INTERIM PROTOCOL

for determining the

AEROBIC DEGRADATION POTENTIAL

of

HAZARDOUS ORGANIC CONSTITUENTS IN SOIL

Developed by

BIOSYSTEMS TECHNOLOGY DEVELOPMENT PROGRAM
SCIENTIFIC STEERING COMMITTEE

with assistance from

THE SOIL TREATMENT PROCESSSES COMMITTEE
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1.0 SCOPE AND APPROACH

The selection of technologies for the cleanup of National Priority List Sites using biological treatment is often based, in part, on information obtained from treatability tests. In bioremediation, an individual or company (a vendor) frequently uses treatability information to substantiate their proposed technology and strategy for the biological cleanup of a hazardous waste site. If not provided with guidance, however, a vendor will use a variety of methods and techniques to obtain the treatability information. This can lead to interpretational problems and relevancy concerns by the third party (usually an EPA regional office or state agency representative responsible for the coordination of remedial actions to clean up a hazardous waste site) evaluating the proposed technology and strategy. Thus protocols are required to provide consistency in the development of treatability information and interpretation of the resulting data.

This particular protocol provides a vendor with a standard method for comparing aerobic degradation rates of hazardous organic chemicals in contaminated surface soils. The protocol can be used as a standard guideline for the submission of data in support of their claims of aerobic treatability. Use of the protocol by a third party to evaluate a vendor's technology provides the possibility of an unbiased assessment.

Contaminated surface soils that are appropriate for the treatability protocol include any soils that can be mechanically perfused with water as part of the treatment technology or any subsurface soils that can be excavated and treated in the surface environment.

Data collected from the treatability tests specified in the protocol;

a.) provide a first cut evaluation of the rate and extent by which specific chemicals, chemical mixtures and /or toxicity of a waste are removed in conjunction with a proposed bioremediation strategy.

b.) insure, through the use of a mass balance principle, that the removal or loss of the hazardous organic chemical(s) is the result of degradation and not some other process such as chemical decay, volatilization, stripping or sorption.

c). are not intended for predicting the rate or extent of biodegradation at field scale.

d.) and cannot be used in predicting the cost of full scale implementation, or the time required to bring the site to closure.

The protocol provides optional applications. It can ;

a.) compare the rates of degradation under different environmental conditions [pH, oxygen status, moisture, depth zone of contamination, presence of co-occurring contaminants] and/or under different treatment conditions (aeration, supplements, slurries,etc.).

b.) compare rates to determine if the selected environmental condition(s) or the selected treatment conditions affect the degradation rates significantly in unaltered or untreated conditions.

2.0 SUMMARY OF METHOD

The protocol is based on the use of small scale experimental reactors designed to mimic the conditions proposed by the vendor for an aerobic biological treatment (bioremediation) of contaminated soils. Four basic reactor designs are considered:

1. no tillage,
2. periodic tillage,
3. forced ventilation and
4. slurried reactor.

A minimum of two types of tests are required for evaluation of a proposed bioremediation strategy. One, called the complete treatment test uses the reactor design which most closely mimics the proposed bioremediation strategy and includes any proposed biological inocula, nutrient amendments, adjustments or other procedures to stimulate biological activity. The second, called a no treatment test, is standard against which the vendor's proposed strategy is compared. It consists of the reactor with no tillage and involves no additions, adjustments or manipulations.

Other tests can also be set up to determine the necessity of all or some of the adjustments to of the soil proposed in the bioremediation strategy. For example, one may want to evaluate the effectiveness of nutrient additions. In this case, a third test is included in which the nutrient addition is eliminated but all other proposed additions and manipulations are maintained. Or the contaminants to be treated may be very volatile (as judged, for example, from changes in contaminant concentration during handling) and thus a specialized reactor design might be included.

The basic framework of the protocol is to establish treatability efficacy of the contaminants in the soil by determining the rate and extent of disappearance of specific chemicals over time. In addition, a method for the use of a bioassay to follow the loss of toxicity over time is provided

in the protocol to compare with the chemical disappearance data.

Procedures for dealing with hazardous wastes and ensuring the health and safety of laboratory personnel are not addressed in this protocol. Any laboratory using the protocol must comply with the appropriate procedures for handling and disposal of the wastes and the appropriate Good Laboratory Practices.

3.0 COLLECTION AND HANDLING OF SITE MATERIALS

3.1 SAMPLE SELECTION

Samples of contaminated soils will be collected from areas where the vendor has proposed to use bioremediation strategies. Sampling areas should be selected that are representative of conditions most typical of the site. That is, sampling areas should be based on the site characterization data and the treatment strategy. In general, areas with the highest concentrations of contaminants should be selected. If, however, there are any compounds present that are suspected to be inhibitory to the biological treatment process, their concentration and toxicity should be included in the sampling design. For example, the microbes can tolerate concentrations of lead and zinc that are an order of magnitude higher than cadmium or nickel. Therefore sampling should be based on the concentrations of cadmium or nickel even though the concentrations may be considerably smaller than other less toxic metals.

Sampling areas for any particular treatability study, should not differ substantially in terms of soil type and chemical composition (for example, the presence or absence of heavy metals). Sampling plans should be developed in accordance with the recommendations given in the USEPA compendium of methods (SW - 846, 3rd Edition, November 1986).

3.2 Sample Collection

Enough soil must be collected from each sampling area for a minimum of two tests (complete treatment and no treatment) and any additional test proposed. All the soil collected from a sampling area must be composited and thoroughly mixed at the point of collection. Subsamples used in the treatability tests are then taken from this mixture.

Three replicate 200 gm (dry weight) samples of composited soil should also be collected for chemical analysis. These samples should be wrapped tightly in heavy gauge aluminum foil and quick frozen with dry ice. Additional samples should be taken for moisture characterization. The quantities of soil collected should be recorded.

If possible, the soil should be drained at the time of collection. Sampling should not occur after a major climatic event, such as rain, abnormal droughts, or seasonal changes.

3.3 SAMPLE MOISTURE CHARACTERIZATION

A soil moisture curve should be determined on the composited samples from each area of the field site immediately following sample collection. This curve, which relates the capillary tension on water in the soil to the water content on a mass basis, should be determined using the procedures outlined in Appendix C. The moisture information is necessary for properly adjusting the moisture content of the soils in the reactors.

