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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

EDISON, NEW JERSEY 08837

April 8, 1985

MEMORANDUM

SUBJECT: Bioassessment Document for Combs Fill, Chester, NJ

FROM: Royal J. Nadeau, Chief
Environmental Response Branch

TO: Chris Shultz, Regional Project Officer
Environmental Protection Agency, Region II

Enclosed are two copies of the Bioassessment Protocol that we use for assessing impact to ecological systems. This protocol was developed several years ago by the Environmental Protection Agency's Office of Research and Development Laboratory in Corvallis, Oregon several years ago.

We have used this protocol and the bioassays described within at several sites across the country. The results have been very useful for evaluating environmental impacts. In the case of Don Lynch's Friedman Site, the protocol will be used to evaluate the feasibility of the "no action" alternative. However, at Combs Fill, I would expect that these tests would provide useful information for the feasibility study.

These tests are not yet available to the private sector; thus, none of the REM/FIT zone contractors use this information as a matter of course in preparing site management documents.

We will be glad to assist you and the New Jersey Department of Environmental Protection in seeing that the protocol is customized and carried out at Combs Fill with subsequent data evaluation and interpretation. If you have any questions, please call Dr. Spence Peterson, ORD-Corvallis (FTS-420-4791) or me (FTS-340-6740).

Enclosure
cc: Bill Hanson, Washington, D.C.
Dr. Spence Peterson, ORD

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PROTOCOL FOR BIOASSESSMENT OF HAZARDOUS WASTE SITES

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ACKNOWLEDGMENTS

This bioassessment protocol was developed in steps. First, a set of biological test procedures were defined for possible use. The conceptual basis and the specific tests as related to ecological needs and current regulatory requirements were then evaluated at a workshop in Washington, D.C. October 26-27, 1981. The attendees also considered the current status of hazardous waste site prioritization and cleanup and statistical factors, field application and evaluation procedures, and other possible biological tests and procedures.

The workshop participants are listed below and represent federal and state agencies, industry, universities, and consultants. Although this document was prepared and compiled by the author, the workshop participants made substantial contributions and we acknowledge their efforts and subsequent review.

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Robert Yelin	Tetra Tech, Inc.	Pasadena, CA

Introductory papers were presented by Peterson (Problem Description), Kleveno (Problem Setting and Site Priority), Gherini (Chemistry), Herricks (Biology), Murarka (Risk Analysis), Miller (Protocol Methods), Porcella (Sampling Criteria), Thomas (Field Studies), and Klein (Microbial Tests). Becky Boone of AWARE, Inc. provided invaluable coordination of the workshop and subsequent mailing of the protocol.

Special thanks go to Spencer A. Peterson and William A. Miller of the USEPA, Corvallis whom provided overall guidance and specific constructive crition. Also, Clarence Callahan, Joseph Greene, and Ibrahim Hindavi, USEPA, critiqued the procedures and approach. In addition to the above Alan Maki of Exxon and Pat Guiney of Gulf Research provided review.

The document was reviewed by Steven A. Gherini and Thomas M. Grieb of Tetra Tech, Inc. However, any errors are the authors. Ms. Pencie Shrewsbury prepared the manuscript in its final form. The work was done under a USEPA purchase order, P.O. 2B0177NALX.

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EXECUTIVE SUMMARY

The bioassessment protocol is one of several tools, including chemical analysis and field study, that can be used to characterize the potential environmental risk associated with hazardous waste sites. The protocol can be applied to priority ranking for deciding the need for cleanup of a site compared to other sites and to assess cleanup effectiveness by testing for potential hazards at the site boundaries or along a sampling transect.

Bioassessment involves using defined biological tests to determine biological response to concentrations of the biologically active components of soil and water samples from a hazardous waste site. The tests are described in Appendix A and include aquatic and terrestrial tests. The algal, fish and Daphnia tests are used for water and soil leachate samples, and seed germination-root elongation, earthworm, and soil microorganism tests are used for soil samples. The tests are standardized and each has a background of literature citations which include some field evaluation. Because of occupational risks, field and laboratory procedures must be used to minimize hazard to staff during the application of the protocol (Guidelines, Appendix B).

The key to defining site priority or cleanup effectiveness is in the experimental sampling design. Careful definition of general and site-specific issues is necessary. With these issues carefully in mind, the design should be evaluated in terms of cost-benefit so that costly errors in environmental risk and economic risk are minimized. Important points about

how these concepts relate to sampling design are discussed in the main text. The bioassessment protocol is designed to be a set of tools that are applied as appropriate to a specific site. Necessary samples are collected to address the specific issues that occur at the site. Data from chemical analyses and field studies may be available or may be required based on the results obtained from bioassessment.

The bioassessment protocol will be improved for future use with field application and with further research. It is promulgated at this time because there is a need for biological tests and ongoing field application will lead to improvement directly.

INTRODUCTION

BACKGROUND

The potential hazard of planned, existing or abandoned waste disposal sites depends on their risk to human health and the environment. Generally, these hazards fall into four categories: toxicity, persistence, bioaccumulation, and mobility. To minimize these hazards, cleanup and control actions are being taken based on data describing site characteristics.

The identification, characterization, and cleanup of hazardous materials, sites and spills is a high priority of the administration and society. Numerous potential cleanup sites have been identified throughout the United States and the 115 priority sites have been selected (HMIR, 1981). Further characterization and cleanup of approximately a dozen of the very worst sites is expected to proceed shortly. However it is not entirely clear how these characterizations and cleanups will be conducted. Early drafts of the National Contingency Plan indicated that chemical characterization of sites would have a high priority. It was proposed that the chemical characterizations would be applied to existing water and air criteria to determine when cleanup was necessary, how much to cleanup, and when to terminate the cleanup. This approach has been criticized for various reasons. Among the reasons is that criteria applications would tend to be overly protective and thus overly restrictive. Another criticism is that insufficient numbers of adequate criteria exist. Yet another criticism is that single pollutant, constant concentration, laboratory derived

criteria are not applicable to environmentally released complex hazardous waste materials which may be encountered at numerous disposal sites. If water and air quality criteria are not applied then, what tools will be employed to assess the need for and degree of cleanup required?

The multi-media biological testing protocol presented in this document is an alternative or supplement to other assessment techniques. It is not without problems, but currently it is felt that the advantages outweigh the disadvantages.

The overall objective of this report is to develop a reasonably acceptable bioassessment protocol that can be used immediately while being tested over a broad range of pollutant and geoclimatic conditions. The idea is to develop or modify a procedure such that it might be applied in a general way. Interpretation of the data will be on a case by case basis depending on the potential for environmental impact and the intended use of the area in question. The bioassessment protocol consists of a set of specifically defined biological tests. Sampling design and statistical analysis concepts are contained in the protocol. The tests consist of short term aquatic and terrestrial acute test for toxicity. Except for the soil litter test, the biological tests utilize only a single species but they do include plants, invertebrates, vertebrates, and decomposers.

PURPOSE OF THE BIOASSESSMENT PROTOCOL

The bioassessment protocol has the purpose of assessing the potential for ecological harm from hazardous waste sites. The bioassessment protocol is one set of tools, along with chemical and field studies, that can be used to minimize the risk of hazardous materials by knowing more about their potential hazard. The responses of a range of test species to exposure from water and soil samples are used to determine whether toxicity exists at a site. The biological tests that make up the protocol cover a range of biological taxa, are standardized, and have been used for a variety of environmental assessments. They are to be used with water samples or soil samples. The relationship between the issues being decided for a site and the bioassessment protocol depends on the sampling program.

The bioassessment protocol is applied at two levels: site prioritization and cleanup evaluation. Depending on a variety of factors including concentration, type and availability of chemicals, organisms at risk, exposure routes, and duration of exposure, certain sites have the potential for more or less ecological risk. By incorporating these risk factors, the test results can be used to rank sites in order of priority for cleanup, isolation, or other action. Cleanup evaluation is the application of the biological tests to determine: "How clean is a site?"

States, industries, and federal agencies will be interested in using these tests for these purposes. The purpose of this report is to describe the biological tests and protocol. The biological tests are presented in a stepwise manner in Appendix A while the protocol is presented in the text.

A BIOASSESSMENT ANALOGY

The ecosystem is an entity in which humanity and society are an integral part. Protecting natural communities will guarantee protection in most cases for human communities. The state of the natural community can be viewed as an indicator of potential risk to society and effects on important processes can be viewed as proxies for those processes that directly as well as indirectly relate to society. By protecting the ecosystem, we protect man. The bioassessment protocol can be viewed as a quantitative means of estimating biological impacts of hazardous waste sites. The individual tests are bio-transducers for environmental protection, providing an estimate of potential hazard to organisms caused by chemicals that vary in their availability and toxicity in water or soil.

The potential for damage to human health and the ecological integrity of hazardous waste sites can be perceived similarly to a dying canary in a coal mine. The analogy applies to this protocol because bioassessment methods are intended to provide a measure of potential acute biological damage associated with samples from a particular site. A canary breathes the mine air responds to all biologically active components of the atmosphere, and if an acute response occurs, dies, at which time the miners flee. If the canary sickens over a long period of time, it would be replaced and probably no human response would occur. Thus, chronic effects are not assessed.

The bioassessment procedures are similar to the canary in the mine and provide a rapid screening of all of the biological active components of hazardous waste sites, and if an acute response is obtained, provide a

signal causing an appropriate response by society. The key points are:

- Bioassessment provides a biological response which integrates all of the active components in a sample.

- Bioassessment provides an estimate of the biologically available forms of the sample components.

- In comparison to chemical measurements, biological measurements are more direct, integrative, and meaningful.

PROTOCOL OBJECTIVES

The overall objective of the bioassessment protocol is to provide a more comprehensive measure of potential ecological hazard associated with hazardous waste sites than chemical analyses and comparison to air and water quality criteria can provide. The protocol is designed to provide answers about sites in which few data are available. Protocol results will improve the accuracy of the assessment as well as improve cost-effectiveness. Consequently, the confidence of decision makers will be greater. To achieve the overall objective of the protocol, specific steps must be followed:

- Define safety issues at a specific site.

- Define potential transport and fate of site materials and populations at risk.

- Define containment site boundaries.

- Design a sampling program to meet specified statistical criteria.

- Obtain appropriate soil and surface and ground water samples at the site boundary.

- Obtain appropriate soil and surface and ground water samples along a gradient of waste contamination.

- Select tests appropriate to answering the safety issues defined previously.

- Perform necessary pretreatment of samples and the biological tests.

Some of these steps may be repeated based on biological test results. For example, containment site boundaries might be extended because protocol results show that soil and ground water samples beyond the boundary are excessively contaminated.

BIOASSESSMENT PROTOCOL

PERSPECTIVE

Hazardous wastes have been characterized as wastes from a list of specific industries (Table 1), wastes containing one or more components on the priority pollutants list (Table 2), or a waste that is ignitable, corrosive, reactive, or toxic. Also, wastes are hazardous if they exhibit chemical measurements of a specified leachate that exceed the national drinking water standards by a factor of 100 or more (Table 3). Concepts and controversy relating to the characterization of hazardous wastes have been discussed elsewhere (____, 1981). The tests described in this protocol may be extremely useful in refining these definitions.

To gain more perspective on the purpose of bioassessment, it is instructive to ask which potential hazards are not being assessed with biological tests. The bioassessment protocol only addresses the toxicity issue. In addition, volatile materials will probably not be assessed accurately. Chronic toxicity, carcinogenesis, mutagenesis, and teratogenesis are not assessed using the bioassessment protocol. A Level 2 protocol could be defined for assessing these hazards.

Although the bioassessment protocol does not measure all facets of hazardous waste site problems, it does provide a measure of those factors that directly affect environmental processes, i.e. toxicity. In this regard they are useful for assessing the potential ecological hazard of hazardous

Table 1. EPA Primary Industry Categories

Adhesives and sealants
Aluminum forming
Auto and other laundries
Battery manufacturing
Coal mining
Coil coating
Copper forming
Electric and electronic components
Electroplating
Explosives manufacturing
Foundries
Gum and wood chemicals
Inorganic chemicals manufacturing
Iron and steel manufacturing
Leather tanning and finishing
Mechanical products manufacturing
Nonferrous metals manufacturing
Ore mining
Organic chemicals manufacturing
Paint and ink formulation
Pesticides
Petroleum refining
Pharmaceutical preparations
Photographic equipment and supplies
Plastic and synthetic materials manufacturing
Plastic processing
Porcelain enameling
Printing and publishing
Pulp and paperboard mills
Rubber processing
Soap and detergent manufacturing
Steam electric power plants
Textile mills
Timber products processing

Table 2. EPA's Priority Toxic Pollutants

1. *acenaphthene
2. *acrolein
3. *acrylonitrile
4. *benzene
5. *benzidine
6. *carbon tetrachloride (tetrachloromethane)

*chlorinated benzenes (other than dichlorobenzienes)

7. chlorobenzene
8. 1,2,3-trichlorobeinzene
9. hexachlorobenzene

*chlorinated ethanes (including 1,2-dichloroethane, 1,1,1-trichloroethane and hexachloroethane)

10. 1,2-dichloroethane
11. 1,1,1-trichloruethane
12. hexachloroethane
13. 1,1-dichloroethane
14. 1,1,2-trichloroethane
15. 1,1,2,2-tetrachloroethane
16. chloroethane

*chloroalkyl ethers (chloromethyl, chloroethyl and mixed ethers)

17. bis(chloromethyl) ether
18. bis(2-chloroethyl) ether
19. 2-chloroethyl vinyl ether (mixed)

*chlorinated naphthalene

20. 2-chloronaphthalene

*chlorinated phenols (other than those listed elsewhere; includes trichlorophenols and chlorinated cresols)

21. 2,4,6-trichlorophenol
22. parachlorometa cresol
23. *chloroform (trichloromethane)
24. *2-chlorophenol

*dichlorobenzenes

25. 1,2-dichlorobenzene
26. 1,3-dichlorobenzene
27. 1,4-dichlorobenzene

*dichlorobenzidine

28. 3,3-dichlorobenzidine

*dichloroethylenes (1,1-dichloroethylene and 1,2-dichloroethylene)

29. 1,1-dichloroethylene
30. 1,2-trans-dichloroethylene
31. *1,3-dichlorophenol

*dichloropropane and dichloropropene

32. 1,2-dichloropropane
33. 1,2-dichloropropylene (1,3-dichloropropene)
34. *2,4-dimethylphenol

*dinitrotoluene

35. 2,4-dinitrotoluene
36. 2,6-dinitrotoluene
37. *1,2-diphenylhydrazine
38. *ethylbenzene
39. *fluoranthene

*haloethers (other than those listed elsewhere)

40. 4-chlorophenyl phenyl ether
41. 4-bromophenyl phenyl ether
42. bis(2-chloroisopropyl) ether
43. bis(2-chloroethoxy) methane

*halomethanes (other than those listed elsewhere)

44. methylene chloride (dichloromethane)
45. methyl chloride (chloromethane)
46. methyl bromide (bromomethane)
47. bromoform (tribromomethane)
48. dichlorobromomethane
49. trichlorofluoromethane
50. dichlorodifluoromethane
51. chlorodibromomethane
52. *hexachlorobutadiene
53. *hexachlorocyclopentadiene
54. *isophorone
55. *naphthalene
56. *nitrobenzene

*nitrophenols (including 2,4-dinitrophenol and dinitrocresol)

57. 2-nitrophenol
58. 4-nitrophenol
59. 2,4-dinitrophenol
60. 4,6-dinitro-o-cresol

*nitrosamines

61. N-nitrosodimethylamine
62. N-nitrosodiphenylamine
63. N-nitrosodi-n-propylamine
64. *pentachlorophenol
65. *phenol

*phthalate esters

66. bis(2-ethylhexyl) phthalate
67. butyl benzyl phthalate
68. di-n-butyl phthalate
69. di-n-octyl phthalate
70. diethyl phthalate
71. dimethyl phthalate

*polynuclear aromatic hydrocarbons

72. benzo(a)anthracene (1,2-benzanthracene)
73. benzo(a)pyrene (3,4-benzopyrene)
74. 3,4-benzofluoranthene
75. benzo(k)fluoranthane (11,12-benzofluoranthene
76. chrysene
77. acenaphthylene
78. anthracene
79. benzo(ghi)perylene (1,12-benzoperylene)
80. fluorene
81. phenanthrene
82. dibenzo (a,h) anthracene (1,2,5,6-dibenzanthracene)
83. indeno (1,2,3-cd)pyrene (2,3-o-phenylenepyrene)
84. pyrene
85. *tetrachloroethylene
86. *toluene
87. *trichloroethylene
88. *vinyl chloride (chloroethylene)

pesticides and metabolites

89. *aldrin
90. *dieldrin
91. *chlordane (technical mixture & metabolites)

*DDT and metabolites

92. 4,4'-DDT
93. 4,4'-DDE (p,p'-DDX)
94. 4,4'-DDD (p,p'-TDE)

*endosulfan and metabolites

95. a-endosulfan-Alpha
96. b-endosulfan-Beta
97. endosulfan sulfate

*endrin and metabolites

98. endrin
99. endrin aldehyde

*heptachlor and metabolites

100. heptachlor
101. heptachlor epoxide

*hexachlorocyclohexane (all isomers)

102. a-BHC-Alpha
103. b-BHC-Beta
104. r-BHC (lindane) -Gamma
105. g-BHC-Delta

*polychlorinated biphenyls (PCB's)

106. PCB-1242 (Arochlor 1242)
107. PCB-1254 (Arochlor 1254)
108. PCB-1221 (Arochlor 1221)
109. PCB-1232 (Arochlor 1232)
110. PCB-1248 (Arochlor 1248)
111. PCB-1260 (Arochlor 1260)
112. PCB-1016 (Arochlor 1016)
113. *toxaphene
114. *antimony (total)
115. *arsenic (total)
116. *asbestos (fibrous)
117. *beryllium (total)
118. *cadmium (total)
119. *chromium (total)
120. *copper (total)
121. *cyanide (total)
122. *lead (total)
123. *mercury (total)
124. *nickel (total)
125. *selenium (total)
126. *silver (total)
127. *thallium (total)
128. *zinc (total)
129. **2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

* Specific compounds and chemical classes listed in the NRDC consent decree and referenced in the Clean Water Act.

