride. A known test response also was established using a white silica sand spiked with sodium fluoride.

Although considerable progress was made toward a standardization of this procedure, some difficulties remain for collaborative testing. The known test response provides a value with a fairly high standard error, which, in some ways, probably detracts from the future application of this technique when accuracy and/or systematic error estimates are needed. The current evaluation also revealed an apparently poor capability for sensitivity and a somewhat limited range of reliable measurement. However, the procedure will definitely detect the presence of certain chemicals and, thus, can be effectively used as a screening technique. Consequently, the observed method sensitivity should not necessarily be considered detrimental to further method standardization or to future application as part of a monitoring network.

This Project Summary was developed by EPA's Environmental Research Laboratory, Athens, GA, 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

Assessing the significance of current and potential problems at uncontrolled hazardous waste sites has been very difficult because (1) complex chemical mixtures are found at most sites and (2) toxicity and environmental fate data are limited for many of the compounds present at the sites, especially for those that are by-products of either organic synthesis or of organic degradation. Biological tests can be important components of waste site monitoring programs. Benefits include their ability to predict the potential hazard posed by unknown mixtures of chemicals, to identify the bioactive fractions of unknown chemical mixtures, and similarly, to detect toxic substances not normally reported by standard chemical analysis.

If biological methods are to be used as part of the waste site monitoring effort, the selected procedure(s) must be capable of

providing reliable and repeatable results. Consequently, it is necessary to determine the procedure's capability for data quality in a single laboratory and, by way of a collaborative test, in multiple laboratories.

This research concerns an evaluation of a modified freshwater assay. The assay is based on a procedure developed by EPA's Environmental Research Laboratory, Corvallis, OR, as a bioassessment method for hazardous waste sites. The assay method has been tested at actual waste sites and found to be one of the better screening techniques for detecting the presence of potentially hazardous chemicals. An aqueous eluate is acquired initially from the original soil sample, and this eluate is subsequently filtered and diluted prior to toxicity testing. This algal assay procedure addresses neither non-water soluble chemicals nor volatile compounds. In addition, procedures associated with the collection of soil samples (e.g., representative sampling of a waste site area) are not addressed. Based on the ecological importance of unicellular algae, however, this relatively simple technique can provide an environmental hazard assessment for those chemicals that are most likely to be transported to surface and ground waters.

This work seeks to establish the data quality that can be achieved within a single laboratory. The objective of this evaluation also provides a basis for deciding whether this modified algal procedure merits collaborative testing and to define more clearly the assay's potential for inclusion as part of an operational monitoring network.

Procedure

Single Laboratory Evaluation

Phases of the single laboratory test include identification of procedural variables that must be carefully controlled (ruggedness testing), evaluation of method sensitivity, identification of the limits of reliable measurement, evaluation of systematic error (bias), and identification of method precision and accuracy.

Ten successive analyses (i.e., a series that yields ten valid responses by following the method protocol) are typically conducted for several phases of the single laboratory test. The determination of method precision, for example, requires that ten successive independent analyses be conducted on the same sample material. Multistage calculations to determine the required number of analyses might be conducted during the single laboratory test as more information becomes available on the expected variance. However, 10 analyses will allow the test laboratory to estimate the standard deviation to within 45 percent of its true value (at a 95 percent confidence interval). The single laboratory test cost will increase rapidly as more of these successive analyses are required because each additional value must represent a valid test response and, therefore, will include whatever quality control analyses (blanks, replicates, etc.) are required in the original method protocol to ensure a valid test response.

Consensus Review

The method protocol was reviewed by persons familiar with both this specific assay and with many of the problems encountered at uncontrolled hazardous waste sites. A consensus review is always necessary as an attempt to achieve consensus agreement prior to beginning the method standardization process. The protocol was slightly revised based on the review comments and reviewed a second time by the same scientists. However, as is often the case, complete consensus agreement for each step of the assay was not totally achieved.