3.4 SAMPLE TRANSPORTATION

Soil samples must be transported to the laboratory in containers capable of;

a.) preventing loss of volatile organic compounds,
b.) protecting the soil sample from light, and
c.) minimizing adsorption of chemicals to container and cap surfaces.

Screw-capped, wide mouth, glass bottles having a lid with a Teflon TR liner are recommended (cf. Chapter Four - Organic Analyates, Section 4.1.2 SW-846). Collected samples will be maintained on wet ice or in a refrigerator at less than 10 C during transport and until the soil is used in the reactors. The soil samples should be kept at a moisture content representative of the field or specified by the vendor. Caution should be exercised to prevent wet soils from becoming anaerobic during shipment. If a sample container is broken or opened before the soil is to be used in the reactors, the sample must be discarded and if necessary, the area resampled.

3.5 Sample Preservation

The addition of chemical preservatives is prohibited. The sample must be iced down prior to any shipment.

3.6 Sample Holding Times

The soil samples to be evaluated in the treatability test can be held for a maximum of 14 days if the samples are refrigerated (not frozen). Soil samples that are to be chemically analyzed, can be held indefinitely as long as they are frozen. No holding time is allowed for soils that are used to determine moisture content.

4.0 APPARATUS AND MATERIALS

4.1 REACTOR COMPONENTS

The basic reactors, which are drawn from the soil flask system of USEPA (1984, 1986a, 1986b, 1988) and Sims et al. (1982, 1986), are shown in Fig. 1. The reactor is designed to determine both the rate of loss by volatilization and biodegradation. The reactor consists of a 500 ml Erlenmeyer flask (Kontes cat. No. K-617000-0624 or equivalent) with a standard taper ground glass joint that accepts an aeration cap (Inlet Adapter, Kontes cat. No. K-1881000-2440 or equivalent) modified to deliver air to the flask as depicted in the figure. The joint is protected with a Teflon TR sleeve. The aeration cap admits chemically clean air through Teflon TM tubing. The soil flask can be supplied with breathing quality compressed air, or laboratory air can be cleaned by pumping it through an appropriate filter trap. The purge air flows over the surface or through the soil-waste mixture within the flask and exits the aeration cap through an effluent tube close to the top of the flask. Split stream sampling is conducted through glass tees in the flask effluent line. An air splitter can be used to divert the appropriate flow to the sorbent tube, or the tube can be connected to a constant flow vacuum pump.

4.2 Reactor Design

The different treatment types are mimicked as follows:

a.) Treatment without tillage is obtained by incubating reactors in a static fashion with uniform air flow above the soil surface.

b.) Treatment with tillage is obtained by turning the reactors on their side and thumping them gently to stir the soil. This is performed periodically following a schedule supplied by the vendor.

c.) Treatment with forced ventilation is obtained by layering the soil in the reactor on top of a layer of pea gravel or sand and then delivering humidified air to the gravel or sand to purge the air through the soil. This modification is shown in Fig. 2.

d.) A stirred reactor is obtained by adding water to the soil, to bring it to the soil-to-water ratio specified by the vendor, and then adding the slurry to the reactor. The slurry is aerated by delivering the air to the bottom of the reactor; if the slurry is not to be aerated, the air should be delivered above the slurry. The slurry can be stirred with a magnetic spin bar. This arrangement is depicted in Fig. 3. Other mechanical mixing devices can be selected by the vendor depending on soil and slurry texture.