** This compound was specifically listed in the consent decree; however, due to its extreme toxicity EPA recommends that laboratories not acquire an analytical standard for this compound.

Table 3. Primary Drinking Water Quality Standards

Parameters	Annual Average Maximum Daily Air Temperature °F	°C	Maximum Level*
Inorganic Chemicals			
Arsenic			0.05
Barium			1.
Cadmium			0.010
Chromium			0.05
Lead			0.05
Mercury			0.002
Nitrate (as N)			10.
Selenium			0.01
Silver			0.05
Fluoride			
	53.7 and below	12.0 and below	2.4
	53.8 to 58.3	12.1 to 14.6	2.2
	58.4 to 63.8	14.7 to 17.6	2.0
	63.9 to 70.6	17.7 to 21.4	1.8
	70.7 to 79.2	21.5 to 26.2	1.6
	79.3 to 90.5	26.3 to 32.5	1.4
Chlorinated Hydrocarbons			
Endrin (1, 2, 3, 4, 10, 10-hexachloro-6, 7-epoxy-1, 4, 4a, 5 6, 7, 8, 8a-octahydro-1, 4-endo-5, 8-dimethano naphthalene)			0.0002
Lindane (1, 2, 3, 4, 5, 6-hexachlorocyclohexane, gamma isomer)			0.004
Methoxychlor (1, 1, 1-Trichloroethane) 1, 1-bis (p-methoxyphenyl)			0.1
Toxaphene ($C_{10}H_{10}Cl_8$-Technical chlorinated camph ene, 67-68 percent chlorine)			0.005
Chlorophenoxys: 2,4-D, (2, 4-Dichlorophenoxyacetic acid)			0.1
2, 4, 5-TP Silvex (2, 4, 5-Trichlorophenoxy-propionic acid)			0.01
Turbidity (for surface water sources	1 TU up to 5 TU*		
Coliform Bacteria			
Membrane filter technique:	1/100 ml mean/month 4/100 ml in one sample if <20 samples/month 4/100 ml in more than 5% if >20 samples/month		
Fermentation tube with 10 ml portions:	no coliforms in 10% of portions/month no coliforms in >3 portions/sample if <20 samples/month no coliforms in >3 portions of 5% of samples if >20 samples/month		
Fermentation tube with 100 ml portions:	no coliform bacteria in >60% of portions/month no coliform in 5 portions in one sample if <5 samples/month no coliform in 5 portions in 20% of samples if >5 samples/month		

Radioactive Material	Level
Combined radium 226 and radium 228	5 pCi/l
Gross alpha particle activity**	15 pCi/l
Beta particle and photon radioactivity from man-made radionuclides	4 millirem/year
Tritium for total body	20,000 pCi/l
Strontium-90 in bone marrow	8 pCi/l

*mg/l unless otherwise stated.

**Includes Ra^{226} excludes Radon, Uranium.

***If meet special requirement.

Source: EPA (1977)

wastes. Ignitability, corrosivity and reactivity have impacts that are catastrophic and overshadow long-range environmental concerns and therefore require more immediate action. However, the bioassessment tests provide responses to the toxicity of such materials. Volatile materials generally do not exert toxicity unless they are bioaccumulated. Thus, research is needed to develop procedures to evaluate those bioaccumulated materials that become toxic to higher trophic level organisms.

It is important to an accurate perspective on bioassessment tests to consider the role of chemical analysis and field ecological studies in evaluating hazardous waste sites.

Chemical analysis is important for actual cleanup procedures and for evaluating fate and effects of materials from hazardous waste sites. An extensive list of chemicals such as those in Table 2 and 3 can be measured in water, soil, and soil leachate samples. The measured concentrations can be compared to standards or criteria extrapolated from laboratory bioassay procedures. Appropriate actions are taken then according to published regulations. However, considerable uncertainty remains. The decision maker is never certain whether all toxic chemicals are on the list, whether they are measured adequately, or whether the mixture exerts different toxicity than the sum of the individual chemicals. Also, not all of the chemicals are equally available to organisms under a particular set of conditions. Although chemical results are administratively easy to supervise, they are not as meaningful in an ecological context as biological measurements. Although the relative precision of biological tests compared to chemical tests is often questioned, biological tests have been found to be as precise

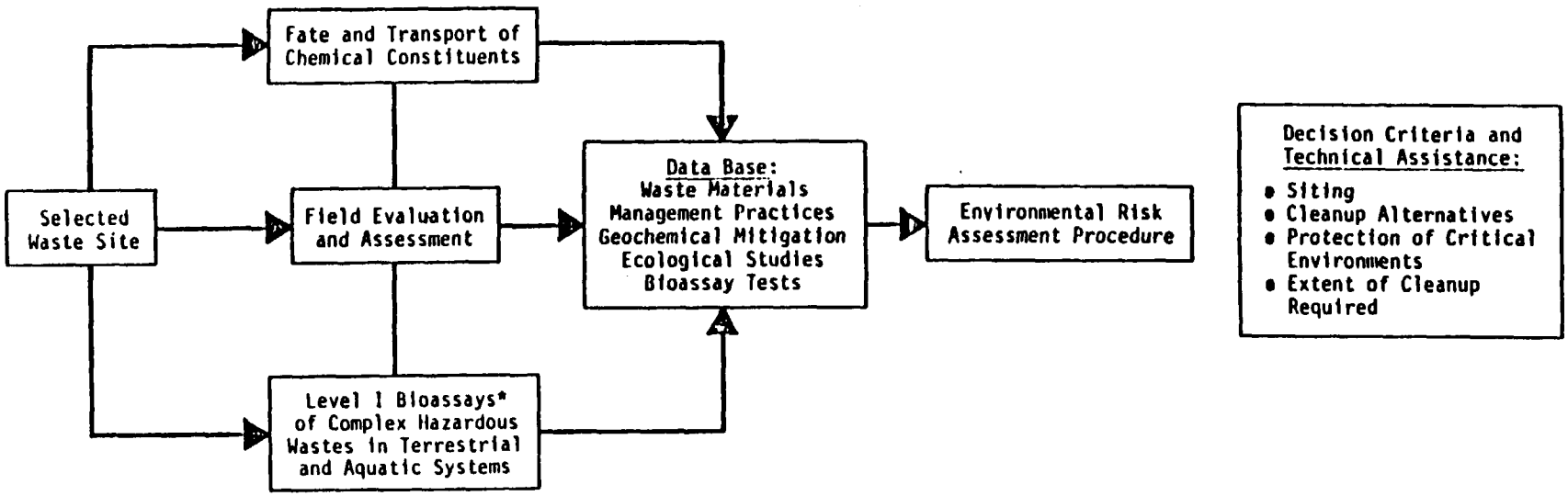
as chemical tests if proper procedures are followed. Moreover, the question does not concern precision as much as accuracy, that is integrating the effects and bioavailability of compounds to organisms.

The most direct approach for evaluating the ecological hazard of an hazardous waste site is to observe effects on the ecosystem by field studies. However, several disadvantages for this approach exist. To observe effects the ecosystem must be damaged, and that is what we wish to avoid. Field studies are expensive, time consuming, are not predictive and in fact are retrospective. However, because field data are direct, they are necessary for many purposes.

We conclude from the above discussion that biological tests are a necessity for evaluating potential ecological effects of hazardous waste sites. However, for two purposes, biological tests should be complemented by chemical measurements and field studies.

First, the biological tests must undergo testing by comparing biological test results to chemical and field study data. This process is being conducted by the U.S. EPA, Corvallis Environmental Research Laboratory, Oregon (Figure 1). As experience is gained in applying the bioassessment protocol, information on field conditions, chemistry, and biological testing must be obtained and reported so that the protocol can be improved.

Second, the bioassessment protocol cannot supplant all chemical and field data for regulatory reasons. Many of these data will be available at



*Application of Bioassessment Protocol.

Figure 1. Evaluation of Environmental Testing Protocols for Hazardous Waste Assessment (Peterson, 1982)

the initiation of site studies or are obtained by observation and measurement at that time. Coordination of this information with the bioassessment protocol results is an important need in protocol development.

As an example of how the bioassessment protocol can be incorporated into assessment of a hazardous waste site, we have included excerpts from a site response management plan produced by Mathis (1981) of USEPA's Region IV.

The following discussion is taken directly from Mathis (1981) with minor modification including provision for applying the bioassessment protocol. The EPA Site Tracking System is portrayed in a flow chart (Figure 2). A principal investigator with responsibility for site evaluation is assigned. The major milestones are as follows:

1. SITE IDENTIFICATION This represents the entry of a potential uncontrolled site into the system, and may be initiated through any of the many methods of information gathering. General guidance is that in the absence of an affirmative showing that no hazardous material is involved, all reported potential sites will be entered into the system.

2. PRELIMINARY ASSESSMENT At this point the investigator completes a search of available files in Federal and State Agencies, usually accomplished by telephone contact outside the agency, and will also complete telephone interviews with identified persons having knowledge of this site. The purpose

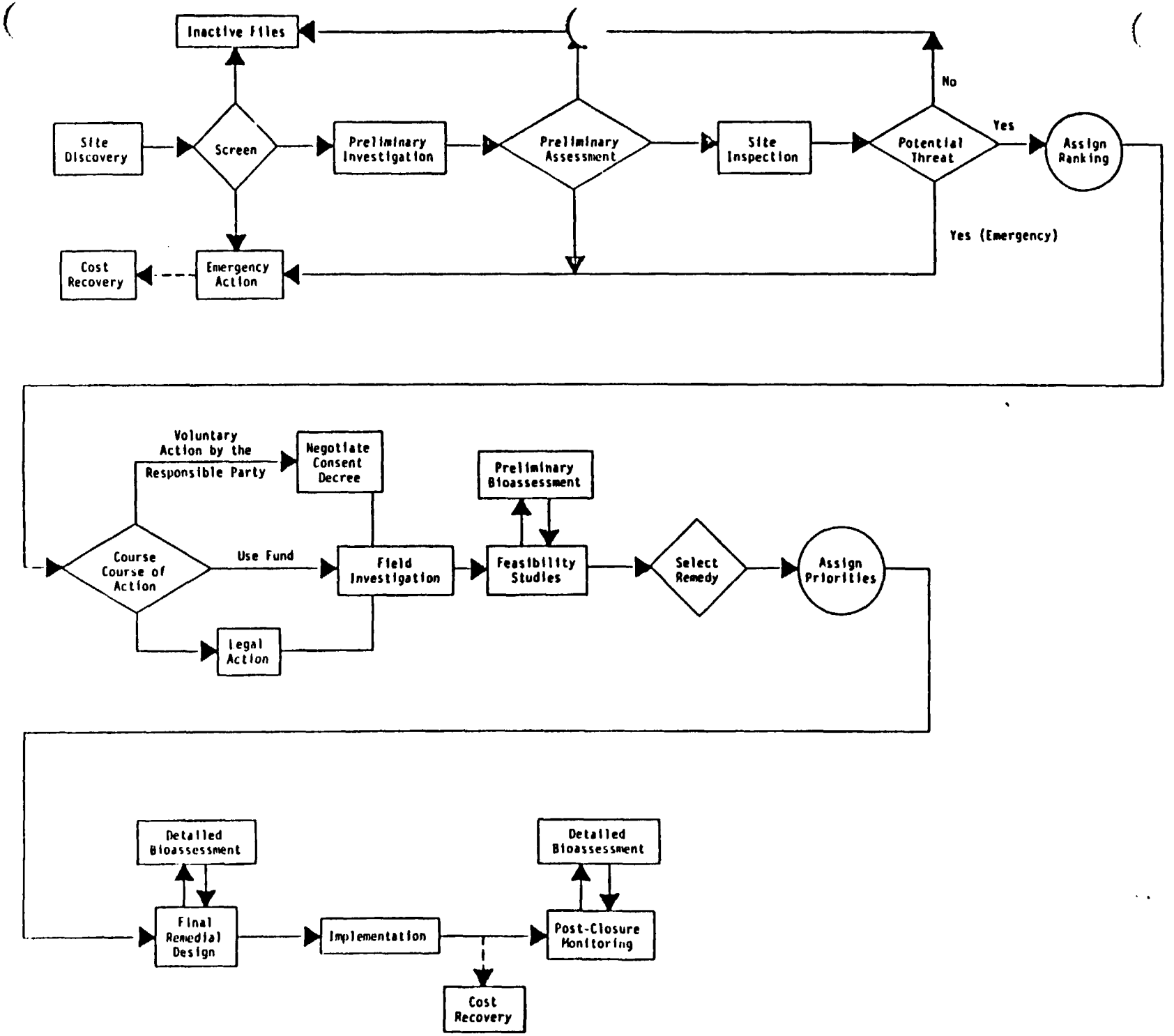


Figure 2. Site Response Management Plan (from Mathis, 1981)

of this action is to discern possible releases which would require emergency containment action to mitigate an imminent hazard to health or the environment, with immediate investigation (response time in hours); and to permit a subjective evaluation of the degree of hazard of other sites as HIGH MEDIUM, LOW, NONE, or UNKNOWN. This judgment becomes the basis for prioritizing the site for the next level of investigation. Resources expended at this point range from 0.5 to 1.5 days.

3. SITE INSPECTION This activity involves a visit to the site by a team of at least two investigators. Their function is to observe the potential site prior to entry, assess risk for site entry, interview knowledgeable indigenous personnel, appraise the population at risk, identify potential exposure routes, and if justified, enter the site to observe and subjectively evaluate topography, geology, quantity and type of material present, conditions of storage or disposal, evidence or probability of release or migration, and resources needed to quantify or objectively measure these parameters. Due to safety considerations, no sampling is conducted at this time, however considerable information is collected and recorded in the form of observations and photographs. Resources expended in this activity range from 2.0 to 4.0 work-days exclusive of travel. The purpose of this activity is to produce a more certain evaluation of the potential hazard as HIGH, MEDIUM, LOW, or NONE, to prioritize the site

for field investigation as needed and to permit preparation of a TENTATIVE DISPOSITION.

4. TENTATIVE DISPOSITION This activity comprises a decision point from which the site is tentatively classified as most appropriate for one of four courses of action. The decision is recommended by the principal investigator, and is reviewed by appropriate section leaders for concurrance. Basis for this recommended decision is a review of all assembled information on the site. Resources required are approximately 1.0 work-days plus review. The four alternative decisions possible are:

   a. Enforcement Action by State or Federal Agency.