Outline of Assay Procedure

Soil Preparation

A blender is initially used to homogenize the soil which is then smoothed to an even layer in a ceramic pan. The soil is then air dried before a 125-gram portion is used for the assay. Rocks or gravel are manually removed prior to drying. The 125-gram soil sample is transferred to a 1-liter teflon-lined container and 500 ml of deionized water are added. The container then is capped and mechanically shaken. The liquid is decanted from the container and transferred to 50-ml centrifuge tubes and then centrifuged. The total amount of soil eluate recovered from the container will vary somewhat between samples but at least 400 ml should be available for analysis.

Eluate Dilutions

This leachate (soil eluate) is filtered, and the pH is adjusted prior to testing. Several preliminary filtering steps (i.e., 2.7 μm; 1.6 μm; 1.0 μm; etc.) may be required depending on the characteristics of any precipitate noted in the resulting soil eluate. After centrifugation, the eluate typically is transferred to a Buchner funnel that contains a 0.7-μm, nonsterile glass fiber filter. Subsequently, the eluate is filtered through a 0.45-μm nylon filter to obtain a sterile eluate. Although the growth response of *Selenastrum capricornutum* is not affected over a wide range of pH values, the potential array of chemicals and soil types encountered when using the procedure occasionally will require pH alterations for the leachate. Therefore, several pH determinations are routinely conducted and, when required, pH adjustments are made using 1N sodium hydroxide and 1N hydrochloric acid.

In the next step, the soil eluate is diluted to a series of three separate concentrations using the growth medium preparation. Algal cells are transferred from the stock culture to each dilution flask. A negative control also is prepared using the growth media preparation and the algal cell addition. Zinc chloride is added in addition to the growth medium and algal cells when preparing the positive control. Ninety-six hours after the flasks are inoculated, the cell concentration in each flask is determined using a Coulter counter.

Duplicate flasks are prepared routinely for the negative control, positive control, 80% soil eluate, 10% soil eluate, and 1% soil eluate, respectively. In the case of the positive control, separate response values are tabulated. The first step of the final calculation is to determine the eluate effect for each of the three dilutions and the second step is to extrapolate an EC_{50} value which then serves as the final test result. The eluate effect is tabulated using the initial inoculum cell count and the 96-hour cell count determined for each eluate dilution and for the negative control. The proportion of growth medium used in a given dilution flask also is included in the tabulation. A positive control response also is routinely calculated for each separate assay.

Cell Culture

A pure monoculture of algal cells is required for the assay. The algal cells are first transferred from the stock culture to an inoculum flask, which should contain 50 ml of previously sterilized deionized water. When the assay flasks are prepared, the cell transfer should contain a sufficient number of cells so that the resulting concentration (in the 50 ml of soil eluate plus growth medium) will be $1 \times 10^4 \pm 1 \times 10^3$ cells/ml.

Nutrient Composition

Total consensus was never reached concerning the composition of the nutrient medium. The nutrient media used obviously will affect the algal response, but persons experienced with the assay simply do not agree on the concentration of some medium components. The growth medium used throughout this evaluation, and the medium composition specified in the "consensus" method protocol, is given in Table 1. The medium is prepared by adding 1.0 ml from each of the Table 1 solutions (macronutrient and micronutrient) to 900 ml of sterilized deionized water. The final volume is taken to 1 liter.

Table 1. *Composition of Algal Growth Media*

Macronutrients

Stock Solution [a]		Nutrient Composition Prepared Medium	
Compound	Concentration (g/l)	Element	Concentration (mg/l)
	Stock Solution I		
$NaNO_3$	25.500	N	4.200
$NaHCO_3$	15.000	Na [b]	11.001
		C	2.143
	Stock Solution II		
K_2HPO_4	1.044	K	0.469
		P	0.186
	Stock Solution III		
$MgSO_4\ 7H_2O$	14.700	S	1.911
	Stock Solution IV		
$MgCL_2\ 6H_2O$	12.164	Mg [c]	2.904
$CaC1_2\ 2H_2O$	4.410	Ca	1.202