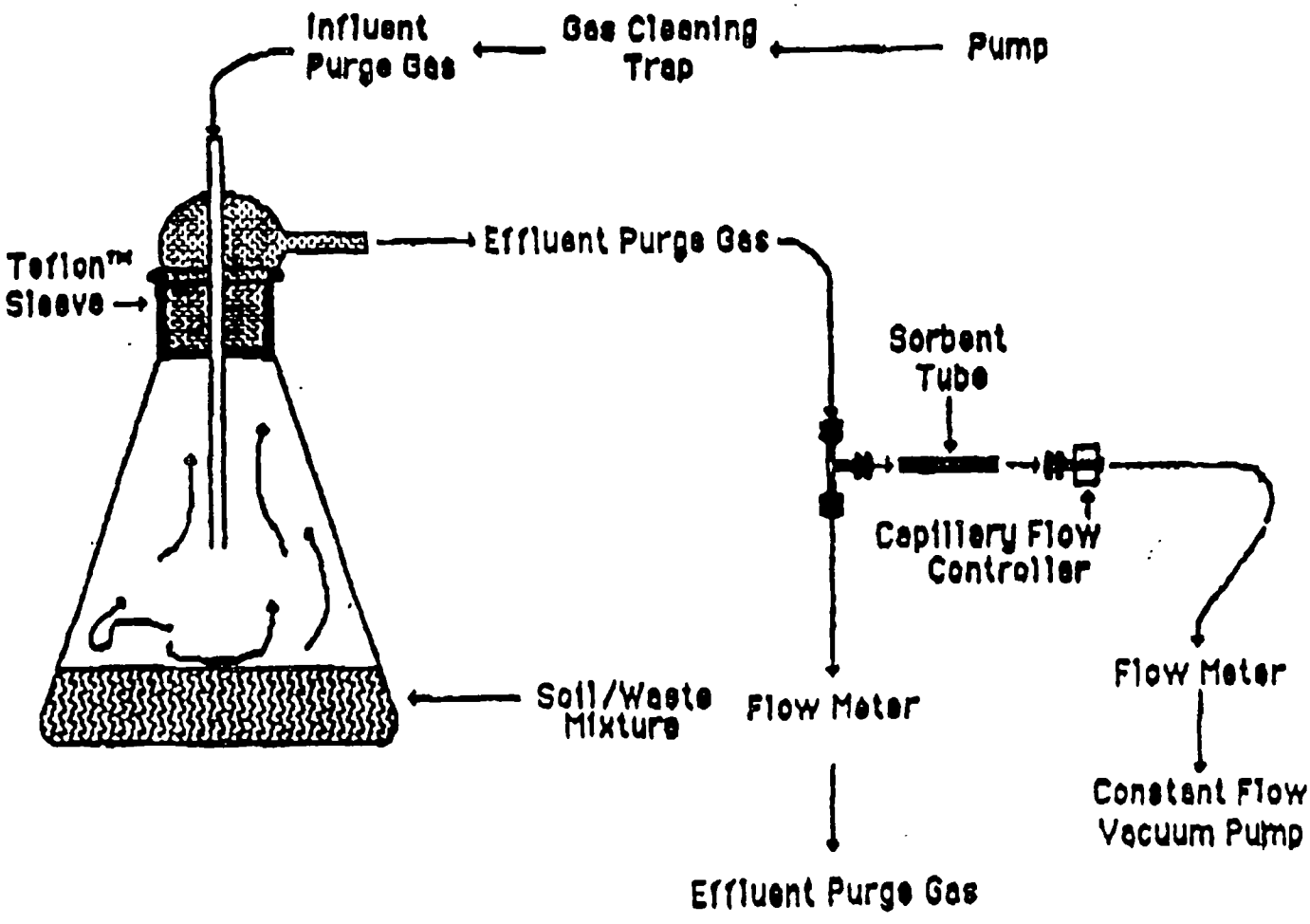

Figure 1. LABORATORY FLASK APPARATUS USED FOR MASS BALANCE MEASUREMENTS

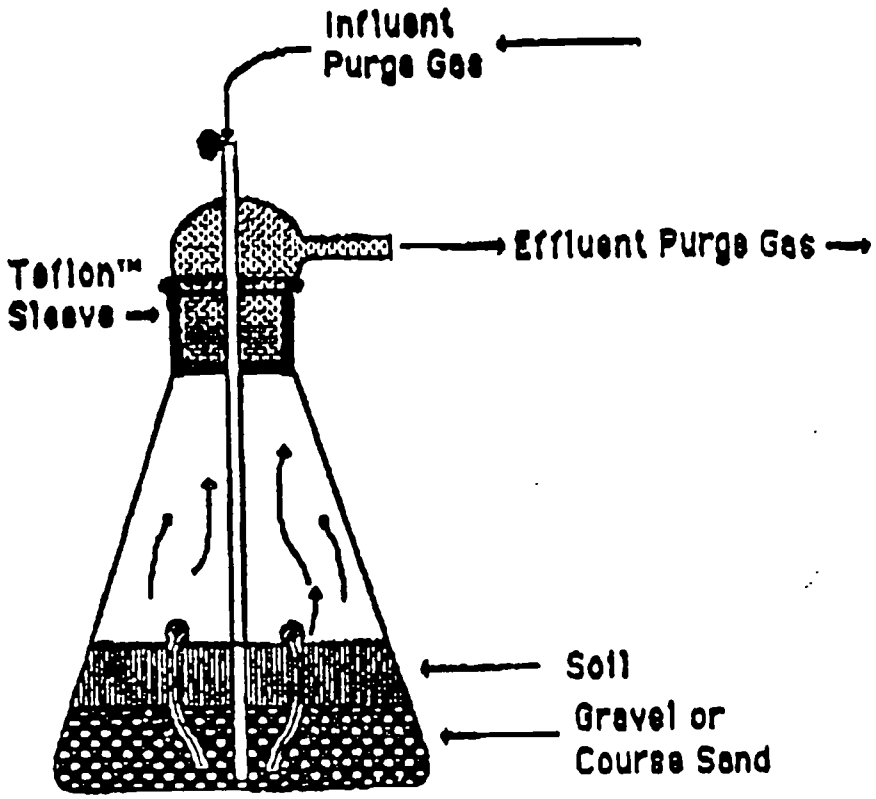

FIGURE 2. LABORATORY FLASK APPARATUS USED FOR SOIL VENTING EVALUATION

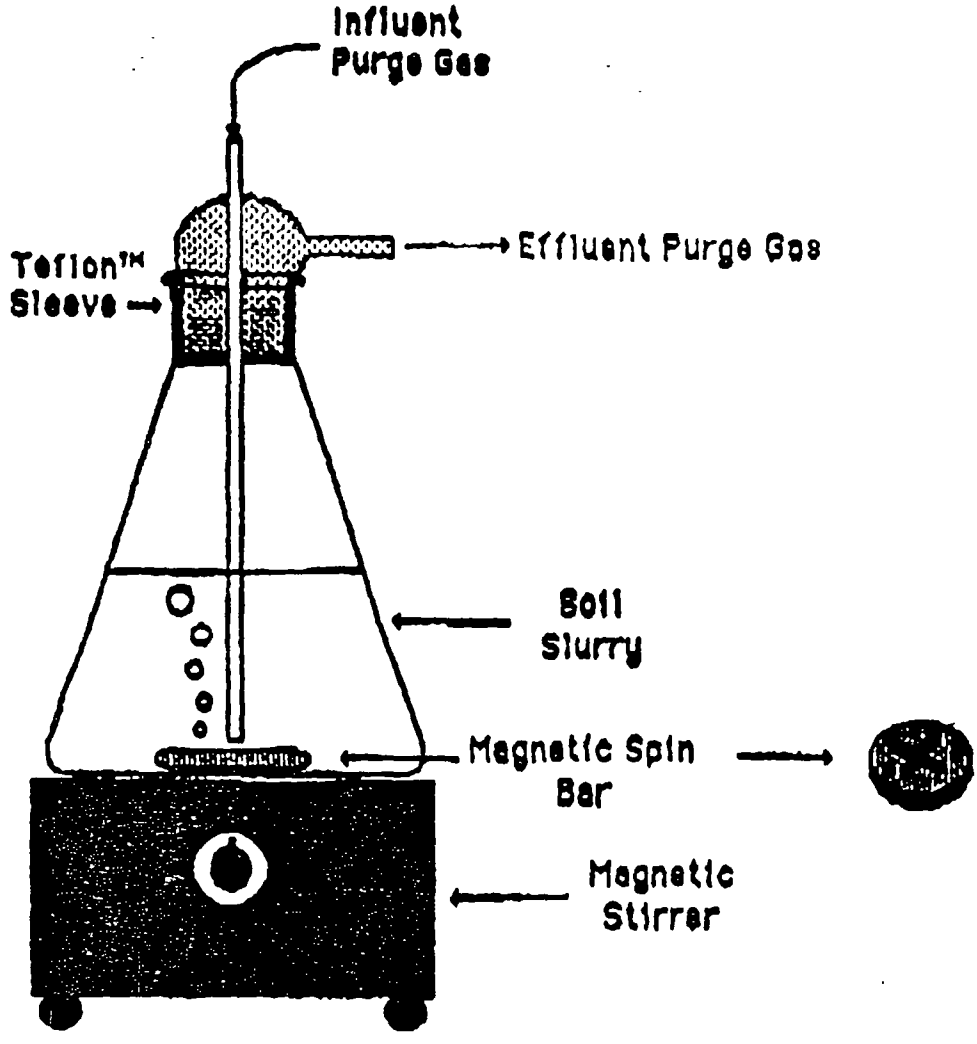

FIGURE 3. LABORATORY FLASK APPARATUS USED FOR SLURRY TAPE MATERIAL

5.0 PROCEDURE

5.1 REACTOR SET-UP

The composite field sample is brought to room temperature in the laboratory and thoroughly mixed. This step is extremely important if the composite soil sample was brought to the laboratory in more than one container. The mixed soil is then divided into portions equalling the number of tests to be performed. Portions will be required for a minimum of two treatability tests; a.) a no treatment test consisting of a reactor mimicking treatment without tillage and containing an unamended/unprepared subsample of the composited field sample and b.) a complete treatment test consisting of a reactor most closely mimicking the treatment strategy proposed by the vendor for cleanup of the contaminated site and containing a subsample of the composite field sample treated according to the inoculations, amendments, adjustments, and/or manipulations proposed by the vendor. Further portions of soil may be required depending on other tests designated by the site manager or suggested by the vendor. The intent of the protocol is to provide enough flexibility to mimic the proposed treatment as closely as possible.

Treatment of the soils should be accomplished after addition of the soil to the reactor. For example, if tillage of material(s) (i.e., inocula of bacteria, nutrients, chemicals, etc.) into the soil is part of the treatment strategy, then the material(s) should be till into the composited soil samples before it is added to the reactors. If, on the other hand, the proposed treatment strategy requires that material(s) be applied only to the soil surface without mixing, then the material(s) should be applied to the surface of the soil in the reactor without mixing. Similar considerations should be given for other types of the treatment strategies.

The recommended quantity of soil for each reactor is equivalent to 200 gm (dry wt), however, as little as 50 gm can be used and larger amounts are also appropriate if the size of the reactor is also increased. All weights of soil added to the reactors should be recorded. Moisture content of the soils should be adjusted before addition of soil to the reactors.

The reactors are arranged in triplicate sampling sets for analysis at a minimum of four sampling times, geometrically spaced (the first time being zero). Additional sampling times will be negotiated between the vendor and the third party, depending on such factors as the compounds of interest, the soil used, and the environmental conditions,etc. With a minimum of four sampling intervals in triplicate, a minimum of two treatability tests required

(complete treatment test and no treatment test), and complete sampling of the contents of a reactor with each sampling time, a minimum of 24 reactors will be have to be set up for each treatability study.

5.2 REACTOR OPERATION

An experimental test is initiated when a reactor is filled with the proper amount of contaminated soil, capped and the purge gas flow begun at approximately 200 ml/min.

Time zero analysis of concentration of contaminants in the soil (initial reactor concentration) is performed by sacrificing triplicate reactors immediately after the soil is placed in the reactor and the reactor capped, but prior to commencement of the purge gas flow.

The frozen samples collected in the field for chemical analysis (field concentration) should also be analyzed at this time.