      This implies that a viable defendant and an imminent hazard are both present.

   b. Remedial Action using Federal, State or Other Resources.

      This implies that either a responsible party may be willing to undertake necessary action, or that no viable defendant is apparent and direct action using the authority of CERCLA seems appropriate. It also implies that an imminent hazard is present.

   c. Further Investigation Needed.

      This indicates that collection of field data through sampling, geophysical studies, the bioassessment protocol, or other means is required to ascertain the presence or absence of an imminent hazard, or to quantify

and delineate the extent of that hazard. This mandates a resource-intensive investigation, and requires a review to set the priority for this effort. Prioritization may be aided by an initial preliminary assessment using the bioassessment protocol.

d. No Further Action Required.

This implies that uncontrolled hazardous material is not present at this time, and no significant hazard exists.

5. FINAL STRATEGY DETERMINATION This Activity represents the coordinated timetable for a recommended Enforcement Action of Remedial Action, the timetable for the required investigations, or the final concurrance in a finding of No Further Action Required. Final Strategy Determinations are tracked, and are amended as progress is made on the respective timetable. When a Final Strategy Determination of No Further Action is reached the site may be placed in the inactive file of the system. Resource requirements in this stage are highly variable. Cleanup procedures can be evaluated using the bioassessment protocol. It is possible that litigation may take several years to complete, at great cost, and remedial measures may entail costs in the millions. Extensive investigation effort may cost hundreds of thousands of dollars and involve many months of effort. In contrast, a determination of no further action may be processed in a single work-day.

It is notable that at each milestone in the process there is an opportunity to reappraise the priority of each site for the next level of activity, and that at each milestone, limited resources are focused on those sites which are most significant in terms of:

1. Seriousness of hazard to health.
2. Seriousness of hazard to the environment.
3. Presence of a viable defendant or responsible party.
4. Existence of a technically feasible remedy.
5. Availability of uncommitted and appropriate resources.
6. Other factors which tend to raise priority; EG: State Opinion.

It is also generally the case, though not infallibly so, that as a high priority site advances toward the point at which no further action is needed, resource requirements increase. This situation has resulted in the appearance of several resource-related limitations to the speed with which potential uncontrolled hazardous material sites are processed.

The first of these limitations is the availability of analytical support for the program. Analytical capability, and the associated quality assurance support, is a finite resource due to the high cost, and is also finite in terms of physical capacity. Furthermore, the pursuit of Enforcement Options places an even greater demand on this resource than investigation or

remedial design, due to the need for elaborate amounts of data of unimpeachable quality. It may also be projected that where remedial actions are conducted in anticipation of a possible legal action to recover costs, the desirability of documenting the findings, the action taken, and the benefits accrued thereby, will all necessitate a significant increase in analytical effort beyond that minimum necessary to design and implement the remedy.

The second limitation which has emerged is the need for intensive expenditures of manpower to perform on-site tasks in a safe manner. To approach a totally unknown site in what is generally agreed to be a conservative, safety conscious method requires a team of 5 to 6 men in order to cope with all contingencies. Furthermore, this team requires elaborate and costly equipment to detect possible agents in real time, while providing protection against a wide spectrum of toxicants. The bioassessment protocol could provide an excellent solution for this problem. This equipment, in turn, requires recurrent training of personnel and periodic maintenance if it is to be used effectively. Finally, the team and its special equipment are not readily transportable by many commercial carriers when prepared for operation. While many regulatory agencies and private laboratories acknowledge similar safety criteria for site investigation, very few of them are staffed or funded to allow operation in accordance with recommended procedures. Thus, specialized contractors may often be the most feasible choice for site sampling.

APPLICATION OF BIOASSESSMENT PROTOCOL

At least three scenarios regarding hazardous waste sites in relation to the potential application of bioassessment can be defined:

- The site is uncharacterized and unprioritized. In this case an assessment of potential hazard to site workers, neighborhood public, downstream users, and the natural ecological community is needed. First, the National Ranking Model (e.g., Caldwell et al., 1981) should be applied and then, if the potential hazard is high enough, a rapid physical-chemical screening approach should be taken (e.g. Turpin et al., 1981). Other data would be obtained as indicated in the discussion above by Mathis (1981).

- The site is characterized but the extent of contamination is unknown. In this case the waste site is not well defined and it is probable that the bioassessment protocol applied at the screening level would be very useful to evaluate the extent of contamination and provide input to the experimental design for more detailed assessment.

- The site is characterized and cleanup or other remedial action is being taken. In this case bioassessment would provide an estimate of remaining hazard, monitoring of incipient hazard,

and would help in establishing or monitoring the boundaries of a required containment zone. Criteria related to bioassessment results would be based on samples collected at the boundaries of the containment zone or, for water samples, samples appropriate to projecting the effects of flow beyond the containment zone boundaries.

The first and second scenarios lead to the site priority or screening line of the bioassessment protocol. These data are needed before proceeding to the more detailed assessment. The screening assessment has the intent of providing a rapid survey of potential problems. The third scenario leads to the detailed assessment. The key step is to develop a sampling design appropriate to the site and potential hazards of the site.

Samples are collected at essentially three stations for the preliminary assessment. The core station (most impacted station), the site containment boundary, and a reference station (off site). Appropriate surface or ground water and soil samples are collected for bioassessment. Bioassessment results for core and boundary stations are compared to a control consisting of artificial media as well as to the reference site. Essentially, three replicate samples randomly selected at each site, are used for the preliminary assessment.

Detailed assessment consists of transects and multiple stations designed to evaluate question concerning the spread of toxic materials and the relative hazard associated with the samples. Generally, the transect would begin at the core station. Multiple stations along the containment

boundary would be required. The important question will concern allocation of manpower and laboratory resources. Optimum allocation must be based on the Experimental Design and concepts are discussed under that heading.

The other major question concerns the biological response obtained from applying the protocol. Response levels for each organism: high, intermediate, low, can be obtained for each sample. Points can be assigned for the response levels, summed, and priorities assigned according to relative biological hazard. Then the sites can be further ranked according to other risk criteria having to do with fate and human effects considerations. However, at this time, we are primarily concerned with developing a scale related to the biological test results and fate and human effects will be reported at a later date.

BIOLOGICAL TESTS FOR THE BIOASSESSMENT PROTOCOL

Two types of samples are tested, water and soil. Water samples are collected from surface and ground water sites or obtained as leachates from soil samples. Soil samples are collected from appropriate sites using grab sample apparatus. Core samples are not required but may be used. Descriptive data, methods of collection, and sample treatment and storage requirements for water and soil samples are described in the sampling chapter. These samples are tested with either aquatic or terrestrial organisms.

Organisms for use with water samples include algae, daphnids, and fish. Organisms for use with soil samples include decomposers, plant seeds and earthworms. These tests are summarized in Table 4 and discussed in detail in Appendix A. Besides showing the tests, the target variables, and appropriate sample type, the response levels for each biological test are listed. These response levels are provisional at this time and provide guidance on the relative hazard associated with a given sample. The response levels will be refined as more data are obtained.

The tests included in the bioassessment protocol were selected based on criteria related to the validity of tests and their feasibility for use. Validity factors are the accuracy, precision, and sensitivity (precision at low concentration) of a test. Accuracy concerns how well the results can be extrapolated to a natural system. Precision and sensitivity are statistical factors and vary with the organisms and the type(s) of toxicants. Feasibility factors concern the cost of the test, the degree of expertise needed by the operator, the convenience of performing the test, and the speed with which a result is obtained. Although these data are available for some of the tests, ongoing research will provide a complete compilation of the data. This information will be necessary in order to design sampling programs with the optimal allocation of sampling and testing resources.

Table 4

DEFINITION OF TOXICITY CATEGORIES FOR AQUATIC AND TERRESTRIAL ECOLOGICAL ASSAYS

Assay	Activity Measured	Sample Type[a]	MAD[b]	Units	Response Levels for LC_{50} or EC_{50} Concentrations[c]		
					High	Moderate	Low or Not Detectable
Freshwater Fish	96-hr LC_{50} (lethality)	S	1	g/L	<0.01	0.01-0.1	0.1-1
		L	100	percent	<20	20-75	75-100
Freshwater Invertebrate	48-hr EC_{50} (immobilization)	S	1	g/L	<0.01	0.01-0.1	0.1-1
		L	100	percent	<20	20-75	75-100
Freshwater Algae	120-hr EC_{50} (growth inhibition)	S	1	g/L	<0.01	0.1-0.1	0.1-1
		L	100	percent	<20	20-75	75-100
Seed Germination and Root Elongation	115-hr EC_{50} (inhibit root elongation)	L	100	percent	<20	20-75	75-100
Earthworm Test	168-hr LC_{50}	S	500	g/kg	<50	50-500	500
Soil Respiration Test	336-hr EC_{50}	S	500	g/kg	<50	50-500	500
		L	100	percent	<20	20-75	75-100

[a] S = solid, L = aqueous liquid, includes water samples and elutiate or leachate. Nonaqueous liquids are evaluated on an individual basis due to variations in samples such as vehicle, percent organic vehicle, and percent solids.

[b] MAD = Maximum applicable dose.

[c] LC_{50} = Calculated concentration expected to kill 50 percent of population within the specified time interval.

LC_{50} = Calculated concentration expected to produce effect in 50 percent of population within the specified time interval.

POTENTIAL BIOASSESSMENT METHODS

A matrix of experimental design is shown in Figure 3. The protocol methods cover a wide spectrum but there are obvious gaps. An important gap, which is not shown, concerns bioaccumulation. Since bioaccumulation requires a long-term exposure, it is not included in the protocol. A possible research effort on octanol:water coefficients related to microbial responses or direct microbial bioaccumulation tests could be made to assess bioaccumulation. Although some specific processes, photosynthesis and respiration, are not included in the protocol, it is feasible to develop rapid tests for evaluating them. Other gaps such as a vertebrate soil test should be disregarded because the benefits are small compared to the costs of developing and performing such tests. Existing tests such as mutagenetic response of the plant, Tradescantia, the Ames test, and Microtox (Patent Pending) may have application as part of a protocol but more development of these techniques is required. Microbial enzyme tests exist that are rapid, inexpensive, and may provide ecologically accurate and meaningful results. Adaptation of these tests to evaluate hazardous waste sites could be an extremely cost-effective study goal.

Organism Type	Physiological Process	Laboratory Biological Test*	
		Soil	Water**
Plant	Photosynthesis	-	-
	Growth	Germination, Root Elongation	Selenastrum
	Genetic	(Tradescantia)	-
Invertebrate	Respiration	-	-
	Lethality	Earthworm	Daphnids
	Growth	(Earthworm)	-
	Genetic	-	-
Vertebrate	Respiration	-	-
	Lethality	-	Fathead Minnow
	Growth	-	-
	Genetic	-	-
Decomposer	Enzyme	-	(Microtox)
	Growth	Litter Decomposition	-
	Genetic	(Ames Test)	(Ames Test)

*Parentheses indicate test needs more study before broad use; dash indicates no candidate test; otherwise the tests are in the protocol, Appendix A.

**Water includes surface and ground water samples or soil leachates.

Figure 3. Matrix of Tests as Related to Types of Organisms and Physiological Processes.

EXPERIMENTAL DESIGN

SCOPE

The sampling program must be designed so that answers to the two critical issues--site prioritization and cleanup evaluation--are obtained. These issues can be phrased as questions: In terms of relative toxicity how does the site rank compared to other sites in the state (or other defined region)? Are levels of toxic materials in soil or water samples sufficiently low that potential environmental hazard beyond the site boundary is minimal?

To begin answering these questions, risk, statistical design, sampling constraints, and the characteristics of the tests themselves must be considered. Each of these topics will be briefly discussed before providing a step-by-step sampling protocol.

RISK ASSESSMENT

Some element of risk assessment is involved in all decision making. To evaluate the potential risk to ecosystems from hazardous waste sites, a basic understanding of the concepts of risk assessment can help in defining the issues and significance of the method. In this section, the major concepts of risk assessment are discussed in general and then addressed specifically to the bioassessment protocol.

There are three broad steps in making a risk assessment: analyzing the system, determining the "dose-response" relation, and integrating these factors to estimate the risk. Note that defining the level of acceptable risk is essentially a political decision. Acceptable risk may be more a question of perception and decisions may be left either to the individual, for example, cigarette smoking, or to society, for example, nuclear radiation.

In analyzing the hazardous waste system, the composition and quantity of material released to the environment need to be estimated. Then, transport and migration should be defined for materials including any environmental transformations of compounds that would occur under the specific conditions at the site.

The dose-response relationship refers to the concept of relating some response to the concentration of material and, in most cases, the duration of exposure. A target variable must first be defined that bears a functional relationship to exposure. This is a key step since knowing the amount of material that is present is not meaningful without knowing that there is an effect, that a quantitative relation exists between concentration and effect, and that the effect is important ecologically and on a significant scale. The biological tests play an important role in developing these relationships.

After the above two factors are known within reasonable confidence limits, the risk can be assessed by determining the quantity of material, the exposure to key parts of the system, and estimating the risk using the concentration-duration-effect relationship. This integration step requires

many assumptions which must be clearly identified. Often low-dose extensions are difficult, inaccurate, and for statistical reasons, most costly to obtain.

The bioassessment protocol as it presently exists in this report does not deal explicitly with specific hazardous materials, their quantity, fate, or transformation. Instead, the biological tests measure responses to the mixture of materials obtained in a sample. The responses are directed at the concentrations available to organisms and at the mixture which may be different than the sum of the individual effects because of interactions. By analyzing transect samples, a response gradient can be determined that relates to transport and migration processes. However, the most cost-effective application will occur at the containment boundary of the site.

The biological tests were developed using the concentration-duration-effect relationship (Table 5). This relationship was developed using one toxicant at a time. However, studies using multiple pollutants and complex waste mixtures as in Table 5 have shown the concentration-duration-effect concept is valid. Thus, the concept of using dilutions of a complex waste in a synthetic or natural receiving medium is valid.

There is one other issue of risk that must be considered. What are the effects of error? Two kinds of error exist; saying that there is a certain level of risk when in fact there is none and saying that there is none, when in fact a risk exists. In the first case, significant unnecessary expenditure might occur. In the second, considerable environmental damage might occur. It is important to evaluate both errors in a cost-benefit

Table 5. Raw Data for an Acute Toxicity Test Exposing Juvenile Mysid Shrimp to a Simulated Refinery Effluent (from Buikema, et al., 1982)

Concentration	Replicate	N	Number dead 24 h	Number dead 48 h
Control	1	10	0	0
	2	10	0	0
10%	1	10	0	2
	2	10	1	1
18%	1	10	1	3
	2	10	1	3
25%	1	10	2	5
	2	10	3	4
32%	1	10	5	9
	2	10	7	7
56%	1	10	10	10
	2	10	10	10
100%	1	10	10	10
	2	10	10	10

expenditure might occur. In the second, considerable environmental damage might occur. It is important to evaluate both errors in a cost-benefit manner before defining acceptable risk. For example, if there is significant additional cleanup cost with little environmental benefit, it may be possible to accept a higher level of test response at the containment boundary. Conversely, the no-effect risk level might be appropriate for a lower cleanup cost associated with high hazard. These questions must be explored in more detail in specific projects.

These questions have substantial effects on the sampling design. The variance in the biological test results needs to be known as well as the variance in sampling. This information is used to allocate sampling and test resources. Without proper design, money is wasted and proper results may not be obtained. These factors are discussed qualitatively in the next section.

Some general approaches to risk analysis that provide further information on risk assessment have recently been discussed (Ricci and Molton, 1981; Stan and Whipple, 1980; Squire, 1981). A detailed review of the literature on carcinogenic risk assessment is contained in Krewski and Brown (1981) which has general applicability in the context of this report.

STATISTICAL CONSIDERATIONS AND EXPERIMENTAL DESIGN

The goal of using bioassessment procedures as a tool for the evaluation of the potential environmental hazards among existing sites is to detect differences within and among sites and to distinguish among groups of sites on the basis of this potential. To achieve these objectives, appropriate test procedures must be identified and experiments must be designed in such a manner that differences in the test results, which reflect the potential hazards, will be identified. Three main categories of statistical considerations which relate to these requirements are discussed herein. These are: the location of sample collection sites, experimental procedure specifications, and analytical techniques. Although discussed separately, these topics are not independent and decisions concerning each of these aspects affect the options available in the other levels of the sampling design.