Micronutrients

Stock Solution		Nutrient Composition Prepared Medium	
Compound	Concentration (mg/l)	Element	Concentration (µg/l)
	Stock Solution V		
H_3BO_3	185.520	B	32.460
$MnC1_2\ 4H_2O$	415.610	Mn	115.374
$ZnC1_2$ [d]	3.271	Zn	1.570
$CoC1_2\ 6H_2O$ [d]	1.428	Co	0.354
$CuC1_2\ 2H_2O$ [d]	0.012	Cu	0.004
$Na_2MoO_4\ 2H_2O$ [d]	7.260	Mo	2.878
$FeC1_3\ 6H_2O$	160.000	Fe	33.051
$Na_2EDTA\ 2H_2O$	300.000	–	–

[a] *Other forms of the salt may be used if the resulting element concentrations are the same as those listed.*
[b] *Includes Na from $NaNO_3$.*
[c] *Includes Mg from $MgSO_4\ 7H_2O$.*
[d] *Prepare at 100 times concentration and add in designated amount to stock solution V.*

Positive Control

Zinc chloride was used as the positive control compound but many other chemicals undoubtedly could be selected. The purpose of the positive control is to provide information on whether the algal assay is responding to a known toxic material and, after several assays have been conducted, the various positive control responses can provide data that are used to assess the variability occurring between assays. The positive control response is not used when tabulating the EC_{50} value for the final test result, but the positive control values obviously are used to help determine whether a test response is accepted as a valid test result.

The positive control contains the growth medium solution, the algal cell inoculum, and the zinc chloride. It should be emphasized that the positive control compound is added directly to the assay flask and not to a soil sample. Two positive control flasks are prepared for each assay. The average response of the two positive controls must show at least a 15 percent inhibition of cell concentration as compared to the negative control. In addition, the cell counts from the two positive control flasks must be within 50 percent of each other in order to achieve a valid test result. If these conditions do not exist for the positive control result, the assay results will not constitute a valid test response. The suggested zinc chloride concentration of the positive control is 128 µg/l. The suitability of this concentration, however, should be verified by the testing laboratory prior to beginning an actual series of assays.

Sample Material

Water eluates were prepared from a series of soil types and tested using *Selenastrum capricornutum* prior to the selection of a clay loam soil (characterized as being 22% sand, 51% silt, and 27% clay) for use during the evaluation. After the soil was selected, a

suitable quantity (approximately 275 kg) was collected so that enough soil would be available for the entire single laboratory effort. The soil was homogenized thoroughly, first by grinding and then by mixing in a mechanical soil mixer for several hours. The soil and the subsequently prepared soil eluate were chemically analyzed primarily to determine whether a sufficiently homogenized preparation had been achieved for use in the method evaluation. Analyses also were conducted to confirm the transport of fluoride from the spiked soil (sodium fluoride) to the eluate and to compare this transport with the eventual EC_{50} test result. Results from this analytical effort are shown in Tables 2 and 3.

Results and Discussion

Ruggedness Test

The first step in the evaluation was to identify those procedural steps that must be carefully controlled. If the Chlorophyta procedure is "rugged" it will not be susceptible to the inevitable, modest departures in routine that occur, and the final test result will

Table 2. *Element Analysis of Soil Subsamples Taken from the Homogenized Soil Collection Used for the Chlorophyta Evaluation*

	Element Composition (µg/g)								
Subsample Number	Mg	K	Zn	Fe	Mn	Cu	Ca	SO_4-S	pH
1	27.3	21.5	7.5	76.2	71.8	0.76	195.5	7.5	5.5
2	29.5	22.3	7.5	73.6	71.6	0.48	202.5	7.0	5.5
3	27.5	20.8	7.5	71.0	67.4	1.24	180.5	7.0	5.4
4	27.3	20.1	6.8	68.9	64.1	1.53	184.5	6.8	5.4
5	24.7	19.0	6.4	65.4	59.8	1.52	170.0	7.0	5.4
6	26.7	20.3	6.8	66.0	59.4	0.49	176.0	7.0	5.4
7	27.6	20.2	7.1	68.7	67.0	1.05	199.5	9.3	5.6
8	26.3	20.0	7.7	73.6	71.6	0.48	182.5	7.5	5.5
9	27.7	21.8	6.6	64.9	63.5	0.55	189.5	7.3	5.5
10	29.5	22.8	7.4	74.6	60.2	0.63	185.2	5.5	5.5
$\bar{X}$	27.4	20.9	7.1	70.5	65.6	0.9	185.3	7.2	5.5
±S.D.	1.4	1.2	0.5	4.4	5.0	0.4	11.3	0.9	0.1
C.V.[a]	5.2	5.7	6.3	6.2	7.6	49.4[b]	6.1	12.9	1.2