The addition of extraction solvent directly to the reactor at any sampling time, will terminate any biological activity.

Gas flow measurements and analysis should be initiated at this time, according to instructions given in Appendix B.

The reactors should be incubated in the dark (or protected from the light with aluminum foil) and at a constant temperature that reflects the average temperature of the field site when it is to be biologically remediated.

The test soil in the reactors should be maintained at a soil moisture tension between -0.3 and -1.0 bar unless soil moisture is a variable to be evaluated (not applicable to slurry reactors). The reactors will be weighed on a daily basis, and water added to the reactors as needed to keep them within the specified moisture range (see Appendix C). The moisture tension in all the reactors in the protocol may vary from -0.3 to -1.0 bar, but all the replicates in all the experimental treatments may not vary from each other by more than +/- 0.2 bar.

5.4 Analysis of Reactor Contents

Reactors will be sampled by sacrificing the contents of an entire reactor. All soil in the reactor will be extracted and analyzed according to chemical procedures given in Appendix A. If the reactor contains a slurry, the soil and water can be extracted together if extraction efficiency of the contaminated soil is not affected by the presence of water. Otherwise, the water and soil should be extracted separately.

6.0 DATA REPORTING AND ANALYSIS

6.1 DATA TO BE REPORTED

The following data will be reported for each of the treatability tests performed.

- A record of all sampling transactions including sampling procedure, map of the site showing sampling areas (identified on a plan map to within +/- 2 m and vertical depth of sample to +/- 2 cm), time of sampling, sample size, and storage of samples.

- Concentration of chemicals in the frozen samples at the time of sampling (field concentration) and before the samples are added to the reactors (time zero reactor concentration).

- Amount of soil used in the reactors and a description of all modifications to the reactors.

- Quantity of chemical(s) in samples taken in the field and in the same samples at the time of their preparation for addition to the reactors

- Quantity of residual chemical(s) in each of the reactors at each sampling time.

- Quantity of chemical(s) in the traps for volatile organics at each air sampling time.

- Quantity of the chemical(s) in the solvent washings of the reactor glassware, tubing, and other associated equipment at each sampling time.

- Information on the presence of toxic materials, such as heavy metals, in the samples taken from the field site.

- The soil moisture curve for the soils sampled for the tests.

- Written log (indicating type, extent and time of any action) of the temperature profile over the entire experiment.

- Written log (indicating type, extent and time of any action) of the sample pump rate and purge gas flow rates and time interval that the trap is on line for each reactor at each sampling time.

- Written log (indicating type, extent and time of any action) of all the additions of water to the reactors, reactor weights as measured for moisture loss.

- Concentration of chemical(s) in the reactor head space at equilibrium from the volatility test.

- Written log (indicating type, extent and time of any action) of any other additions, removals, changes, manipulations, or mishaps which occur during the course of the experiment.

- Written log of all cited analytical procedures (see Appendix A).

- Hard copies of all GC/HPLC recorder tracings.