The selection of sampling locations within the individual hazardous waste sites will be a function of the type of biological test to be performed, as well as uniformity of the site with respect to edaphic characteristics and habitat type. It is known that the relationship between hazardous waste concentrations and morphological, chemical and biological sampling area determinants will vary widely within the sampling sites. Yet at the same time, it is essential to the overall study design for the comparison among sites, that these variations be kept at a minimum; i.e. an assumption of many statistical models which can be utilized to test for differences among sites is that random deviations of an observation from its expectation are constant. In this manner the effect of the individual hazardous waste sites is isolated and can be identified.

The feasibility of two procedures which will minimize the influence of within site variability on the estimated site effect should be evaluated. The first is the characterization of sample type-sample site relationships. The ability to specify sampling site selection criteria based on morphological, edaphic or biological characteristics for each proposed type of biological sample should be addressed. Certainly, tradeoffs must be made in the process of the specification of sampling area characteristics. This is because the very criteria which narrow the possibilities of sampling locations within waste sites can, at the same time, restrict the applicability of the biological test by restricting the number of hazardous wastes sites throughout the United States at which it can be utilized. However, for the purpose of reducing the sample variance within sites and equilibrating sample variances among sites, site-sample characterization must be discussed.

Secondly, the feasibility of random sampling within the acceptable sampling area should be addressed. Random sampling is essential to the assumptions of many appropriate analytical techniques and, as discussed below, the larger the number of replicates the greater is the ability to distinguish among differences in the target variable or variables among sampling (hazardous waste) sites. In the overall study design, a stratified random sampling plan should be adopted with the strata identified on the basis of the set of descriptive criteria discussed above and random sampling within the strata.

Related to the question of the selection of sample location within individual waste sites is the specification of control samples. Each bioassessment procedure selected should be conducted with control

experiments at each site. These samples will provide the means to assess the effects of the hazardous waste within the sites and will serve as quality control checks for the bioassessment program. Procedures for control samples (experiments) should be designed such that they can be repeated in the same manner at each site.

The second major area of statistical considerations which must be addressed is the appropriate level of sampling effort. Decisions on the level of sample replication or sampling effort in general cannot be made independently of careful consideration of the minimum level of difference in selected biological parameters that it is desired to detect and the precision with which differences should be detected. It is crucial to carefully plan field experiments in order to define these levels, to establish the number of samples required, and to specify the appropriate analytical approach.

Taking the analysis of variance (ANOVA) technique as an example of the type of statistical technique that might be used to identify differences in the mean values of specified measurements among the waste sites, sampling specifications and their implications to the overall study objectives will be discussed in the following paragraphs.

In considering the use of the ANOVA model, two significant criteria are specified. These are the probability of rejecting the null hypothesis when it is true (commonly called the alpha probability, or Type I error) and the probability of accepting a null hypothesis when it is false (commonly called the beta probability, or Type II error). Respectively, as stated previously, these errors are the probability of concluding there is a risk

when no risk exists (Type I) and no risk when a risk does exist (Type II). The first error can result in excessive cost while the second causes excessive environmental damage.

Another statistical parameter, referred to as the power of a test is important in this context since it defines the probability of correctly detecting experimental effects (e.g. differences among waste sites) in a particular bioassessment procedure. Results of statistical tests are often summarized by stating that no significant difference among stations was found at the 0.05 significance level. This refers to the alpha probability criterion which embodies the risk of mistakenly rejecting a null hypothesis that no differences exist. However, the above conclusion provides only a part of the necessary information. The significance level of the beta parameter should also be specified, especially in the case of comparisons to determine the relative environmental risk from hazardous waste sites, because decision makers are also interested in the probability that the test was unable to detect a difference that did exist.

Closer examination of the beta probability and its complement, the power of a test, is instructive since these probabilities can be defined as a function of sample size. In this manner, the probability of the level of difference that can be reliably detected with alternative allocations of sampling resources (stations and replication) can be determined.

In addition to the value of such investigations in a posteriori analyses of results, the probability of detecting differences in the selected biological parameters between waste sites and the levels of differences which can be detected with proposed sampling designs should be

addressed in the bioassessment program specifications. In order to achieve this objective, the population variance of selected bioassessment parameters must be estimated. This can be accomplished during a feasibility or pilot survey which can be designed to simultaneously address many other aspects of the proposed bioassessment program.

Consideration must be given to the methods as well as the criteria which will be utilized to distinguish among waste sites or to categorize them according to enviromental risk. Quantitative methods are recommended, and a large array of parametric and nonparametric statistical techniques are available for this purpose. However, in order to fully utilize the discriminating ability of these methods, the experiments must be planned carefully. As indicated above in the discussion of sample replication, the allocation of sampling resources should be made so as to optimize the level of difference among sites that can be reliably detected.

Also, before the application of the parametric models, it must be determined if the underlying assumptions (i.e., homogeneity of variances, independence, and normality) can be met. In the case that the assumptions of parametric tests cannot be maintained by data transformations, nonparametric tests can be substituted. Although nonparametric tests lack the distributional assumptions of parametric tests, they have less power and they lack the ability to evaluate interactive effects.

The analysis of variance (ANOVA) is an example of a parametric statistical model which can be used to make univariate comparisons among waste-site samples. The purpose of the statistical model is to estimate true differences among the sample means. To describe possible effects of

the waste site, a simple linear model is proposed by which any single observation can be decomposed as follows:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

where:

Y = biological observation for site i and replicate j

μ = mean value over all sites and relicates

α_i = effect of site i on Y

ε_{ij} = random deviation of the observation from its expectation $(\mu + \alpha_i)$.

The ANOVA is used to test the hypothesis that there is no difference in the biological observations made at different sites, i.e. that differences do not exist in the component due to site location ($\alpha_1 = \alpha_2 = ... = \alpha_n$).

In cases where significant differences are found to exist among sampling sites, multiple range tests (such as the Student-Neumann-Keuls or Duncan's) can be used to identify subsets of samples (sites) having equal mean values for the variable under examination. An example of the use of a multiple range test to demonstrate treatment differences among algal bioassays and to identify subsets having equivalent mean values is shown in Table 6.

In conclusion, decisions concerning the methods which will be used to distinguish among the waste sites sampled must be made in advance of the adoption of any bioassessment procedure. In this manner the bioassessment

Table 6. Duncan's Multiple Range Test of Complex Additions to Scenedesmus[a] (from Cleave, et al., 1980)

max standing crop, $\bar{X}$

treat. no.	concn, mL	treatment
1	20	AR elutriate
25	20	BP elutriate
27	10	BP elutriate
42		control
44		control
45		control
26	15	BP elutriate
21	20	BR salts
15	10	AP salts
7	10	AR salts
22	15	BR salts
32	5	BP salts
20	5	BR elutriate
4	5	AR elutriate
28	5	BP elutriate
24	5	BR salts
30	15	BP salts
43		control
33	20	AP column leachate
10	15	AP elutriate
6	15	AR salts
9	20	AP elutriate
23	10	BR salts
31	10	BP salts
3	10	AR elutriate
39	10	AP column salts
5	20	AR salts
14	15	AP salts
8	5	AR salts
12	5	AP elutriate
2	15	AR elutriate
41		control
16	5	AP salts
38	15	AP column salts
29	20	BP salts
35	10	AP column leachate
13	20	AP salts
19	10	BR elutriate
18	15	BR elutriate
37	20	AP column salts
36	5	AP column leachate
17	20	BR elutriate
34	15	AP column leachate
40	5	AP column salts
11	10	AP elutriate

max specific batch growth rate, $\hat{\mu}_b$

treat. no.	concn, mL	treatment
1	20	AR elutriate
41		control
32	5	BP salts
15	10	AP salts
36	5	AP column leachate
45		control
44		control
16	5	AP salts
20	5	BR elutriate
6	15	AR salts
31	10	BP salts
40	5	AP column salts
42		control
12	5	AP elutriate
21	20	BR salts
25	20	BP elutriate
13	20	AP salts
30	15	BP salts
34	15	AP column leachate
43		control
29	20	BP salts
9	20	AP elutriate
5	20	AR salts
7	10	AR salts
23	10	BR salts
4	5	AR elutriate
26	15	BP elutriate
18	15	BR elutriate
17	20	BR elutriate
24	5	BR salts
14	15	AP salts
27	10	BP elutriate
28	5	BP elutriate
10	15	AP elutriate
8	5	AR salts
35	10	AP column leachate
19	10	BR elutriate
38	15	AP column salts
33	20	AP column leachate
39	10	AP column salts
11	10	AP elutriate
22	15	BR salts
37	20	AP column salts
2	15	AR elutriate
3	10	AR elutriate

[a] Treatments are ranked from the lowest value at the top of the listing to the highest value at the bottom of the listing. Any groups of treatments that are not significantly different ($P < 0.05$) from each other are connected by a line of stars to the right of the ranking list.

program can be optimized in order to provide the level of precision to assess potential hazards for the minimum costs.

GUIDELINES FOR SAMPLING

The primary reason for a sampling design is to allocate sampling and testing resources in an optimal manner.

In the preliminary assessment, a minimal design of three replicate samples at each of three stations is specified, obviating the necessity for a statistical design. However, if resources permit and potential hazard is great enough, a statistical design should be used. The number of stations and the number of sample replicates are defined during statistical design.

Three types of samples are obtained: soil samples, ground water, and surface water. A preliminary survey to observe physical-chemical-biological factors such as surface waters and flow directions, topography, types of habitats, site boundary conditions, aquifer locations and underground flow nets, and other ecological variables is an invaluable aid to selecting stations.

Each site will have specific characteristics that will cause a specific set of stations to be selected. The most samples and stations would arise from a situation where a groundwater aquifer underlies a disposal site, surface water is within the site containment boundary, and the area of direct disposal is large. Thus, the reference station, the core station, and the site boundary station would each require three types of samples:

surface water, ground water, and soil. A total of 18 water samples and 9 soil samples would be obtained at that site using three replicate samples at each of the three stations. In addition, leachates from the soil would be tested. Such a hazardous waste site would have the experimental design shown in Table 7.

The final selection of sampling stations depends on the user-defined heirarchy of needed information related to the available resources. The methods for selecting stations and criteria for defining the number of replicates are based on the statistical analysis to be performed. Selected Type I error (alpha) levels and the desired power of the test (1-beta) then fix the number of stations and replicates required. Needed resources should be based on potential risk not on a budget figure. For example, if potential risk to ecosystems or society is relatively large or the cost of cleanup is relatively large, greater testing resources should be made available to insure that cleanup will be effective, that is the risk will be substantially reduced, and monitoring should be implemented to insure cleanup does occur.

Preliminary Assessment

The preliminary assessment is used for initial prioritization and to determine the range of probable responses to be obtained with the biological tests. A minimum program consisting of the baseline control (synthetic media), reference station, core (most impacted) station, and the site containment boundary station should be sampled. At least 3 replicate randomly selected samples should be obtained at each station.

Table 7. Minimal Experimental Design Showing Tests Dilution Series Required

Biological Test	Station	Number of Samples to be Tested: Water Samples Surface	Water Samples Ground	Soil Soil	Soil Leachates*
Algal	Reference	3	3		(3)
	Core	3	3		(3)
	Boundary	3	3		(3)
Invertebrate	Reference	3	3		(3)
	Core	3	3		(3)
	Boundary	3	3		(3)
Fish	Reference	3	3		(3)
	Core	3	3		(3)
	Boundary	3	3		(3)
Plant Seeds	Reference				(3)
	Core				(3)
	Boundary				(3)
Earthworms	Reference			3	(3)
	Core			3	(3)
	Boundary			3	(3)
Soil Litter	Reference			3 or	(3)
	Core			3 or	(3)
	Boundary			3 or	(3)

* Additional samples would not be collected because leachates would be obtained from soil samples.

If the biological tests uniformly give low or nondetectable response levels (Table 4), it is assumed that the site will be relatively risk free. Any test that shows a response at the intermediate or high level for a particular sample is cause for review and, probably, further analysis. Then, the appropriate steps and decision points shown in Figure 2 would be followed.

It may be desirable to perform a statistically based sampling program at this level of assessment. A sampling design based on an ANOVA to analyze the data incorporating the variance of each biological test should incorporate an error level of: alpha = 0.2. The power of the test (1-beta) should be estimated. Expected variance due to a biological test might be relatively high (coefficient of variation = 50 percent). The positive result might require samples to show a difference at the intermediate response level (Table 4) for the site containment boundary sample compared to a reference station.

Detailed Assessment

Statistical methods should be used to evaluate the differences in the results of the bioassessment tests among the sampling stations. The analytical procedures must be specified in advance so that adequate sampling is conducted. Guidelines for selecting sampling locations within the individual sites should be developed. For example, attempts should be made to characterize optimal locations for individual sample types. In this manner sample variances will be reduced. Transects along major exposure routes or along site containment boundaries could be used.

A pilot or feasibility study for the purpose of determining the number and types of samples required and optimum allocation of resources should be done. The preliminary assessment might serve for this purpose.

The error levels would be more conservative than those described for the preliminary assessment. For example, based on potential risk the following level could be selected, alpha = 0.1. Again the power of the test (1-beta) should be estimated. Then the sampling stations and number of replicates would be specified according to resources needed to characterize the site and the expected results from the biological tests.

APPENDIX A

BIOLOGICAL TESTS FOR

BIOASSESSMENT OF HAZARDOUS

WASTE SITES

CONTENTS OF APPENDIX A

OVERVIEW OF
BIOASSESSMENT PROTOCOL

TEST PROCEDURES

A set of aquatic and terrestrial biological tests have been compiled to aid in assessing potential environmental hazard of hazardous waste sites. Users would select all tests or those appropriate to their needs and use them according to the procedures contained in this report.

The three tests directed at aquatic ecosystems are the algal bioassay, and the fish and macroinvertebrate toxicity tests. The three terrestrial tests are the root elongation test, the earthworm acute toxicity test, and the soil litter microorganism test.

The aquatic tests are applied as appropriate to surface or ground water samples, leachates of soil samples, and nonaqueous samples where appropriate. The terrestrial tests are applied to soil leachates and soil samples.

Two levels of testing are defined, range finding and definitive, but more detailed sampling and testing designs can be devised for specific needs. Range finding tests are a geometric series of dilutions by factors of 10, for example, 1.0, 0.1, 0.01, 0.001. At least three dilutions should be performed. Definitive tests are a geometric series of dilution by factors of 2, for example, 1., 0.5, 0.25, 0.125, 0.0625, 0.03125. At least six dilutions should be performed. For the definitive test, concentrations

usually bracket the intermediate value of the range-finding test or utilize the most effective value as the highest concentration. Soil or water are diluted in synthetic media (algal assay medium (AAM), reconstituted water, artificial soil) and, if appropriate, in unimpacted natural waters or soils from the site environs.

Control biological tests should always be performed. These include, where appropriate, negative, positive, solvent and reference controls (defined in the following section). The tests have been designed to be as reproducible as possible using carefully standardized test media and organisms. Quality assurance should follow the "Guidelines and Specifications for Implementing Quality Assurance Requirements" (1). Safety procedures should be practiced to prevent exposure to staff (Appendix B).

The tests described in this appendix were taken from several important sources and readers should be aware of the references. The three aquatic tests and the root elongation test are from Brusick et al. (2), and the earthworm test is in development (contact C. Callahan, Corvallis ERL). The soil respiration test was largely provided by Lighthart (3). Other major sources include the Committee in Methods for Toxicity Tests with Aquatic Organisms (4), USEPA Methods (5), Standard Methods (6), Greene et al. (7), and the Review of Statistical Methods by Stephan (8).

GLOSSARY

Response Variables

Effective Concentration (EC) - concentration that produces the desired effect at a specified level in a percent of the exposed population within a specified time.

96 hour EC_{50} - concentration that produces the desired effect in 50 percent of the population within 96 hours of exposure. Typical levels are 10, 20, 50, 100 percent.

Lethal Concentration (LC) - concentration that produces death as the effect at a specified level in a specified time.

Stimulating Concentration (SC) - concentration that causes growth to increase at a specified level in a specified time.

Controls

Negative Control - a test in which no toxicant is added to 100 percent dilution medium.