[a] *In addition to the results shown above, a few nitrogen analyses were conducted (Kjeldahl nitrogen) using the soil eluates. These analyses revealed an essentially consistent concentration (µg/ml) occurring between eluates from different soil subsamples. Some attempts at organic compound identification also were conducted, i.e., eluate samples were analyzed using GC/MS and LC/MS procedures. Few specific organics were detected and those that were identified were detected at low concentrations (ng/ml for eluates and ng/g for the actual soil). Tentatively identified organic compounds included benzoic acid, hydroxy methoxy benzaldehyde, and methyl hexanone. The various chemical analyses have helped to confirm that a reasonably homogenized soil was selected for the current Chlorophyta evaluation.*

[b] *With the exception of potential analytical error at these relatively low concentrations, no explanation is presented for this unusually high coefficient of variation.*

Table 3. *Approximate Transfer of Sodium Fluoride from Soil to Eluate Using the Chlorophyta Assay Procedure*

	Amounts of sodium fluoride added to 125-g soil sample (mg)				
	556	900	1090	1112	2224
Sodium fluoride concentration in soil (mg/g)	4.5	7.2	8.7	9.0	17.8
Sodium fluoride concentration in eluate (µl/ml)	378	442	770	693	1714
Total recovery in eluate (mg)	151.2	176.8	308.0	277.2	685.6
Approximate recovery of spike (%)	27	20	28	25	31
$\bar{X}$ EC_{50} for assay	-0.7	56.6	50.9	44.6	32.7
Standard deviation ±	—	6.5	7.5	2.6	8.2
Coefficient of variation (%)	—	11.4	14.8	5.8	25.1

not be altered by these slight variations. 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 indicate the limits of allowable variability. The experimental design used during the ruggedness test (based on the design by W. J. Youden In The Collaborative Test) did not seek to study each separate variable in an individual sequential fashion, but rather to provide for the simultaneous introduction of multiple protocol variations (Tables 4 and 5).

As noted in Table 4, eight complete assays were conducted as part of the ruggedness evaluation. Each test sample contained 100 mg of the organic compound sodium dodecyl sulfate that had been added to the 125-gram aliquot soil samples immediately prior to the test. The "protocol directed" conditions were designated as A through G, and the varied conditions were designated as a through g. The evaluation was concerned with identifying respective variations in the final test result due to the specific procedural differences, i.e., A-a, B-b, C-c, D-d, E-e, F-f, and G-g. Each of the eight trials consisted of a single analysis conducted using eight aliquots of the test material (100 mg of sodium dodecyl sulfate). The final test results were indicated as s, t, u, v, w, x, y, and z.

Table 4. *Experimental Design for a Seven Variable Ruggedness Test* [a]

Test Number	*Combination of Variables*	*Test Result*
1	*A B C D E F G*	*s*
2	*A B c D e f g*	*t*
3	*A b C d E f g*	*u*
4	*A b c d e F G*	*v*
5	*a B C d e F g*	*w*
6	*a B c d E f G*	*x*
7	*a b C D e f G*	*y*
8	*a b c D E F g*	*z*

[a] *Based on W.J. Youden, 1969, The Collaborative Test, p. 151-158. In Precision Measurement and Calibration. H.H. Ku, Editor. U.S. Department of Commerce, National Bureau of Standards. 436 pp. For the Chlorophyta evaluation, each test sample contained 100 mg of the organic compound sodium dodecyl sulfate that had been added to the 125-gram aliquot soil samples immediately prior to the test.*