6.2 INTERPRETATION OF REPORTED DATA

The change in concentration of the test chemical(s) over time will determine the degradation rate. The chemical(s) concentration can change as a result of either;

a.) decreased extraction efficiency during chemical analysis,
b.) volatilization,
c.) chemical decomposition, and/or
d.) biodegradation.

Changes in extraction efficiencies over the course of the experiment and chemical decomposition are both unlikely but their contribution to the disappearance of the contaminants can not be directly determined with the information given in this protocol. Volatilization is readily determined by the amount of chemical detected in the volatilization traps assuming that a good mass balance (greater than 90%) is obtained. Biodegradation is the most likely process affecting the contaminants if the addition of inorganic or organic nutrients and/or oxygen results in a substantially faster decrease in concentration of the contaminants than without the additions. If the additions do not affect the disappearance rates, then some combination of biological, chemical and physical may be controlling the rates.

Time	No Treatment				Complete Treatment			Partial Treatment	
	R	V	D	F	V	D	R	V	D
T(0)									
T(1)									
T(2)									
T(3)									
t(n)									

R - The fraction remaining in the reactor is determined by dividing amount of chemical remaining in the reactor (including chemical found on reactor walls and tubing) at (0,1,2,3,n) by the amount in the reactor at T(0).

V - The fraction of the chemical lost from volitilization and stripping is determined by dividing the amount of chemical in the reactor at time T(0) by the amount of chemical accumulated in the volatile organic traps over incubation period T(0) to T(n).

D - The fraction of chemical lost to degradative processes is then assumed to be the fraction not accounted for in R and V. D is determined by combining R and V and subtracting this value from 1.0.

FIGURE 4. DATA TABLE FOR RECORDING DEGRADATION RATE INFORMATION

The relative importance of biodegradation and volitilization to the loss of hazardous chemicals from the reaction vessels can be determined from a pseudo mass balance illustrated by the table in Figure 4. The data from the treatability test showing changes in chemicals concentration over time, is first transformed to determine the fraction of chemical (R) remaining in the reactor at any time (T), the total amount of chemical lost by volitilization and stripping (V) over the time period from T(0) to T(n), and the amount of lost from apparent degradation processes (D). Methods for determining R, V, and D are described in the footnote of Figure 4.

The relative importance of volitilization and stripping over degradation is determined by comparing mean values in the V and D columns within each treatment (No Treatment, Complete Treatment, Partial Treatment, etc.). The relative rates of loss due to degradation and volitility can be determined by comparing the rates with either the V or D columns between treatments (No and Complete, No and Partial, Partial and Complete, etc.)

An indication of degradation can be determined by comparing the mean of three replicates at any given sampling time to any other mean with a t-test for the differences of two means. If the data follow a normal distribution, they may be subjected to statistical analysis as collected. If the data follow a log-normal distribution, as is frequently the case with concentrations of organic chemicals in soils and geological materials, a log transformation may be taken before the data are subjected to statistical analysis.

Biodegradation is assumed if removal of the contaminant(s) of interest in the experimental test mimicking the treatment proposed by the vendor, is greater than 20% per month at 95% confidence, <u>after</u> correcting for removal in the controls. If an adaptation or acclimation process is expected, then the test should be incubated for a long enough period to see greater than a 20% change; i.e., to cover the acclimation period.

In some instances, the concentration of test chemical(s) at the beginning and end of a treatability study, as depicted in the actual chromatographs, can be compared for a quick estimate of the effectiveness of the biological treatment. In other cases, a decrease in concentration of a component(s) within a chemical mixture may be observed and eventually quantified.

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APPENDIX A

CHEMICAL ANALYSIS OF TEST CHEMICALS AND/OR WASTE SAMPLES

The selection of a suitable extraction procedure for a given combination of analyte(s) and soil matrix generally requires some method development (Coover et al. 1987). For example methods that successfully recover a compound from one medium may not adequately recover the same chemical from similar media (Albro 1979). Also, extration recoveries from a given set of structually similar media may vary (Albro 1979).

Where possible we recommend that the existing and established analytical methods described in Test Methods for Evaluating Solid Waste (USEPA SW-846 3rd Edition November 1986) be used.

The recommended SW-846 methodology for selected analytes are:

Gas Phase Volatiles

Method 0010	Modified Method 5 Sampling Train
Method 0020	Source Assessment Sampling System (SSAS)
Method 0030	Volatile Organic Sampling Train (VOST)
Method 5040	Protocol for Analysis of Sorbent Cartridges from Volatile Organic Sampling Train

Soil Phase Volatiles

Method 5030	Purge and Trap
Method 8010	Halogenated Volatile Organics
Method 8015	Non-Halogenated Volatile Organics
Method 8020	Aromatic Volatile Organics
Method 8030	Acrolein, Acrylonitrile, Acetonitrile

Selected Non-Volatiles

Method 8040	Phenols
Method 8060	Phythalate Esters
Method 8080	Organic Pesticides and PCB's
Method 8090	Nitroaromatics
Method 8100	Polynuclear Aromatic Hydrocarbons
Method 8120	Chlorinated Hydrocarbons
Method 8140	Organophosphorous Pesticides
Method 8150	Chlorinated Herbicides

GC/MS analytical methods are not recommended for this protocol due to the reltively high cost. Analytical

methodology using gas chromatographic and liquid chromatographic analysis is sufficient for the use of this protocol. For a select few analytes GC/MS, or other specialized techniques, may be the only means to correctly identify and analyze for their presence.

Recommended extraction/concentraiton techniques (soils and sediments) are:

Method 3540 Soxhlet Extraction
Method 3550 Sonication Extraction

Other published methods for Soxhlet extraction (Anderson et al. 1985, Bossert et al. 1984, Coover et al. 1987, Eiceman et al. 1986, Kjolholt 1985, Grimalt et al. 1986), sonication extraction (de Leevw et al. 1986, Sims 1982) and homogenization and extraction(Coover et al. 1987, Fowlia and Bulman 1986, Lopez-Avila et al. 1983, Sims 1982, Stott and Tabatabai 1983, and U.S. EPA 1982a, and extraction of materials from treatability studies (Brunner et al. 1985, Russell and McDuffle 1983) are available for reference and special applications.