Positive Control - a test in which an effective concentration of a known toxicant is added to 100 percent dilution medium.

Solvent Control - a test in which the solvent used to extract the toxicant from a sample is evaluated. Solvent is added to 100 percent dilution medium at the same concentration as would occur with the extract.

Reference Controls - tests using natural water or soil samples collected from unimpacted areas of the site environs.

Sample Descriptions

Aqueous Sample - a receiving water (surface or ground water) or a soil-leachate obtained by extracting with water.

Non-Aqueous Sample - a water sample with more than 0.1 percent organic material (>1000 ppm), or any soil-leachate obtained by extraction with organic solvents.

Solid Sample - any solid phase material; generally, a soil sample.

Dilutions of Samples - a solid, aqueous or non-aqueous sample which is mixed homogeneously with natural soils or waters (receiving system) or synthetic soils or waters (artificial or reconstituted).

Test Descriptions

Definitive Test - test used to establish the effective concentration of a substance or material. As used herein, the concentrations are in a geometric series with a ratio of 2.0.

Range-Finding Test - test used to determine the appropriate range of concentrations in which to apply the definitive test. The range-finding test can be entirely different from the definitive test, a slight modification of the definitive test, or an application of the definitive test over a broader range of concentrations. Generally, as used herein, the concentrations are in a geometric series with a ratio of 10.0.

Bioassessment Protocol - a combination of bioassessment tests for assessing potential environmental hazards at a site.

Bioassessment Procedure - a bioassessment test applied to a sample, for example, methods of evaluating algal growth in a soil leachate.

Bioassessment Test - a specific biological population for assessing a biological response to a mixture of toxicants or a single toxicant. For example, the canary in the coal mine and the fish toxicity tests are Bioassessment Tests.

Bioaccumulation - uptake and retention of environmental substances by an organism from its environment, as opposed to uptake from its food.

Bioconcentration - uptake and retention of environmental substances by an organism from all sources. A bioconcentration factor (BCF) can be calculated as the quotient of the concentration of chemical in the tissue (or whole) of an aquatic organism divided by the concentration in the water in which the organism resides.

TEST SAMPLES

Tests are conducted on water, soil leachates or extracts, and soil samples collected as appropriate to the experimental design from the hazardous waste site. Dilutions are made into standard or reference water and soil samples. Sampling design should follow guidelines discussed in the text. In all cases, including actual sampling, transportation of samples, storage, pretreatment, dilution, and actual testing, procedures designed to provide protection of personnel safety and safety of the general public and of the environment must be carefully followed. Safety guidelines are discussed in Appendix B.

REPORTING

The test record should include where applicable:

- name and address of test laboratory
- date or period of testing

- name of person responsible for testing
- number of tests carried out (rangefinding and definitive tests)
- exact description of test conditions
- details of any variation of test materials and conditions from protocol
- details of test organism (age, maintenance and breeding conditions, source of supply)
- average live weight and range and number of organisms per dose at start and end of test
- description of obvious physical or pathological symptoms or distinct changes in behavior observed in test results
- graph showing concentration/effect curve
- mortality and changes in weight for control animals
- where appropriate, mortality and changes in weight for animals used as control, reference, or test animals
- date and signature of the person performing the test

- any other documents on test conditions

RESPONSE LEVELS

Generalized response levels for the 3 aquatic and 3 terrestrial tests are summarized in Table A-1. The three qualitatively defined levels are presented as guidance. They are intended to help users to evaluate relative toxicity of specific samples. However, the response levels are not fixed values and further results will be incorporated to obtain better qualitative estimates of toxicity. Furthermore, the low or not detectable levels may be misleading since a lack of strong toxicity does not necessarily mean a sample is "safe"; it only is an indication of the immediate potential hazard due to toxicity.

Table A-1

DEFINITION OF TOXICITY CATEGORIES FOR AQUATIC AND TERRESTRIAL ECOLOGICAL ASSAYS

Assay	Activity Measured	Sample Type[a]	MAD[b]	Units	Response Levels for LC_{50} or EC_{50} Concentrations[c]		
					High	Moderate	Low or Not Detectable
Freshwater Fish	96-hr LC_{50} (lethality)	S	1	g/L	<0.01	0.01-0.1	0.1-1
		L	100	percent	<20	20-75	75-100
Freshwater Invertebrate	48-hr EC_{50} (immobilization)	S	1	g/L	<0.01	0.01-0.1	0.1-1
		L	100	percent	<20	20-75	75-100
Freshwater Algae	120-hr EC_{50} (growth inhibition)	S	1	g/L	<0.01	0.1-0.1	0.1-1
		L	100	percent	<20	20-75	75-100
Seed Germination and Root Elongation	115-hr EC_{50} (inhibit root elongation)	L	100	percent	<20	20-75	75-100
Earthworm Test	168-hr LC_{50}	S	500	g/kg	<50	50-500	500
Soil Respiration Test	336-hr EC_{50}	S	500	g/kg	<50	50-500	500
		L	100	percent	<20	20-75	75-100

[a] S = solid, L = aqueous liquid, includes water samples and elutiate or leachate. Nonaqueous liquids are evaluated on an individual basis due to variations in samples such as vehicle, percent organic vehicle, and percent solids.

[b] MAD = Maximum applicable dose.

[c] LC_{50} = Calculated concentration expected to kill 50 percent of population within the specified time interval.

LC_{50} = Calculated concentration expected to produce effect in 50 percent of population within the specified time interval.

GENERAL MATERIALS AND METHODS FOR BIOLOGICAL TESTS OF WATER SAMPLES

GENERAL INSTRUCTIONS

Setup and Preparation

The recommended test organisms in freshwater tests are the algae, Selenastrum capricornutum, the juvenile fathead minnow, Pimephales promelas, and early instars of Daphnia magna. The recommended test period is 120 hours for the algal test, 96 hours for the fish test, and 48 hours for the daphnid test. Thus, the principal finding obtained from an algal study is the 120-hour EC_{50}, EC_{95} or SC_{20}, from the fish study the 96-hour LC_{50}, and from the daphnid study the 48-hour EC_{50}.

The procedures for the fresh water tests have been developed largely from References 2, 5, 6. Modifications to the original protocols have been made where necessary to adapt tests to the requirements of the Bioassessment Protocol.

Materials and methods that are common to all, or nearly all, aquatic tests are presented in this section. The section for each specific test discusses materials and methods unique for that test and identifies which of the general materials and methods are applicable.

Facilities

The facilities should include tanks equipped for temperature control and aeration for holding and acclimating test organisms, and a constant temperature area or recirculating water bath for the test vessels. If the use of reconstituted dilution water is necessary, there should be a tank for its preparation. If air is used for aeration, it must be free of oil and fumes. The test facility must be well ventilated and free of fumes. Illumination should be provided of an intensity and duration that is specified in the Materials and Methods section for each test.

Construction Materials

Materials that come in contact with effluent samples, stock solutions, or test solutions should minimize sorption of any constituents of the test material and not contain any substances that can be leached or dissolved by the water. Glass, #316 stainless steel, and perfluorocarbon plastics must be used whenever possible to minimize leaching, dissolution, and sorption. Unplasticized plastics may be used for holding and acclimation tanks and in the water supply system. Rubber, copper, brass, and lead must be avoided. If stainless steel is used it must be welded, never soldered. Silicone adhesive used to cement glass containers sorbs some organochlorine and organophosphorus compounds which are difficult to remove; therefore, as little adhesive as possible should be in contact with test material solutions and extra beads of adhesive should be on the outside, not the inside, of the containers.

Test Containers

Fish tests should be conducted in 19.6-liter wide-mouth soft-glass jars or in all-glass containers 30 cm wide, 60 cm long and 30 cm high. Daphnids should be exposed in 3.9-liter wide-mouth soft-glass bottles, in 3.3-liter battery jars or in 250-milliliter beakers. Algal tests should be conducted in Erlenmeyer culture flasks of Pyrex or Kimax type of glass. The flask size is not critical, but due to CO_2 limitations the volume-to-volume ratio is. The recommended contents-to-flask-volume ratios for hand shaken flasks are:

25 ml in 125 ml flask

50 ml in 250 ml flask

100 ml in 500 ml flask

Maximum permissible contents-to-volume ratios in continously shaken flasks should not exceed 50 percent.

Cleaning and Preparation of Glassware

Each testing container must be cleaned before use. A new container must be (1) washed with non-phosphate detergent, (2) rinsed with 100 percent acetone, (3) rinsed with water, (4) rinsed with 10 percent nitric acid, (5) rinsed thoroughly with tap or other clean water, and (6) a final rinse with distilled or deionized water (3 volumes). After testing, each container should be cleaned as above unless the container is discarded.

For fish bioassays, disinfect test containers for 1 hour with an iodophor, 200 mg hypochlorite per liter, or a quaternary ammonium salt such as 800 ppm Roccal II (National Laboratories, Montvale, New Jersey 07645) with at least one thorough scrubbing during the hour, then rinse thoroughly. For safety, do not use acid and hypochlorite together.

All glassware used in algal testing is prepared as above. Flasks are dried in an oven at 50° to 70°C. Demonstrably nontoxic plugs (for example, Gaymar white, polyurethane or equivalent, Gaymar Industries, Orchard Park, New York 14127) are inserted and the glassware is autoclaved for 20 minutes at 1.1 kg/cm^2 (15 psi) and 121°C. Cooled flasks are stored in closed cabinets.

Receipt and Quarantine for Fish

Stock fish shipped from outside sources may have been subjected to changes in temperature, dissolved oxygen and pH, handling disturbances, and other stresses, and should be examined carefully for health and vigor. Introduce holding water gradually into the shipping bags, observing the fish for abnormal behavior. When the difference in water temperature between the bag and holding tank is 2° or less, fish from one bag should be introduced into the tank and observed for five minutes for acute stress. If acute stress is not seen, the remaining fish may be introduced into the tank in a similar manner.

To prevent spread of disease, incoming fish for stock should be quarantined for at least 2 weeks and observed for abnormal behavior and parasites. The quarantine tanks should be prepared in advance by thorough scrubbing and cleaning with an industrial cleaner, rinsing with water, sterilizing with a quaternary ammonium salt such as 800 ppm Roccal II, and rinsing with at least three changes of water before filling with dilution water. If after 2-weeks' quarantine they show no signs of infection or abnormal behavior they are transferred to stock holding tanks, otherwise, they are either discarded or treated as described in Disease Treatment for Fish, below.

To prevent initiation and spread of disease, nets, buckets, fish graders, and hands should be routinely disinfected with 200 ppm Roccal II before being placed in the water.

Disease Treatment for Fish

Freshwater fish may be chemically treated to cure or prevent diseases by using the treatments recommended in Table A-2. However, if a group of fish is severely diseased, the entire lot should be destroyed. Generally, the fish should not be treated during the first 16 hours after arrival at the facility because they may be stressed due to collection or transportation and some may have been treated just prior to transit. Tests must not begin with treated fish for at least 4 days after treatment. Tanks and test chambers which may be contaminated with undesirable microorganisms should be disinfected following the procedures outlined in Cleaning, above in this section.

Performing the Tests

Test Material

All samples and test materials must be handled according to safe procedures that protect the workers, society, and the ecosystem. These procedures are described in Appendix B. The test material may be a solid, aqueous liquid, or nonaqueous liquid. For the quantity of sample required to run each test, see Table A-3. Samples are usually tested directly without preparation, however, some test materials require pretest preparation. Except for the algal test, the aqueous sample (leachate or water sample) should be run directly in the dilution water and must not be

TABLE A-2 RECOMMENDED PROPHYLACTIC AND THERAPEUTIC TREATMENTS FOR FRESHWATER FISH[a]

Disease	Chemical	Conc., mg/l	Application
External bacteria	Benzalkonium chloride (Hyamine 1622®)	1-2 AI[b]	30-60 min[c]
	Nitrofurazone (water mix)	3-5 AI	30-60 min[c]
	Neomycin sulfate	25	30-60 min[c]
	Oxytetracycline hydrochloride (water soluble)	25 AI	30-60 min[c]
Monogenetic trematodes, fungi, and external protozoa[d]	Formalin plus zinc-free malachite green oxalate	25 0.1	1-2 hours[c]
	Formalin	150-250	30-60 min[c]
	Potassium permanganate	2-5	30-60 min[c]
	Sodium chloride	15,000-30,000 2000-4000	5-10 min dip[c,e]
	Dexon® (35% AI)	20	30-60 min[c]
Parasitic copepods	Trichlorfon (Masoten®)	0.25 AI	f

[a] These recommendations do not imply that these treatments have been cleared or registered for these uses. Appropriate State and Federal regulatory agencies should be consulted to determine if the treatment in question can be used and under what conditions the uses are permitted. These treatments should be used only on fish intended for research. They have been found dependable, but efficacy against diseases and toxicity to fish may be altered by temperature or water quality. Researchers are cautioned to test treatments on small lots of fish before making large-scale applications. Prevention of disease is preferred, and newly acquired fish should be treated with the formalin-malachite green combination on three alternate days if possible. However, in general, fish should not be treated on the first day they are in the facility. This table is merely an attempt to indicate the order of preference of treatments that have been reported to be effective. Before a treatment is used, additional information should be obtained from such sources as Davis (9), Hoffman and Meyer (10), Reichenbach-Klinke and Elkan (11), Snieszko (12), and van Duijn (13).

[b] AI - active ingredient.

[c] Treatment may be accomplished by (1) transferring the fish to a static treatment tank and back to a holding tank; (2) temporarily stopping the flow in a flow-through system, treating the fish in a static manner, and then resuming the flow to flush out the chemical, or (3) continuously adding a stock solution of the chemical to a flow-through system by means of a metered flow or the technique of Mount and Brungs (14).

[d] One treatment is usually sufficient except for "Ich", which must be treated daily or every other day until no sign of the protozoan remains. This may take 4 to 5 weeks at 5 to 10°C and 11 to 13 days at 15 to 21°C. A temperature of 32°C is lethal to Ich in 1 week.

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[e] Minimum of 24 hours, but may be continued indefinitely.

[f] Continuous treatment should be employed in static or flow-through systems until no copepods remain, except that treatment should not be continued for over 4 weeks and should not be used above 27°C.

aerated or altered in any way, except that it may be filtered through a sieve or screen with holes 2mm or larger to remove large particicles. Aqueous samples should be filtered (0.45 micrometer cellulose acetate filters) to remove indigenous algae for the algal assay. This should be done as soon after collection as possible (on site is preferable) and must be done before sample storage. Solid and nonaqueous samples may be added directly by weight or volume respectively, diluted with dilution water and small subsamples of equal volume added to each test container. Samples must be covered at all times and violent agitation must be avoided. Undissolved materials must be uniformly dispersed by gentle agitation immediately before a portion of the sample is taken for use.

TABLE A-3 SAMPLE SIZE REQUIREMENTS
FOR AQUATIC ECOLOGICAL ASSAYS (2)

Type of Test	Solid (grams)	Liquid (liters)	
		Aqueous	Nonaqueous[a]
Freshwater Fish	100 (75)[b]	100L (75L)	0.100 (0.075)
Freshwater Invertebrate	10 (4)	10L (4L)	0.010 (0.004)
Freshwater Algae	2 (1)	1L (0.6L)	0.010 (0.005)

[a]Nonaqueous liquids include aqueous samples with greater than 0.2% organics, nonaqueous liquids, solvent exchange samples, and extracts or leachates in a nonaqueous (organic) vehicle.

[b]The first value given is the requested sample size for routine testing. The value in parentheses is the minimum feasible sample size to conduct the test.

If testing is to be done on-site, the tests should begin within 8 hours of collection. If testing is to be done at a laboratory, the samples should be placed on ice for preservation during transportation. Testing should be performed as soon as possible after laboratory receipt of the samples. Samples should be stored at $4^{o}C$ if testing is not initiated upon sample receipt. The temperature of the sample should be adjusted to that of the test ($\pm 2^{o}C$) before portions are added to the dilution water. Solid materials may be added directly to dilution water.

When diluting samples containing highly volatile substances, it may be desirable to add the test sample below the surface of the dilution water. Complete and accurate records of collection methods, treatments, and addition techniques must be maintained.