Table 5. *Test Variables Used to Evaluate Ruggedness of Chlorophyta Procedure*

Directed Instruction	*Altered Instruction*
A. 125-g soil aliquot used as sample material for assay.	*a. 110-g soil aliquot used as sample material for the assay.*
B. at start of assay, each flask contains 1 x 10^4 algal cells per ml.	*b. at start of assay, each flask contains 0.75 x 10^4 algal cells per ml.*
C. original soil collection is first air dried for 24 hours (room temperature).	*c. original soil collection is first air dried for 20 hours (room temperature).*
D. 500 ml of water added to the aliquot sample of air dried soil.	*d. 550 ml of water added to the aliquot sample of air dried soil.*
E. soil eluate is pH adjusted to between 6.0 - 8.5 prior to the assay.	*e. soil eluate is pH adjusted to pH 10 prior to the assay.*
F. eluate dilution series consists of 1%, 10%, and 80% soil eluate.	*f. eluate dilution series consists of 1%, 30%, and 80% soil eluate.*
G. algal assay terminated at 96 hours.	*g. algal assay terminated at 92 hours.*

The average of A = (s + t + u + v)/4, compared with the average of a = (w + x + y + z)/4, serves as a rapid means of assessing the effect of changing variable A to a. Because each of the two groups of four determinations contains the other six variables (twice at the upper case level and twice at the lower case level), the effect of these variables (if present) tends to cancel out, leaving only the effect of changing variable A to a. The relative effect of the other variables was estimated by examining the following averages:

$$B = \frac{(s + t + w + x)}{4} \qquad b = \frac{(u + v + y + z)}{4}$$

$$C = \frac{(s + u + w + y)}{4} \qquad c = \frac{(t + v + x + z)}{4}$$

$$D = \frac{(s + t + y + z)}{4} \qquad d = \frac{(u + v + w + x)}{4}$$

$$E = \frac{(s + u + x + z)}{4} \qquad e = \frac{(t + v + w + y)}{4}$$

$$F = \frac{(s + v + w + z)}{4} \qquad f = \frac{(t + u + x + y)}{4}$$

$$G = \frac{(s + v + x + y)}{4} \qquad g = \frac{(t + u + w + z)}{4}$$

After tabulating the above averages, the differences between each respective variable was computed, e.g.,

$$A - a = \frac{(s + t + u + v)}{4} - \frac{(w + x + y + z)}{4}$$

Most of the modest procedural alterations should typically have little or no effect on the test result. However, a comparison of the respective differences (A-a, B-b, etc.) provides considerable information on which variables, if any, are having the greater effects.

The analysis showed that moderate procedural variations definitely altered the final Chlorophyta test result when they occurred at certain critical steps. Steps shown to be most sensitive to variation involved variables D, E, and F, i.e., the amount of water added to the dry soil sample, the pH of the resulting soil eluate that is actually used during the assay, and the specific eluate concentrations prepared for the assay dilution series. The Chlorophyta protocol consequently was revised to emphasize strict adherence to these critical instructions.

Precision

The precision evaluation was conducted using the ruggedness tested, and ruggedness revised, Chlorophyta method protocol. Each sample contained 8.9 mg of sodium fluoride per gram of homogenized clay loam soil. This sodium fluoride concentration was selected after several concentrations were tested and after it was shown that the assay response was close to the calculated EC_{50} concentration. The final result seemed to represent the best attainable precision when using the Chlorophyta procedure.

Ten separate tests using aliquots of the same sample were conducted. Each separate test shown in Table 6 represents a valid test as required by the Chlorophyta method protocol. Based on the 10 separate determinations using 8.9 mg sodium fluoride per gram of soil, the assay's single laboratory capability for precision (expressed as a coefficient of variation) is presented as 5.8 percent. An attempt was made to conduct the individual determinations on alternate days, i.e., an interval of at least one day between the completion of one assay and the start of the next. While an exactly identical interval was not maintained between individual assays due to the eluate preparation time and problems of routine laboratory scheduling, the individual analyses shown in Table 6 were conducted sequentially over a total time interval that covered several days.