Soil spiking and recovery studies should be conducted to determine the effects of soil, test substance(s), and soil test substance(s) matrix on chemical extraction and recovery efficiency. Soil samples should be sterilized using a method such as mercuric chloride, causing minimal change in soil physical and chemical properties. (Fowlie and Bulman 1986). The sterile soil should be spiked with the test substance(s) to achieve a range of initial oil concentrations (Coover et al. 1987). The range of concentration should include the highest concentration and less than one-half of the lowest initial concentration to be used in degradation evaluations. Extractions of the soil/test-substance(s) mixtures using the selected procedure will allow the evaluation of the effect of test substance(s) soil concentrations on recovery efficiency. The effect of soil concentration on recovery efficiency was evaluated and found to be significant for anthracene and benzo[a]pyrene by Fowlie and Bulman (1986).

Extracts of the soil and complex wastes should be spiked with test substance(s) of interest to evaluate the effect of these matrices on chemical identification and quantification. Interferences due to the extract matrix may be identified. Extraction procedures or instrumentation used for identification and quantification may then be changed if necessary.

Standard curves should be prepared using primary standards of the test substance(s), or chemicals in the test substance, dissolved in a suitable solvent that does not interfere with chemical identification and quantification. Standard curves should be generated using at least six points

ranging from the highest concentration anticipated to the detection limit for the chemical.

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APPENDIX B

GAS FLOW MEASUREMENTS AND ANALYSIS

For reactors containing tilled and untilled soil, or stirred reactors that are not aerated, the purge gas should flow at a rate between 200 and 20 ml/minute. This range of flow rates turns the headspace of the reactor over every one to ten minutes. The flow rate is selected at the convenience of the laboratory doing the testing, based on the performance of the trapping system; but one selected the flow rate must be rigidly controlled (within 2%). The flow rate through reactors with forced ventilation or aerated slurried reactors is specified by the vendor.

The concentration of volatile organics should be determined in the headspace of the sealed samples brought back to the laboratory to fill the reactors. This is most easily done by fitting a septum into the lid of the sample jar(s). The headspace gas can be sampled with a syringe and injected directly into a gas chromatograph. The measured concentration of volatile organic compounds multiplied by the flow rate of purge gas and the total time of incubation of the reactors sets an upper boundary on the quantity of each volatile organic compound that can be released from the soil sample. This calculated upper bound must be compared to the total amount of the volatile organic compounds in the samples frozen in the field to determine the initial concentration of contaminants. If the calculated upper bound for volatile loss is more that 20% of the total amount present for any compound of regulatory concern, the loss of volatiles from the reactors must be determined as described below.

Emissions of volatile organic compounds should be monitored in the three replicate reactors that are to be incubated for the longest time increment. The suggested sampling interval for the contents of the reactors is time zero, one month, two months, and a final sampling time selected by the site manager. If this interval is to be followed, a system of traps and flow splitters should be devised that can contain (without breakthrough) the calculated upper bound for volatile loss in a one-day period for any of the compounds of regulatory interest.

The system of traps and splitters should be able to detect (MDL) a quantity of each organic compound of regulatory concern equal to 1% of the amount of each compound originally present, divided by 0.01. This will ensure that error in the estimated contribution of volatilization to the mass balance will be no more than 5% of the amount originally