Sample Test Concentrations

Preparation of Toxicant. Depending on its nature, the test material is prepared by one of two methods. In the first method, solids or non-aqueous liquid materials may be added directly by weight or volume respectively to a stock solution or to the dilution water. The stock solution may be deionized water or a solvent and then equal volume subsamples of a small size are added to each treatment. If it is not possible to prepare a homogenous solution of the toxicant, it must be added directly to the dilution water in each replicate flask or tank.

The second method is for aqueous samples and allows testing by percent volume (volume/volume). Up to 100 percent of the sample with filter-sterilization if required, is used in the test. Additional test concentrations are prepared on a volume-percent basis by mixing appropriate

volumes of sample with control medium. In the algal test, appropriate amounts of the stock solutions should be added to the 100 percent sample to be equivalent to full-strength algal assay medium. Then, algal assay medium is used to prepare each dilution series. This assures known nutrient availability in the test dilutions to calculate algal yield.

Controls should consist of the dilution water or nutrient medium, and a receiving water sample if appropriate. These are called negative controls. It may be necessary to perform a range finding test with broad dilution limits (factors of 10, 100, 1000) before performing the final tests. Specific requirements are listed in the Section on Test Procedure for each test.

Dissolved Oxygen Concentration. Aeration of test solutions during the test should be avoided to minimize loss of highly volatile materials. Dissolved oxygen must be brought to minimum standards (40 percent of saturation) by dilution. If the dissolved oxygen concentration is less than 40 percent saturation in any test chamber for fish or Daphnia tests, this should be noted in the final report. Algal tests do not have defined dissolved oxygen concentration requirements.

FRESHWATER ALGAE 120-HOUR TEST

Introduction and Rationale

Unicellular algae are important producers of oxygen and form the basis of the food web in aquatic ecosystems. Since algal species and communities

are sensitive to environmental changes, growth may be inhibited or stimulated by the presence of pollutants. Therefore, the response of algae must be considered when assessing the potential ecological effects of industrial or municipal discharges on aquatic ecosystems.

A simple screening test for toxicity to algae can be conducted in 120 hours. Algae are exposed to various concentrations of the test material and growth is measured at 120 hours. Results are expressed in terms of the EC_{90} (the lowest test concentration causing inhibition of growth by $\geq$ 90 percent relative to the control), and EC_{50} (the lowest test concentration causing inhibition of growth by $\geq$ 50 percent relative to the control). Stimulatory effects, if any, should be noted and expressed mathematically in terms of SC_{20} and used for estimation of bioactivity of the sample.

Materials and Methods

General procedures listed for all aquatic tests in the GENERAL INSTRUCTIONS are applicable to the static acute toxicity test with freshwater algae. Specific areas discussed in the GENERAL INSTRUCTIONS that should be followed are: facilities, construction materials, test containers, cleaning and preparation of glassware, and test material.

Materials and methods unique to freshwater algal tests are included below.

Equipment

Equipment should include a constant-temperature room or incubator capable of providing temperature control of 24 $\pm 2^{o}C$. Daily hand shaking or a gyrotary shaking apparatus capable of 100 oscillations per minute should be used for test culture flasks. Continuous illumination of 4300 $\pm$430 lumens/m^2 (400 ft-c) is required for freshwater green algae. Overhead cool-white flourescent bulbs should be used. Light intensity is measured adjacent to the flask at liquid level using a light meter capable of being calibrated against National Bureau of Standards lamps. Culture containers for this and other aquatic tests are discussed in the Test Container section.

Freshwater Algal Nutrient Medium

Algal Assay Medium (AAM) is prepared by adding 1.0 ml of each of the macronutrient and micronutrient stock solutions, in the order listed in Table A-4, to 900-ml of filter-sterilized deionized water, with mixing after each addition. Deionized water is filter-sterilized by passing through a 0.45 micrometer porosity cellulose acetate membrane filter (pre-rinsed with 100-ml deionized water) into a sterile container. Then the final volume is brought to 1 liter with filter-sterilized deionized water. Medium should be constituted as needed but can be stored in the dark at $4^{o}C$ to reduce possible photochemical changes and bacterial growth for periods up to one week.

Test Organisms and Culture Maintenance

For freshwater algal assays, the recommended test organism is Selenastrum capricornutum, a unicellular non-motile chlorophyte that is easily maintained in laboratory cultures. Obtain algal cultures (Culture

TABLE A-4 COMPOSITION OF ALGAL ASSAY MEDIUM (AAM)

Macronutrients

Stock Solutions[a]		Nutrient Composition Prepared Medium	
Compound	Concentration (g/l)	Element	Concentration (mg/l)
$NaNO_3$	25.500	N	4.200
$NaHCO_3$	15.000	Na	11.001
		C	2.143
K_2HPO_4	1.044	K	0.469
		P	0.186
$MgSO_4 \cdot 7H_2O$	14.700	S	1.911
$MgCl_2 \cdot 6H_2O$	12.164	Mg	2.904
$CaCl_2 \cdot 2H_2O$	4.410	Ca	1.202

Micronutrients

Stock Solutions[a]		Nutrient Composition Prepared Medium	
Compound	Concentration (mg/l)	Element	Concentration (µg/l)
H_2BO_3	185.520	B	32.460
$MnCl_2 \cdot 4H_2O$	415.610	Mn	115.374
$ZnCl_2$	3.271	Zn	1.570
$CoCl_2 \cdot 6H_2O$	1.428	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.260	Mo	2.878
$FeCl_3 \cdot 6H_2O$	160.000	Fe	33.051
$Na_2EDTA \cdot 2H_2O$	300.000	---	---

[a]Other forms of the salts may be used as long as the resulting concentrations of elements are the same.

No. ATCC 22662) from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852.

Upon receipt of the algal culture, approximately 1.0 ml should be aseptically transferred to the AAM. The rest of the culture can be maintained up to six months in a dark refrigerator at 4°C. Weekly aseptic routine stock transfer is recommended to maintain a continuous supply of "healthy" cells for experimental work. To retain a unialgal culture over a long period of time it is advantageous to prepare a semi-solid medium containing 1.0 percent agar made up with filler sterilized AAM and placed in sterile Petri plates. A portion of a liquid algal culture is streaked onto it and incubated under standard conditions. Algae should be transferred onto fresh plates every four weeks. Fresh liquid cultures should be started by transfer of a single algal colony to liquid medium at four week intervals. For test inoculation, liquid cultures are used.

A 6- to 8-day-old stock culture is used as the inoculum source. Population density in the stock culture is determined by direct counting or spectrophotometry. The culture should always be checked microscopically to insure that it is unialgal and healthy. A volume of inoculum calculated to yield an initial concentration of 10,000 cells/ml is aseptically added to each test flask. The volume of inoculum added should be between 0.1 and 1.0 ml. See the section on Response Monitoring to determine cell counts.

Test Procedure

Six test concentrations and a negative control are normally tested, with three replicates of each. Three replicate flasks are necessary for statistical analyses. Other experimental design features are discussed in that chapter in the main text. Stock nutrient solutions are added to the 100 percent sample as in making up AAM to insure nutrient salts are equivalent to 100 percent AAM. Full strength AAM is used to dilute samples for the dilution series.

A range finding test will probably be necessary before running the actual test. A control plus concentrations of 100 percent, 10 percent, 1 percent and 0.1 percent (W/V or V/V) are usually necessary using 3 replicates each. The actual test will span the moderate response concentration(s) using a geometric series. For example, if 1 percent (0.01) and 10 percent (0.10) gave toxic responses (EC_{50}), a test series would include: 0.1, 005, 0.025, 0,0125, 0.00625, 0.003125. solutions (W/V or V/V).

Controls include the AAM (negative control) to check standard organism response and receiving water if applicable (reference control), a solvent control if applicable (dilution water plus solvent). The positive control is applied with ZnCl in AAM at a concentration of 80 µg Zn^{++}/l to give a range of inhibition of 51-66 percent (long term mean = 58.8).

Response Monitoring

After 120 hours of exposure, algal growth is measured by any of the following methods: (a) microscopic counts, (b) absorbance (c) electronic particle counting or (d) biomass (dry weight). Cursory microscopic observation is desirable to reveal and record any abnormalities in cell shape or condition. Because the algal test is designed to provide a comparative response to varying dilutions of sample dilute, it is sufficient to use absorbance to estimate growth. Other techniques can be used for special purposes (2, 7, 15).

Microscopic Counting

To determine cell numbers in the inoculum or create a standard curve for later absorbance, direct microscopic counts are necessary. A hemacytometer counting chamber and a microscope are used. Two samples are taken from each flask, and two counts are made of each sample. Whenever feasible, 400 cells per replicate are counted in order to obtain ± 10 percent accuracy at the 95 percent confidence level. Generally this method is the least feasible for monitoring test responses.

Absorbance

Measure absorbance with a spectrophotometer or colorimeter at a wavelength of 750 nm. Report instrument make and model, geometry and path length of the cuvette, wavelength used, and the equivalence to biomass (absorbance units per milligram dry weight per liter).

Limit photometric measurement of absorbance to a range of $0.05 \leq D \leq 1.0$, where D represents optical density. Samples can be diluted to obtain the

appropriate range.

Electronic Particle Counting

A Model ZBI Coulter Counter with Mean Cell Volume (MCV or MHR) Computer is used. The particle counter offers the greatest precision and accuracy and is the preferred method if equipment is available and samples are suitable. The MHR Computer must be calibrated with the Organic Calibration material; biomass may be determined indirectly by the following equation:

$$\text{Cell counts (cells/ml)} \times \text{MCV } (m^3) \times (2.9 \times 10^{-7}) = \text{mg dry weight } \underline{S.\ capricornutum}/\text{liter}$$

Note that the conversion factor of 2.9×10^{-7} may differ between laboratories and should be determined by each investigator. The standard reference particle (Part Number 1607081) can be obtained from Coulter Electronics, Inc., Hialeah, Florida.

If there are particles in the test material, it is possible to eliminate counts contributed by other particles. Uninoculated flasks are counted and these counts subtracted from the total counts. Then dry weights of cells can be calculated with the above formula. Particles may clog the aperture, and in such cases, another method should be used. The advantage of this method is that it allows for determination of biomass produced in addition to cell numbers.

Biomass (dry weight)

For this method, a measured portion of algal suspension is filtered through a tared 0.6 micrometer PVC membrane filter. The filters are prepared as follows: Rinse with deionized water and dry for several hours at 60°C in an oven. Place filters in folded sheets of paper or aluminum weighting dishes on which the weights or codes are written. Cool in a desiccator and weigh. Filter a suitable portion of culture (50 ml or less as the cell density dictates) under a vacuum of 51 kPa. Rinse filter funnel with 50 mL distilled water containing 15 mg $NaHCO_3$/l using a wash bottle and let the rinsings pass through the filter. Dry at 60°C, cool in desiccator, and weigh. Subtract tare weight, divide by volume (liters) of culture filtered and express as mg/l dry weight.

Results and Data Interpretation

Calculations. Percent inhibition (I), or stimulation (S), is calculated for each concentration according to the following formula:

$$\frac{(T\text{-}IN) - P(MSC\text{-}N)}{P(MSC\text{-}IN)} \times 100 = \begin{matrix}(+)\% = \text{Stimulation} \\ (-)\% = \text{Inhibition}\end{matrix}$$

where

P = percent volume of AAM used to dilute the test sample.

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MSC = maximum standing crop (mg/l) obtained in the AAM control.

T = maximum standing crop (mg/l) obtained in the test sample.

IN = dry weight (mg/l) of inoculum used at start of test.

Three endpoints may be calculated from the percent response vs. concentration data. For samples which are inhibitory, an EC_{90} (defined as the lowest test concentration causing growth inhibition of 90 percent relative to control) and an EC_{50} (defined as the lowest test concentration causing growth inhibition of 50 percent relative control) are calculated. For samples which are stimulatory, an SC_{20} (defined as the lowest concentration causing growth stimulation of 20 percent relative to control) is calculated. For all samples, the EC_{50}, EC_{90}, and SC_{20} are calculated using any of several statistical methods.

The 120-hour EC_{50} results are evaluated according to criteria defined in Table A-1 which will permit the test material to be ranked by toxicity category. While the EC_{90} endpoint may be the most meaningful biological effect for long-term impact on the environment, the more sensitive EC_{50} is used in this assay to rank samples.

STATIC ACUTE TOXICITY TESTS WITH FRESHWATER FISH AND *DAPHNIA*

Introduction and Rationale

The static toxicity tests with freshwater fish and *Daphnia* utilize juvenile fathead minnows. *Pimephales promelas*, and early instars of *Daphnia magna*. The static acute exposure period is 96 hours for the fathead minnow and 48 hours for the daphnid study. The 96-hour mean lethal concentration (96-hour LC_{50}) is calculated for the fathead minnow. Because death is not always easily determined in *Daphnia*, the 48-hour effective concentration (48-hour EC_{50}) is calculated for *Daphnia*.

Materials and Methods

Procedures listed for all aquatic tests under GENERAL INSTRUCTIONS are applicable to the static acute toxicity tests with freshwater fish and *Daphnia*. Sections that should be followed are: facilities, construction materials, test containers, cleaning and preparation of glassware, receipt and quarantine for fish, disease treatment for fish, test material, and dissolved oxygen concentration. Materials and methods unique to freshwater fish and *Daphnia* tests are included below.

Dilution Water

Dilution water can be from the site (upstream of possible contamination) local dechlorinated tap water, or reconstituted water. A minimal criterion for an acceptable dilution water is that healthy organisms will survive in it for the duration of acclimation and testing without

showing signs of stress such as discoloration or unusual behavior. Water in which daphnids survive and reproduce satisfactorily should be an acceptable dilution water for tests with freshwater organisms.

The dilution water should be of constant quality and should be analyzed by the methods given in References 17 - 20 to ascertain that none of the following substances exceeds the maximum allowable concentration shown:

Pollutants	Maximum Concentration
Suspended solids	20 mg/l
Total organic carbon	10 mg/l
Un-ionized ammonia	20 g/l
Residual chlorine	3 g/l
Total organophosphorus pesticides	50 ng/l
Total organochlorine pesticides plus PCB's	50 ng/l

The dilution water is considered to be of constant quality if the monthly ranges of the hardness, alkalinity, and conductivity are within 10 percent of their respective means and if the monthly range of pH is less than 0.4 units. Reconstituted dilution water may be prepared according to the method shown in Table A-5. For comparability of results between tests, the hardness should be as close as possible to 100 mg/l as $CaCO_3$.

TABLE A-5 RECOMMENDED COMPOSITION FOR RECONSTITUTED FRESH WATER THAT IS MODERATELY HARD (calculated from 4)

Salts Added to Distilled Water*, mg/l		Water Quality	
$CaSO_4:2H_2O$	70	pH (air equilibrated)	8.3
$MgSO_4$	70	Hardness, mg/l as CaCU3	100
KCl	4.5	Alkalinity, mg/l as $CaCO_3$	100
$NaHCO_3$	168	Total dissolved solids	250

* Stock solutions of individual salts can be prepared so that 10 ml in one liter produces the desired final concentration. Store stock solutions in the dark at 4^{o}.

Species

The juvenile fathead minnow, Pimephales promelas, and early instars of Daphnia magna are the species to be used in Level 1 freshwater static acute toxicity tests. The fathead minnow is a warm-water fish of ponds, lakes, and sluggish streams. Daphnids occur in nearly all types of freshwater habitats. Both species, have been recommended as bioassay organisms by the Committee on Methods for Toxicity Tests with Aquatic Organisms (4) because of their wide geographic distribution, important role in the aquatic food web, temperature requirements, wide pH tolerance, ready availability, and ease of culture.

Source

Fathead minnows may be obtained from private, state, or federal fish hatcheries, or captured from wild populations in relatively unpolluted areas. However, collecting permits may be required by local and state agencies. Fish collectred by electroshocking should not be used. Daphnia

should be reared in the testing facility from laboratory cultures.

Sizes, Life Stages

Fathead minnows used in testing should weigh between 0.5 and 1.0 g each. All fish in each test should be from the same year class, and the standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than twice that of the shortest fish. Weights and lengths should be determined by measuring representative specimens before the test or the control fish after the test. Very young fish (not yet actively feeding), spawning fish, and spent fish should not be used.

Daphnia magna used in testing should be in the early instar stages (stages 2-4) of their life cycle. All organisms in a test must be from the same source and as healthy and uniform in size and age as possible.