Table 6. *Chlorophyta Assay's Single Laboratory Capability for Precision*

(Sample Material Consisted of 8.9 mg Sodium Fluoride per Gram of Soil. Valid Test Result Was Achieved for Each Assay.)

		Positive Control			
Assay Number	Calculated EC_{50}	#1	#2	% Difference	Negative Control
1	41.0	-53.0	-59.3	8.9	2.9×10^6/ml
2	47.4	-51.0	-69.0	26.1	2.8×10^6/ml
3	41.1	-51.0	-59.5	14.3	2.7×10^6/ml
4	42.1	-58.8	-50.2	14.7	2.6×10^6/ml
5	46.6	-62.9	-72.9	13.9	2.6×10^6/ml
6	44.8	-57.7	-65.7	12.2	2.6×10^6/ml
7	46.0	-72.0	-80.4	10.0	2.7×10^6/ml
8	43.5	-89.5	-91.1	2.0	2.6×10^6/ml
9	45.5	-93.6	-94.8	1.3	2.5×10^6/ml
10	48.2	-72.0	-76.1	5.4	2.5×10^6/ml
$\bar{X}$	44.6				
±S.D.	2.6				
C.V.(%)	5.8				

Note: Each individual soil sample weighed 125 grams and 1112 mg of sodium fluoride was added to each sample. As directed by the method protocol, the assay result was not considered to be a valid test result unless (1) the negative control had achieved at least 1.0×10^6 cells/ml at the 96-hour time interval, (2) an average of the two positive control results demonstrated at least a 15 percent inhibition in cell growth, and (3) the two positive control results did not differ from each other by more than 50 percent.

Response to Organic Compound

Soil containing 2,4-dichlorophenol also was tested using the Chlorophyta procedure. Chlorinated hydrocarbons frequently are present at hazardous waste sites and they definitely represent one of the waste site chemical groups that cause environmental concern. This particular chemical was tested as a representative organic pollutant that is somewhat resistant to microbial degradation.

The dichlorophenol was added to the clay loam test soil to achieve a concentration of 80 μg/g. Separate determinations were conducted using ten identical soil samples that contained this concentration of dichlorophenol. Each of the ten determinations represented a valid test response as directed by the Chlorophyta method protocol. It has already been established that the Chlorophyta procedure was responsive to organic pollutants as well as to actual waste site samples that contain low concentrations of several chemicals. This method responsiveness was certainly present when the soil samples contained dichlorophenol. As is noted in Table 7, however, the observed precision is not very impressive when compared to the test results achieved using sodium fluoride. Since preliminary testing preceded selection of the 80 μg/g dichlorophenol concentration, these data may suggest a responsive procedure but a somewhat less precise assay than expected when the sample contains an organic pollutant (i.e., 46.5 percent coefficient of variation when testing dichlorophenol).

Method Sensitivity

For purposes of a single laboratory evaluation, a method's sensitivity is defined as the method's capability to detect (or distinguish between) small changes in sample concentration, i.e., concentrations of analyte. The specific concentrations for the sensitivity phase usually are selected sequentially based on results from previously selected concentrations. The same sample material (sodium fluoride in clay loam soil) used during the precision test phase was used for the method sensitivity evaluation. Table 8 presents a series of assay results that were ultimately used to assess the method's capability not only for sensitivity but also for the limits of reliable measurement.

The single laboratory evaluation typically uses ten independent assays for each new concentration, i.e., ten separate valid responses acquired by following the written protocol. Under routine operating conditions, however, the assay would only be conducted one time per sample. Consequently, when tabulating data for sensitivity, non-overlapping standard deviations (rather than non-overlapping standard errors) are used to indicate whether the method can distinguish between the different samples.