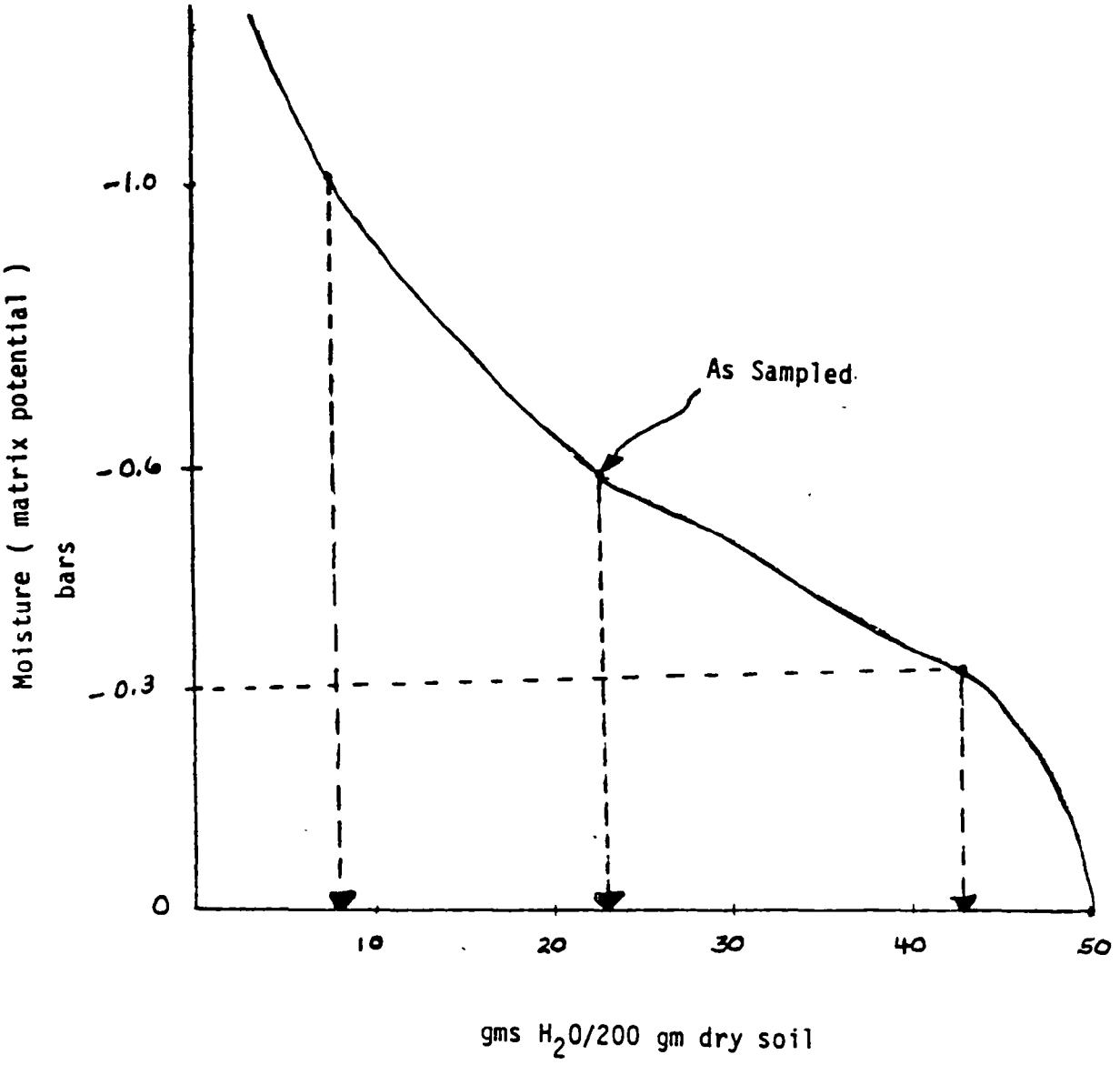

FIGURE 5. SOIL MOISTURE CHARACTERISTIC CURVE

The volatile emissions should be trapped and quantified in the 24 hour period representing the first day of the second week in incubation, the last day corresponding of the one-month incubation, the last day of the final incubation. If an alternate sampling schedule is used, the sampling of volatile emission should be compressed accordingly; however, the entire first 24 hours must be monitored.

The total loss of each volatile compound should be estimated by fitting a curve through the data points (zero-order or first-order on the time of incubation, depending on which is the best statistical fit), then determining the area under the curve.

APPENDIX C

SOIL MOISTURE DETERMINATIONS

Prior to setting up the bioreactors, the soil moisture characteristic curve should be determined on a sample of the material that will be subjected to the protocol. This procedure requires several days and should be started as soon as possible after the sample arrives in the laboratory. A portion of the sample should be air-dried at 150°C for 24 hours to determine its water content. Then the moisture characteristic curve should be consulted to determine the moisture tension in the sample. If the tension is greater than -0.3 bar, water should be added to the bioreactors. The moisture characteristic curve should be consulted to determine the amount of water in the soil at -0.3 bar. The difference between the amount of water in the soil and the amount there at -0.3 is the amount that should be added.

The soil moisture characteristic curve should be consulted to determine the weight of water that can be lost before the moisture tension drops below -0.1 bar. The reactors should be weighed on a daily basis, and water added to the reactors as needed to keep them within the specified range. The moisture tension in all the reactors in the test may vary from -0.3 to -0.1 bar, but all the replicates in the experimental treatments may not vary from each other by more that 0.2 bar at any one time.

The figure shows a hypothetical soil moisture characteristic curve. As sampled the soil contained 23 gm water in 23 + 200 grams of wet soil. After air drying, 100 gm of wet soil weighed 81.3 grams. To attain 200 gm of air-dried soil in the reactors, 223 gm of the soil sample should be added to each reactor.

$$\frac{x}{200} = \frac{100}{81.3}$$

The soil in the bioreactor contains 23 gm water. From the moisture characteristic curve, the soil at -0.3 bar contains 42 gm water, so 20 ml of water should be added to each reactor. From the curve the soil at -0.1 bar contains 8 gm water. The reactors can loose up to 43 -8=35 gm of water before more water needs to be added.

APPENDIX D

EVALUATION OF SAMPLES FROM TREATABILITY TEST FOR TOXICOLOGICAL HAZARD

I. INTRODUCTION

At the time site coordinators consider using the Aerobic Soil Treatability Protocol, available options for remedial action will have been identified and biodegradation should be among those options. If this is the case, two primary questions in risk assessment must be answered;

a. Does the bioremediation process eliminate or significantly decrease the hazard potential of the treated soil?

b. How does the reduction in hazard compare with other remediation options?

The risk assessment procedures described within the Public Health Evaluation Manual (SPHEM) rely upon a review of the toxicity of individual indicator chemicals identified at the site. This manual provides risk assessment managers with appropriate guidance for risk assessments in five areas: carcinogenicity, mutagenicity, reproductive effects, exposure assessments, and assessments of chemical mixtures (U.S. Environmental Protection Agency, 1986a,b,c,d,e). Key within this process is the ability to compare public health risks. Comparisons are not made in necessarily absolute terms but in a comparative manner among the remedial actions developed in other parts of the Remedial Investigation and Feasibility Study process.

The SPHEM guideline, using data and information generated from treatability protocols can be used to help answer both of the above questions. However in its present state, it best applied to situations involving only one or a few pollutant chemicals with known level of toxicity. Inherent within the SPHEM guideline is a health assessment process which is designed to compare results, relative to risk, from bioremediation to other treatment alternatives, including the no action alternative.

Because quantitative genotoxicity (mutagenicity/ carcinogenicity) data is available for only a few of the pollutants at a hazardous waste site, a simple surrogate method for determining the absence or presence of genotoxicants is useful. This appendix provides information concerning such a system.

II. DEVELOPING GENETIC TOXICOLOGY DATA USING THE SALMONELLA BIOASSAY FOR MUTAGENICITY

Since genotoxicity (carcinogenicity and mutagenicity) information is often critical in a risk assessment, the risk assessor may want to use a rapid, relatively inexpensive short-term bioassay. Such an assay should help him/her establish whether a particular treatment process, as initially assessed through the use of a treatability protocol, eliminate, reduces, or increases the genotoxicity of the pollutant organics found in the soil. The bioassay will only be a qualitative indicator of the effect a particular treatment process will have on the hazardous compounds found in the soil samples. In addition, these bioassays can be conducted and the results interpreted without knowledge of the specific chemicals within the mixture and without knowing the mutagenicity of each constituent chemical. Because the bioassay most likely used in conjunction with treatability tests is the Salmonella bioassay, this appendix limits its discussion to this assay.

Mutagenicity tests using bacteria have been available for approximately 30 years. In 1951, Demarec et al. used an Escherichia coli (E. coli) bioassay to test 31 chemicals. In 1971, Ames and Yamasaki published a mutagen detection system using histidine requiring mutants of Salmonella typhimurium (S. typhimurium). This test later gained the pseudonym "Ames test." Dr. H. V. Malling (1971) made a significant improvement to in vitro tests, including the Ames test, by incorporating a mammalian metabolizing system into in vitro tests. This allows these assays to detect promutagens (substances that can be metabolized to mutagens) directly.