Culturing, Care, and Handling

Fathead minnows are maintained at 20-22°C in a flow-through system with a turnover of at least two volumes daily, or in a recirculating system in which the water is passed through a carbon filter and an ultraviolet sterilizer (4).

Daphnia magna are maintained in a static system at 19-22°C. Tanks must be cleaned with a siphon periodically to remove debris, and water should be added as necessary to maintain volume. Cultures must be maintained under optimum conditions at all times to prevent formation of ephippial eggs; daphnids from cultures in which ephippia are being produced must not be used in testing. Generally, periodic subculturing of cultures, elimination of crowding, and adequate food prevent problems in Daphnia cultures.

Both species should be fed at least once a day, at which time careful observations should also be made for mortality and for signs of disease, stress, and injury. Dead and abnormal individuals should be removed as soon as they are observed.

Water quality should be held constant as described above and temperature changes should not exceed $3^{o}C$ in any 12-hour period. Fish tanks should be scrubbed at least twice a week.

The organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible so that the organisms are not needlessly stressed. Small dip nets are best for handling fish and wide bore pipettes ($\leq$ 0.5cm) for Daphnia. Organisms that touch dry surfaces or are dropped or injured during handling should be discarded.

Test organisms should always be shielded from disturbances, and overcrowding should be avoided.

Holding and Acclimation

After collection or transportation, the fish should be held in and acclimated to the dilution water for at least 2 days before beginning a test under the same holding conditions as described in Care and Handling, above.

A group of animals must not be used for a test if individuals appear to be diseased or otherwise stressed or if more than 5 percent die within 48 hours prior to beginning the test. If a group fails to meet these criteria, they must be discarded or treated and held an additional 4 days.

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Fathead minnows should not be fed for 48 hours prior to the beginning of a test. However, the Daphnia may be fed up to the beginning of the test.

Test Procedures

Unless the approximate toxicity of the sample is already known, at least six concentrations of test material should be prepared. The highest dose should be at the maximum applicable dose (MAD) for that sample type (see Table A-1) unless physical characteristics of the sample or other previously gathered toxicity data contravenes this.

In fathead minnow tests, at least 20 fish must be exposed to each test concentrations per replicate with two replicates per concentration used in the test. For Daphnia magna tests, five organisms per replicate with three replicates per concentration should be used. The use of more organisms and replicate test containers and random assignments of test organisms to containers is desirable.

The fathead minnow tests should be conducted at 22 $\pm 2^{o}$C, and those with Daphnia at 19 $\pm 2^{o}$C. A photo period of 16 hours light and 8 hours dark is used for both tests. Neither type of test animal should be fed during exposure. The test conditions are summarized in Table A-6.

In the fathead minnow test there should be 15 liters of test solution or control water in each 19.6-liter jar. If 30 x 30 x 60 centimeter containers are used, the solution should be between 15 and 20 centimeters deep (about 30-35 liters).

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TABLE A-6 SUMMARY OF TEST CONDITIONS,
FRESHWATER FISH OR MACROINVERTEBRATE TEST

	Fathead Minnow, *Pimephales promelas*	*Daphnia magna*
Temperature, °C	22 ± 2	19 ± 2
Photoperiod, hours light:dark	16:8	16:8
Water quality, hardness[a] mg/l as $CaCO_3$	100	100
Container size	19.6 liters	250 ml
Test volume	15 liters	200 ml
Organisms per container	10	5
Replicates	2	3
Feed	No	No
Duration, hours	96	48
Measurements of D.O. and pH, hours	0, 24, 48, 72, 96	0, 48

[a]For dilution water only; the investigators add salts in Table A-5 as appropriate to obtain 100 µg/l as $CaCO_3$.

In the daphnid test there should be 2 to 3 liters of solution or control water in each 3.9 liter wide-mouth bottle or 3.3-liter battery jar, or 200 milliliters in each 250-milliliter beaker.

Test organisms should be placed in the test and control vessels not more than 30 minutes after the test solutions are prepared. Ten fish in each vessel and five daphnids in each replicate are recommended. Chemical,

physical, and biological data are taken and recorded as described below for the duration of the test.

If no toxicity is detected at any concentration and the MAD dose was tested, then no further testing is required. The test material may be reported as having no detectable toxicity. Test materials that kill or immobilize all or nearly all the test organisms at all dilutions should be retested with a lower dose range.

The biological loading in each test and control vessel should not exceed 0.8 g of test organism per liter or be so high as to (1) reduce dissolved oxygen concentration in the control tanks below acceptable levels, (2) raise the concentration of metabolic products above acceptable levels, or (3) stress the organisms by overcrowding, any of which may invalidate the test results.

Results and Data Interpretation

In the fathead minnow test, dissolved oxygen concentration, and pH should be measured for each replicate at the beginning of the test and every 24 hours thereafter in the controls and in the high, medium, and low concentrations. Conductivity and hardness should be measured at the beginning of the test in the control and each test concentration for each replicate. Meters can be used but must be standardized. Temperature of the water bath or controlled-temperature area should be recorded continuously or every 24 hours.

In the Daphnia test, temperature, dissolved oxygen, pH, hardness, and conductivity on the high, medium and low concentrations, should be recorded initially and at 48 hours.

Mortality is the effect most often used to define acute toxicity to aquatic organisms. Criteria for death are usually lack of movement, especially of gill movement in fish, and lack of reaction to gentle prodding.

Because death is not always easily determined with some invertebrates, an EC_{50} may be calculated rather than an LC_{50}. The principal criterion for effect on Daphnia is immobilization, defined as lack of movement except for minor activity of appendages.

Mortality or immobilization and abnormal behavior should be recorded. Dead or immobilized organisms should be removed as soon as they are observed. For definitions of fish behavior terms, and suggested code for recording and reporting, see Table A-7. If more than 10 percent of test organisms in any control die or are immobilized, the entire test is unacceptable.

The concentration of test material lethal to 50 percent of the population (LC_{50}) and 95 percent confidence limits should be determined at 24-, 48-, 72-, and 96-hour exposures for fish tests, and the EC_{50} and 95 percent confidence limits at 24- and 48-hour exposures for Daphnia magna tests. Any of several methods including moving average, Spearman Karber, Litchfield-Wilcoxin, probit, or binomial may be used. For a discussion of the above methods, refer to the review article by Stephan (8). The results

TABLE A-7 DEFINITION OF FISH BEHAVIOR TERMS

Code	Term	Definition
1.	General Behavior.	Observable responses of the test fish, individually or in groups, to the range of factors constituting their environment.
a.	Quiescent:	marked by a state of inactivity or abnormally low activity; motionless or nearly so.
b.	Hyperexcitable:	reacting to stimuli with substantially greater intensity than control fish.
c.	Irritated:	exhibiting more or less continuous hyperactivity.
d.	Surfacing:	rising and remaining unusually long at the surface.
e.	Sounding:	diving suddenly straight to the bottom; remaining unusually long at the bottom.
f.	Twitching:	moving the body or parts of the body with sudden jerky movements.
g.	Tetanous:	in a state of tetany; marked by intermittent tonic spasms of the voluntary muscles.
h.	Flaccid:	lacking tone, resilience or firmness; weak and enfeebled; flabby.
i.	Normal:	unaffected by or not exposed to a particular experimental treatment; conforming to the usual behavioral characteristics of the species.
2.	Swimming.	Progressive self-propulsion in water by coordinated movement of tail, body, fins.
a.	Ceased:	Broken off or tapered off to a stop.
b.	Erratic:	Characterized by lack of consistency, regularity, or uniformity; fluctuating, uneven; eccentric.
c.	Gyrating:	Revolving around a central point; moving spirally about an axis.
d.	Skittering:	skimming hurriedly along the surface with rapid body movements.
e.	Inverted:	turned upside down, or approximately so.
f.	On side:	turned 90° laterally, more or less, from the normal body orientation.
3.	Pigmentation.	Color of skin due to deposition or distribution of pigment.
a.	Light discolored:	color appearance lighter than usual for the species.
b.	Dark discolored:	color appearance darker than usual for the species.
c.	Varidiscolored:	color appearance abnormally varied; mottled.
4.	Integument.	The skin.
a.	Mucus sheading:	observably losing mucous skin coating to an abnormal degree.
b.	Mucus coagulation:	showing observable clumping or clotting of the mucous skin coating, especially at the gills.
c.	Hemorrhagic:	visibly bleeding as from gills, eyes, anal opening.
5.	Respiration.	Physical action of pumping water into mouth and out through gills so as to absorb oxygen.
a.	Rapid:	observably faster than normal to a significant degree.
b.	Slow:	observably slower than normal to a significant degree.
c.	Irregular:	failing to occur at regular or normal intervals.
d.	Ceased:	broken off or tapered off to a stop.
e.	Gulping air:	swimming at surface with mouth open and laboriously pumping surface water and air through gills.
f.	Labored:	performed with apparent abnormally great difficulty and effort.

No Observed Effect Concentration: The highest test concentration in which fish experience no mortality and exhibit no observable behavioral abnormalities at any time during a specified period of exposure to the test material. Ordinarily determined for periods from the start of testing to the end of each successive 24 hours.

(96 hours for fish and 48 hours for Daphnia) are evaluated according to Table A-1 which defines the toxicity categories.

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GENERAL MATERIALS AND METHODS FOR BIOLOGICAL TESTS OF SOIL SAMPLES

GENERAL INSTRUCTIONS

Setup and Preparation

The recommended test organisms in terrestrial tests are seeds from various angiosperms used in the root elongation test (RE test), earthworms Eisenia foetida, and soil litter microorganisms. The recommended test period is 115 hours for the RE test, 7 days for the worms, and 14 days for the soil litter test. The principal findings are EC_{50} for the seeds measured by percent germination and root elongation, EC_{50} for the worms, and EC_{50} for the soil litter test. Although inhibition of seed germination and root elongation are observable toxic responses and are reported, root elongation inhibition is the preferred endpoint for the RE test. The concentration which inhibits root elongation by 50 percent of the control (EC_{50}) is estimated and used to rank samples.

Containers, Cleaning, and Preparation

Required containers are discussed under the appropriate test. Cleaning and preparation of test containers should follow procedures described in the GENERAL INSTRUCTIONS for the aquatic tests.

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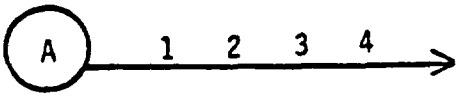
Sampling and Sample Preparation

Samples should be collected randomly at the site boundary or other critical location or along a suspected gradient (identified by any of several methods) such that the most impacted soil can be identified on one end of the gradient and the non-impacted soil on the other end. An example experimental design is shown in the following diagram where A can be a lagoon, an area where leaky drums are stored, or other situation:



A grid at each sampling point is set up, and the surface soil is sampled from a randomly selected quadrant. The points can be uniformly or logarithmically spaced depending on objectives.

Soil samples are returned to the laboratory, air dried and ground. If leachates are to be assessed in any of the aquatic or terrestrial tests, they should be prepared all at one time using the procedures in Table A-8. The reason for splitting the leachate sample for the various tests is to standardize the procedures so that results may be more comparable among the different tests. The split will then be diluted to yield various concentrations of the leachates for the test. Concentrations should be related to the soil extracted.

To perform the soil tests with the earthworms or litter decomposition, the soil samples will be diluted (percent W/W) with standard synthetic soil to produce the desired tests concentrations.

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TABLE A-8 METHODS FOR PREPARING SOIL LEACHATE.

Steps

1) Weigh an adequate amount of air dried soil sample for all desired tests.

2) Add four times the soil weight in distilled water.

3) Place on shaker for one hour at constant temperature (20^{o} $\pm 2^{o}$) in the dark.

4) Allow to settle, decant and filter with Whatman No. 1 to obtain the leachate.

5) Relate all leachates to the original weight of soil. Measure volume of leachate and relate to initial soil weight. For example, if 3100 ml of leachate is obtained from a 1000 gram of air dried soil, there are 3.1 ml/gram. Then, if 25 ml of leachate are added to 100 g of soil for a test, this would be equivalent to 8 g of soil (25/3.1) or a 7.4 percent soil (8/108). This would be the highest concentration and for a geometric series of tests subsequent samples would be decreased by halves. For example, for 7.4, 3.7, 1.85, ... percent, leachate plus sample volumes would be: 25 + 0, 12.5 + 12.5, 6.25 + 18.75, ...

6) Do not concentrate extracts; leachates should be prepared within 24 hours of collection. Leachates should be checked for salinity using conductivity.

To perform the terrestrial tests, the following soil samples are needed (minimum in parentheses):

	Soil, Kg	leachate, l
RE Test	2.5 (1.25)	10 (5)
Earthworm Test	4.0 (2.0)	
Soil Respiration Test	2.0 (1.0)	

These samples would provide sufficient material to perform a second test. If range-finding tests or further repeat assays are performed, additional samples should be collected.

ROOT ELONGATION TEST

Introduction and Rationale

The development of a seed into a mature plant is a series of complex processes. To assess toxic effects requires the selection of a stage in plant development that is sensitive to a broad range of toxicants and is important physiologically. Seed germination and root elongation are critical links in plant development beginning with a dormant embryo and during a period of rapid growth when essential plant structures are formed.

Toxic substances that prevent or reduce germination or root elongation will decrease plant populations and can reduce crop yields. In natural systems those species affected are less able to compete with other species

and tolerant species may be selected, resulting in changes in species diversity, numbers, and population dynamics.

The inhibition of seed germination and root elongation has been used in determining selective toxicities of herbicides (16,17), screening plants for heavy metal (18,19) and salinity tolerance (20,21), and evaluating toxic chemicals (22,23) and allelopathic substances (24,25). The root elongation/seed germination bioassay has several advantages. It is a rapid test germination and root elongation can be observed after 115 hours of incubation. It is a simple test that does not require large expenditures for equipment and facilities or complicated techniques. Personnel required for performing the bioassay do not need to be highly skilled.

The same chemical may cause responses at different doses in different plant species (23). To detect an effect from chemicals of unknown toxicity, several plant species should be selected. The species used in this test -- lettuce (butter crunch), Lactuca sativa L.; cucumber (hybrid Spartan valor), Cucumis sativus L.; and red clover (Kenland), Trifolium pratense l. -- are representative of economically important plants and different plant families. Seed chosen germinates, grows rapidly, contains no natural inhibitors, and requires no special pretreatment. All test organisms are grown under identical environmental conditions (constant temperature, 25°C, constant dark, and enclosed to maintain uniformly high relative humidity).

Although inhibition of root elongation and germination are observable toxic responses, root elongation inhibition is the preferred endpoint in this bioassay. Usually, elongation is inhibited at lower concentrations of toxic substances than is seed germination.

Materials and Methods

Facilities

The facilities must include work areas for planting seed and for measurements, preferably isolated from other activities. There should be a fume hood, a distilled water source and refrigeration available at 5°C. The test facility must have a controlled environmental chamber capable of maintaining a uniform temperature at 25°C within ±2.0°C.

Test Containers

One-piece molded-glass tanks (for example, Anchor-Hocking Glass Co., Lancaster, Ohio 43130), with a 6-quart capacity (approximately 9-1/2 in. (L) x 6-1/4 in. (W) x 7 in. (H)) are used for dosing seeds. Glass plates (5-1/8 in x 6 in.) of single-strength window glass are prepared with polished edges. The glass plates are supported at a 67° angle in the tank with either glass pegs. The pegs are 2 to 3 cm long and 5 mm in diameter. Twenty pegs are attached with epoxy to the inside of each glass tank (Figure A-1). An alternative is a glass rack (for example, Shamrock Scientific Glassware, Little Rock, Arkansas 72205) constructed from two glass rods (approximately 9 in. long) and six half-circles (4-3/4 in. O.D) of glass tubing connected to the rods at right angles at 1-3/8 in. intervals.

Equipment

Items specifically needed include a spray bottle with a fog or mist nozzle, metric ruler, forceps, Soxhlet extraction apparatus, triple beam balance, pH meter, storage bottles, and plastic bags (minimum of 23 in. x 8

in. x 14 in.). An illuminated magnifier may be helpful for planting, seedling examination, and root measurement.