As noted in Table 8, the Chlorophyta procedure does not have a particularly impressive capability for sensitivity. Concentrations of sodium fluoride between 17.8 mg/g and 48.0 mg/g of soil produced the same test response. In addition, concentrations of sodium fluoride between 8.9 mg/g and 7.2 mg/g of soil produced the same test response. Unfortunately, this total concentration range also covers most of the response range. The Chlorophyta procedure, therefore, clearly will detect the presence of the pollutant compound but, for the most part, is only sensitive to order of magnitude concentration differences.

Limits of Reliable Measurement

The same sample material should be used for reliability measurement as was originally used during the method precision and method sensitivity evaluations. The evaluating laboratory typically should select two concentrations of sample material. One of these concentrations should be near the upper extreme of the method's detection range and the other should be near the lower extreme of the detection range. Ten analyses would be conducted on each concentration to provide precision data (expressed as a coefficient of variation). However, the previously acquired data confirming the poor capability for sensitivity and the somewhat limited total assay response range made this stage of the evaluation less difficult than is usually the case.

As was the case for the above-mentioned method sensitivity, the limits of reliable

Table 7. *Assay Response Using Soil Samples Containing Either Sodium Fluoride or 2,4-Dichlorophenol*

(Precision Capability, Expressed as a Coefficient of Variation, in Response to Soil Containing Either an Inorganic or an Organic Pollutant Compound)

	Calculated EC_{50}	
Assay Number	1112 mg NaF per 125 g soil (8.9 mg/g)	10 mg dichlorophenol per 125 g soil (80 μg/g)
1	41.0	55.9
2	47.4	57.3
3	41.1	49.8
4	42.1	25.7
5	46.6	93.4
6	44.8	40.2
7	46.0	94.7
8	43.5	46.1
9	45.5	44.5
10	48.2	116.2
$\bar{X}$ EC_{50}	44.6	62.4
$\pm SD$	2.6	29.0
CV(%)	5.8	46.5

Table 8. Response of Chlorophyta Assay Procedure to Various Soil Concentrations of Sodium Fluoride

mg sodium fluoride per 125 g of soil	mg sodium fluoride per gram of clay loam soil	Mean EC_{50}	±SD	CV (%)	Range of EC_{50}±SD
556	4.5	-0.7	–	–	–
700	5.6	-181.7	455.3	250.6	–
800	6.4	54.3	6.9	12.7	47.4 - 61.2
900	7.2	56.6	6.5	11.4	50.1 - 63.1
1090	8.7	50.9	7.5	14.8	43.4 - 58.4
1112	8.9	44.6	2.6	5.8	42.0 - 47.2
2224	17.8	32.7	8.2	25.1	24.5 - 40.9
3336	26.7	31.9	9.1	28.5	22.8 - 41.0
4448	35.6	22.6	8.1	35.8	14.5 - 30.7
6000	48.0	16.6	10.9	65.5	5.7 - 27.5
7500	60.0	-0.9	29.5	–	–

Note: Each mean EC_{50} value is the result of ten separate assays using the indicated concentration of sodium fluoride per gram of soil. Each separate assay was conducted as directed by the method protocol and included the required control samples. Concentrations of 4.5, 5.6, and 60.0 mg/g apparently are outside the limits of reliable measurement for the Chlorophyta assay.

measurement are not particularly impressive other than that the assay does show a definitive response to the chemical over a concentration range of 6.4 mg/g to 48.0 mg/g (i.e., before complete inhibition/lethality). For this phase, the basic objective was to verify that the method capabilities for sensitivity, precision, and accuracy (when applicable) do not deteriorate at the upper and lower extremes of the detection range.