The term Ames test usually refers to the plate incorporation technique. With this protocol, the investigator mixes the bacteria, the substance under test, and any metabolic activating system in a melted, soft-agar overlay and pours the mixture onto the minimal media plate. After incubation, one examines for toxicity, contamination, and numbers of colonies. Because each substance is tested with 5 to 7 doses using 2 or 3 replicate plates per dose and 2 or more bacterial strains and metabolic activation systems, a single experiment can contain over 200 plates for each substance tested. Although the data can be summarized in a number of ways, a modeled slope value taken from dose-responsive data provides a quantitative method for summarizing results. Generally, the statistical model used is either that of Bernstein et al (1982) or Stead, et al (1981).

The bioassay procedure, developed by Ames et al (1975) and later refined (Maron and Ames, 1983), detects reverse mutations that occur in histidine-requiring strains of S.

typhimurium. The indicator strains used were developed from either spontaneous or induced mutants of the parental strain LT-2 that will not grow on histidine-free medium. When these histidine-requiring strains undergo a reverse mutation to prototropy (normal wild type), the mutants will form countable colonies on a minimal nutrient media deficient in histidine. Since more than one type of gene mutation is inducible in DNA, several indicator strains were developed. Besides the histidine-locus mutation, the most commonly used strains also have additional mutations incorporated. These additional mutations enhance the sensitivity of the assay (Maron and Ames, 1983).

While the plate incorporation test protocol is sufficient to screen most substances, other types of specialized protocols do exist. Maron and Ames (1983) described most of these alternative methods and their uses. Guidance for the performance, quality assurance criteria, data gathering and interpretation of the Salmonella assays are available (Claxton, et al, 1987). In general, an expert in these types of assays should be consulted before the use of bioassays is initiated. The consultant should be made aware of the type of samples to be tested, the types of pollutants that are likely to be present, and how the bioassay information will be used.

Although this assay is not designed to detect all types of genetic damage, it does detect those chemicals that cause small gene mutations. Since gene mutation appears to be one of several possible steps in the process of carcinogenicity, compounds which cause gene mutation and are detected with the Ames test have and increased likelihood of being carcinogens (Clayton, et al, 1988). Since cancer can be induced by non-genetic mechanisms, this bioassay does not detect all carcinogens. An increase in the mutagenicity of a bioremediation sample indicates to the risk assessor that the process being investigated produces additional hazardous substances. On the other hand, a decrease in mutagenicity helps to confirm that the bioremediation process is effective in reducing the genotoxicity of organic compounds in the soil sample. Chemical and bioassay information should supply complementary information and will strengthen the risk assessors evaluation.

III. PROCEDURE FOR EVALUATING BIODEGRADATION PRODUCTS

The purpose of bioassay testing is to determine whether or not an apparently efficacious treatment (the contaminant of concern is removed) decreases, does not change, or increases the overall genotoxicity of the soil sample. This section provides an outline of how to incorporate and interpret the bioassay information.

Selecting Samples for Bioassay. In order to minimize cost, samples are selected after the third (two-month) time-interval of the degradation potential protocol is completed. After the two-month time interval, reactors representing an untreated condition and the most efficacious condition (greatest removal of contaminant) are selected for bioassay. Using this approach, not all reactors need to be bioassayed. These two sets of reactors will represent the two extreme conditions for bioremediation.

Extraction, Concentration, and Solvent Exchange. In many cases, the extracts of the reaction flasks can be aliquated for both chemical analysis and bioassay. If separate reactors are used in preparation for the bioassay, the selected extraction method should be the same as that used for chemical analysis. The extraction solvent for bioassay should also be the same as the chemical analysis extraction solvent unless it prohibits solvent exchange of the extracted mass into dimethylsulfoxide (DMSO) or some other solvent compatible with bioassay. If the same solvent will not allow this, a separate solvent that will allow the appropriate solvent exchange should be used to extract a separate aliquot. After extraction , a small aliquot is used to determine gravimetric mass so the concentration (mass per ml solvent) can be determined. If sufficient mass is available, the remaining sample is solvent exchanged into DMSO at 10 mg/ml concentration. If a precipitate forms at 10 mg/ml, a lower concentration is used.

Bioassay. The chosen samples are tested concurrently at a minimum of 5 doses using two plates per dose with and without a mammalian metabolizing system. In order to conserve sample, an initial range finding test using one strain is performed using 2 mg per plate as the highest dose followed by 4 other doses spaced at half-log intervals. If enough sample is available, definitive testing is done with strains TA98 and TA100. If excess sample is available, other indicator strains also are used. All testing is replicated. If the amount of sample prohibits using at least two strains, the strain chosen should be one that will detect at least some of the known genotoxicants. If it is not known which strain is most appropriate, TA98 with mammalian metabolizing enzymes is used.

It is recommended that the protocol of Maron and Ames (1983) be followed according to the guidelines given by Clayton et al (1987). If either this protocol and/or guidelines are not followed, justification should be given.

Data Analysis and Evaluation. In order to determine whether or not a sample is mutagenic, existing guidelines (Clayton, et al., 1987) should be followed. When the samples are positive and tested concurrently, they can be quantitatively compared using the slope of the dose response curve. If there is less than a two fold difference in slope values, the difference is generally not significant. Slope values can effectively be determined by using either the models of Stead et al (1981) or Bernstein et al (1982) or by doing a least squares linear regression of the linear portion of the dose response curve.

The purpose of bioassay testing is to determine whether or not an apparently efficacious treatment (the contaminant of concern is removed) decreases, does not change, or increases the genotoxicity of the soil sample. If the genotoxicity of the sample after aerobic degradation is decreased, as one would expect with the removal of toxic components, the regulator can have increased confidence that this type of treatment has potential utility. If there is no mutagenicity associated with the samples before or after treatment, the regulator can rely upon the results of the chemical assay for the best available assessment of genotoxicity. If the mutagenicity of the treated samples is greater than the mutagenicity of the original sample (whether or not the chemistry indicates destruction of the contaminant), the biotreatment has produced or made available additional mutagenic compounds. If it cannot be determined in this final situation that the increased mutagenicity is due to artifactual causes, the regulator should assume that the biotreatment is ineffective or detrimental. If the mutagenicity of a sample decreases, the biotreatment is effective. The degree of its effectiveness can be assessed by comparing the slope values (potency) of the untreated and treated samples.

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