Although the procedure's sensitivity is not very good at the extremes of the detection range, it is not any less impressive than the method's best sensitivity. The method's deterioration in precision provides the most information concerning limits of reliable measurement. As mentioned above, ten analyses were conducted at each of several concentrations (Table 8) so that, among other reasons, precision comparisons could be made. The coefficient of variation frequently will show a dramatic increase at the extreme limits of detection and, therefore, precision data provide a distinct indication of the limits of reliable measurement. The results of this effort suggest a very restricted concentration range of 6.4 mg/g to 35.6 mg/g of soil. The coefficient of variation at the greater concentrations increases rapidly, especially when compared with the 5.8 percent coefficient of variation achieved at the 8.9 mg/g concentration. The 65.5 percent coefficient of variation noted at 48.0 mg/g might well be considered as being beyond a range of reliable measurement. However, the assay clearly shows a response to sodium fluoride at the increased concentrations and, considering that the overall procedure is only a screening technique, the increased coefficient of variation should not really affect method usability.

Accuracy

To determine a method's single laboratory capability for accuracy (and for systematic error), the testing laboratory must have both a standard reference material and a known method response (true response) to this reference material. When a method calls for analysis of biological tissue or biological fluid, there will usually be a standard reference material available to the testing laboratory, e.g., samples with certified compound concentrations or perhaps with certified enzymatic activity levels. In these instances, the method's single laboratory capability for accuracy can be assessed by determining the differences between the observed single laboratory result, using the reference sample, and the known true value.

In the case of a method such as this algal assay that uses the soil eluates, no "true response" is available for a reference material; hence the method's single laboratory capability for accuracy (or for systematic error) cannot be determined. Under these conditions, the testing laboratory should first select a reference material and then determine an average test response for a single concentration of the reference sample. Comparison of test data with the results of a reference method can be used in certain situations. For the purposes of this evaluation, however, the use of a reference method is not really possible and is not typically done for a single laboratory evaluation.

Although not technically a standard, this evaluation used a white silica sand that is recommended by the American Society for Testing and Materials (ASTM) for use in certain cement preparations and, more significantly, can be acquired easily by other laboratories that are using the assay procedure. The sand has a particle size of 850 to 600 μm and receives a series of agitation washes prior to use. It is subsequently spiked with sodium fluoride to achieve a 4.8 mg/g concentration.

A greater recovery of toxic chemical will typically occur in the eluate when sand is used and, consequently, the known test response is being established at a different sodium fluoride concentration than was used during most of the current evaluation. The known test response to 4.8 mg of sodium fluoride per gram of washed ASTM silica sand was 43.4 ± 9.7 when expressed as the calculated EC_{50} ± the standard error. Results from the individual determinations that have provided this known value are shown in Table 9. However, these data again emphasize an apparent poor capability for precision which, in some ways, probably detracts from the future application of this response for use in accuracy and/or systematic error estimates.

Conclusion

Although considerable progress has been made with the standardization of this procedure, some difficulties remain if one considers a continuation to collaborative testing. The poor capability for sensitivity and the somewhat limited range of reliable measurement might be considered as being discouraging. However, the greatest problem confronting a continuation to collaborative testing may be the recurring lack of

Table 9. *Determinations to Establish a Known Test Response to a Reference Sample*

(Each Final Result Expressed as Calculated EC_{50})

Assay Number	600 mg NaF per 125 g ASTM silica sand (4.8 mg/g)
1	48.2
2	48.5
3	52.5
4	62.0
5	43.5
6	29.8
7	15.1
8	25.4
9	62.2
10	21.9
11	54.2
12	23.2
13	39.8
14	45.5
15	62.1
16	50.3
17	45.3
18	43.5
19	42.6
20	51.6
$\bar{X}$ EC_{50}	43.4
±SE	9.7
±SD	13.8
CV %	31.8

consensus noted among knowledgeable biologists for several phases of the method protocol. However, many frequently used procedures have never enjoyed total consensus agreement and various organizations such as the Association of Official Analytical Chemists (AOAC) and the American Society for Testing and Materials (ASTM) frequently publish method revisions as well as interim protocols to allow for ongoing improvements. The authors of this report recommend that the current algal assay be collaboratively tested as a next step toward more frequent application of the procedure for hazardous waste monitoring.

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The complete report, entitled "Freshwater Assay Using Soil Eluates as Sample Material (Single Laboratory Evaluation)", (Order No. PB 90-203 456/AS; Cost: $17.00, subject to change) will be available only from:
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