Test Organisms

The seeds used in the test are available from commercial seed companies, State Agricultural Experiment Stations, and laboratories of the U.S. Department of Agriculture. Seed from one seed lot for each species should be purchased in amounts adequate for 1-year's testing. Information on seed lot, the seed year or growing season collected and germination percentage should be provided by the source of seed. Only untreated (not treated with fungicides, repellants, etc.) seed is acceptable for the bioassessment protocol.

Size Grading of Seed

After purchase, size grading is carried out on the entire seed lot for each kind of seed. Small samples of 100-150 g are sized at a time. The seed lot is inspected and trash, empty hulls, and damaged seed are removed. Depending on species, select a series of four screens to separate sample into size classes (see Table A-9). The four screens are nested with the screen containing the largest holes on top and screens with successively smaller holes in sequence below. A blank or bottom pan collects the fraction that passes through all screens. Seed is poured onto the top screen and the whole set of nested screens are shaken (by hand or with a vibrator) until all the seed remains on one screen or reaches the bottom pan. The separated fractions are collected and saved. The procedure is repeated until all the seed in the lot is sized. That size class which contains the most seed is selected and used exclusively for the duration of the tests. The seeds in the size class are divided into small lots placed

TABLE A-9 HAND SCREENS FOR SIZING SEEDS[a]

Species	Perforated Metal Sheet		Wire mesh
	Round Holes	Oblong Holes	
Red Clover	1/19, 1/18, 1/17, 1/15 (Fractions of an inch)		
Radish	6-1/2, 7, 7-1/2, 8 (64ths of an inch)		
Wheat	9, 9-1/2, 10, 10-1/2 (64ths of an inch)		
Cucumber		1/13 x 1/2 1/14 x 1/2 1/15 x 1/2 1/16 x 1/2 (fractions of an inch)	
Lettuce			6 x 28 6 x 30 6 x 32 6 x 34 (fractions of an inch; e.g., 1/6" x 1/28")

[a]Supplied by (for example), A.T. Ferrell and Co., Saginaw, MI 48601, or Seedburo Equipment Co., Chicago, IL 60607.

in separate envelopes or sacks, and stored in moisture-proof sealed containers in a refrigerator at 5°C.

Preparation of Glassware

The glass tanks (fitted with glass pegs or tanks with glass racks) and glass plates are thoroughly washed as described under Cleaning and Preparation of Glassware.

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Tissue Paper Precleaning

Eight to 10 sheets of single-ply cellulose tissue (for example, Kimwipes) are placed in a Soxhlet Extractor and extracted using standard chemical procedures with distilled water for a minimum of 24 hours (4 cycles/hour). After extraction, the tissues are removed, air dried, and stored in a dry glass container.

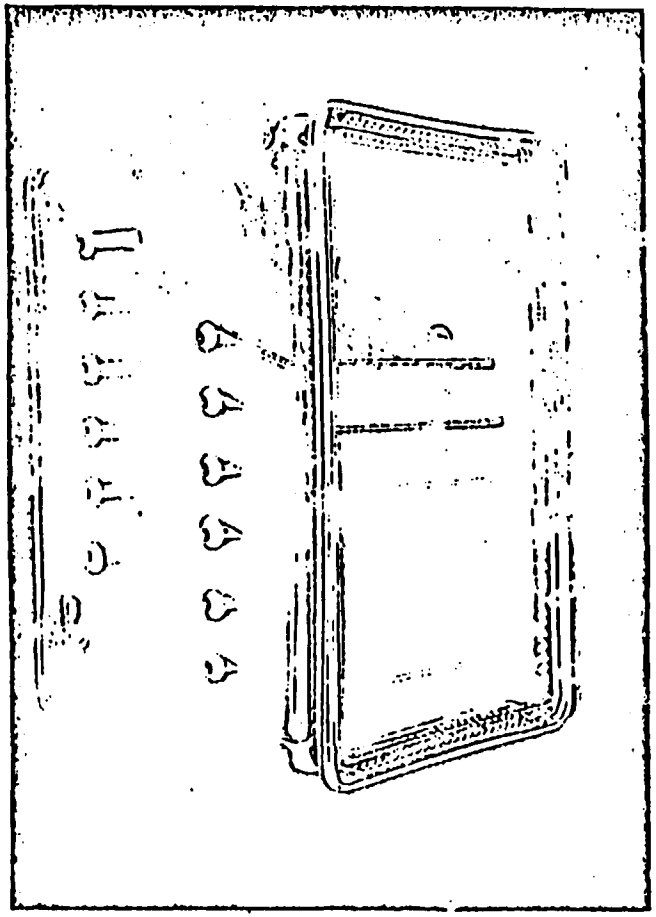
Test Procedures

Test Medium

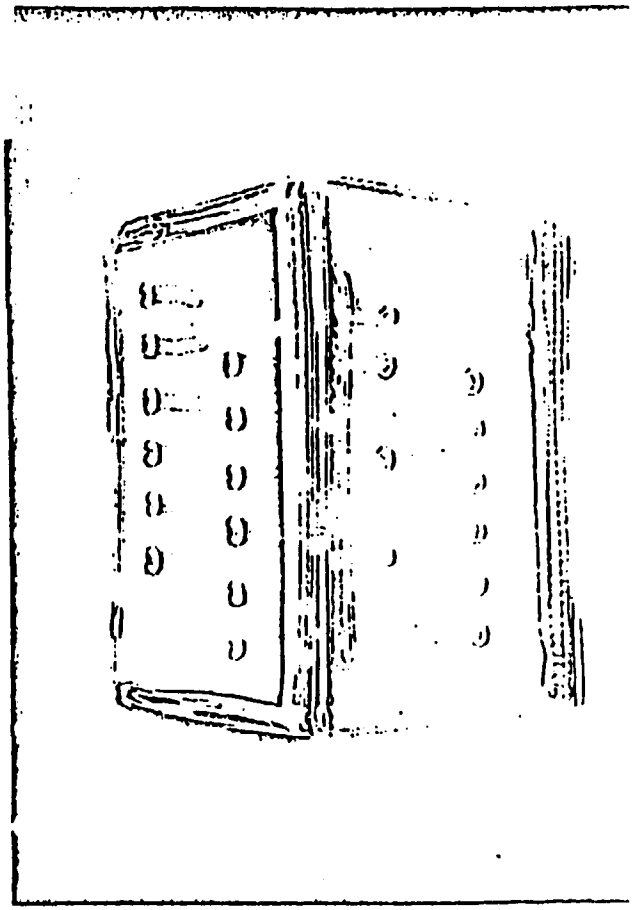
The test medium is an aqueous extract of a particulate or solid sample. Aqueous extracts of solids are prepared using the procedure outlined in Table A-8. Aqueous extractions of solid samples should be tested as soon as possible or the solid sample must be stored in closed polyethylene containers until extraction can be made. Dilutions of the effluent or aqueous extractions should be made without use of solvents or additives except for distilled water, which is used as a negative control. Before testing, the pH of the samples and controls is adjusted to 6.5 using HCL or KOH. Test medium osmotic potential should be greater than -3 bars to avoid osmotic effects which can retard root elongation and seed germination. Generally, leachates with lower than 0.01 N salt will meet this requirement. Saline soils may exert seed elongation toxicity even without toxicants and it may be necessary to compare results to a reference control.

Procedure for Planting Seed

Whatman 3MM chromatography filter paper rectangles (13 kg 15.2 cm) are soaked in the test solution in a shallow tray for a minimum of 5 minutes to saturate. One sheet of filter paper is removed from the test solution, allowed to drain, and placed on a glass plate to which the paper adheres. Trapping air bubbles between the filter paper and the glass plate should be avoided. Using forceps, 15 seeds from one species are placed on the filter paper substrate in a row, equally spaced, across the top of the plate 1-in. down from the top edge. Seeds are placed with the radicle end (embryo or germ) toward the bottom of the plate (Figure A-2) and, in the case of wheat, with embryo side of the seed up. A narrow strip, (1/2 cm wide) of previously cleaned (Soxhlet Extraction) single-ply tissue is placed over the row of seeds to hold them in place and, if necessary, sprayed with just enough fine distilled water mist to cause the tissue to cling to the seeds and filter paper. Test solution, usually 500 ml, is poured into the rectangular glass tank fitted with glass peg guides (empty tank if glass rack is used). The glass plate holding seed and substrate is inserted in the glass tank between the glass peg guides or in the glass rack to support the plates at a 67^{o} angle with the horizontal (Figure A-1). The lower end of the plate opposite the seeds should be immersed in the test solution with a minimum of 2 cm, but not more than 3 cm, of the plate and filter paper in the solution. Solution volumes smaller than 500 ml can be used if clean inert glass beads are added to the solution to displace and raise the liquid level. This procedure is repeated for each seed type (lettuce, radish, wheat, cucumber, red clover).

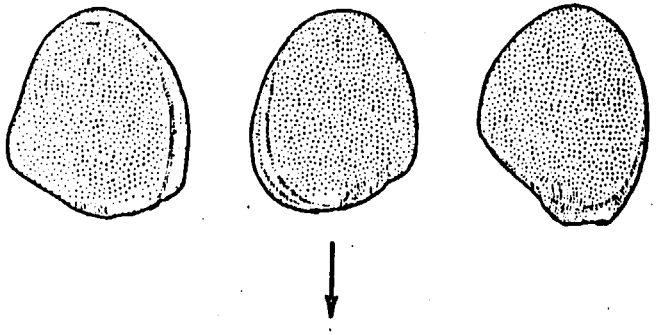


Glass tank with glass plates inserted between rods.

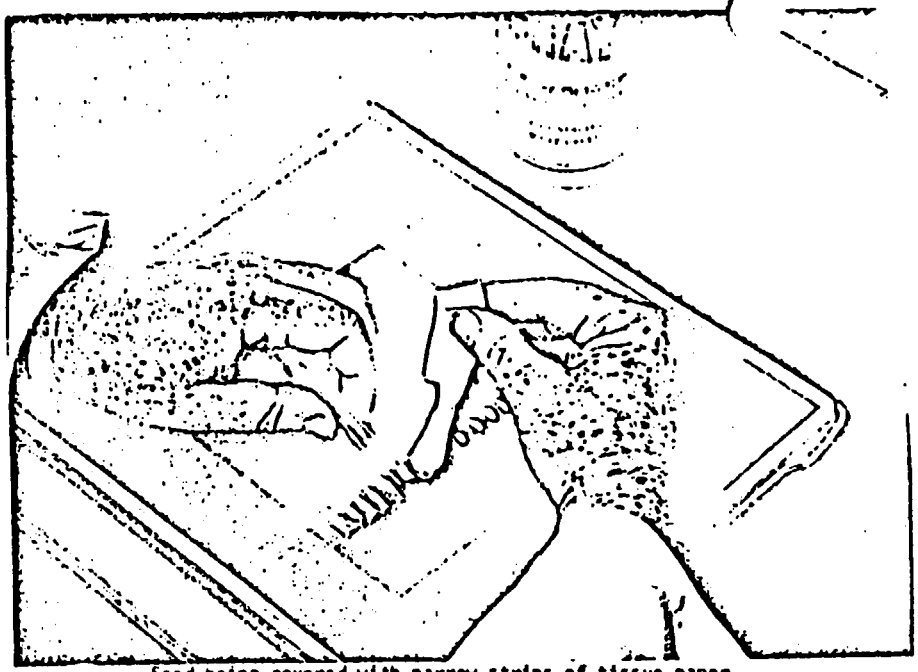


Glass tank fitted with glass rods.

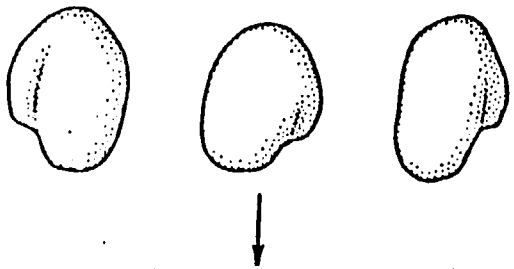
Figure A-1. Example Presentation of Setup of Glass Tanks for RE Test.



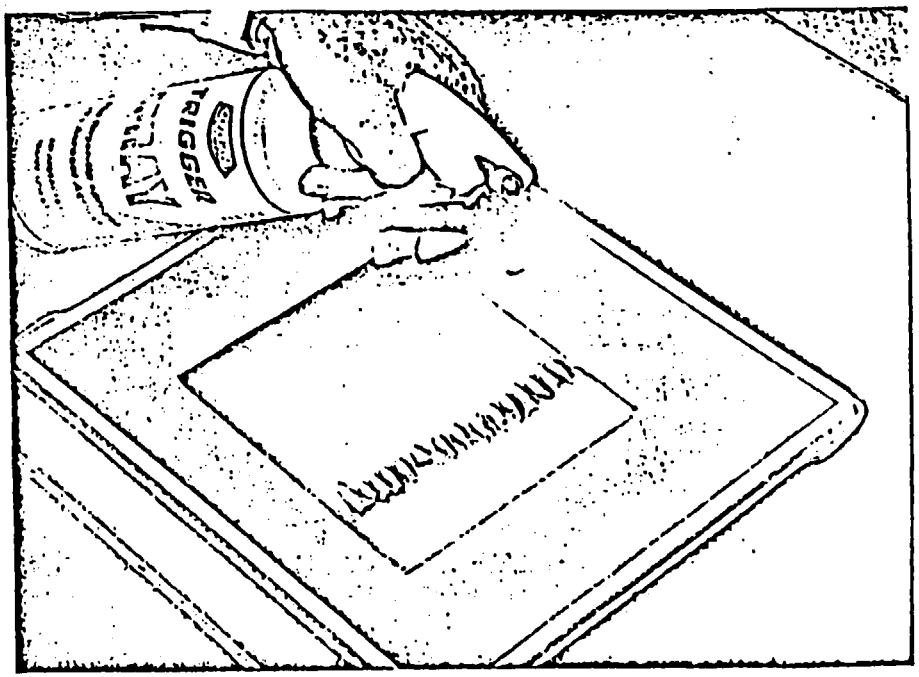
Radish seed (enlarged) with radicle end toward the bottom of plate.



Seed being covered with narrow strips of tissue paper.



Red clover seed (enlarged) with radicle end toward bottom of plate.



Fine distilled water mist causes tissue to cling to seed and filter paper

Figure A-2. Examples of Preparing and Orienting the Seeds for the RE Test.

Incubation

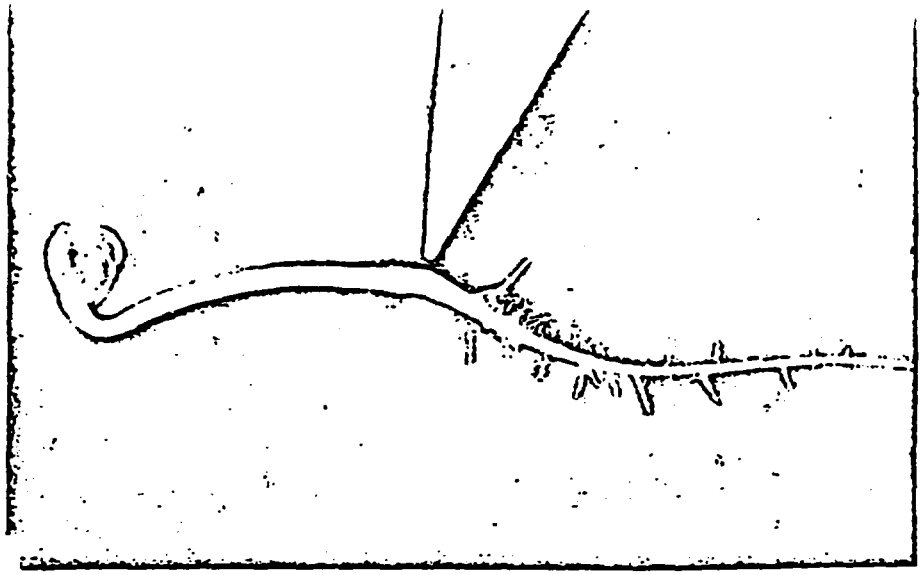
The glass tank containing 5 plates with 15 seeds each and the test solution is enclosed in a heavy plastic bag and tied shut (Figure A-1). The enclosed tank is placed in the dark, 25±2°C controlled chamber. A tank is prepared for each test solution of sample solution, the positive controls (200 mg/l of NaF), and the negative (distilled water) controls.

Measurement of Root Length

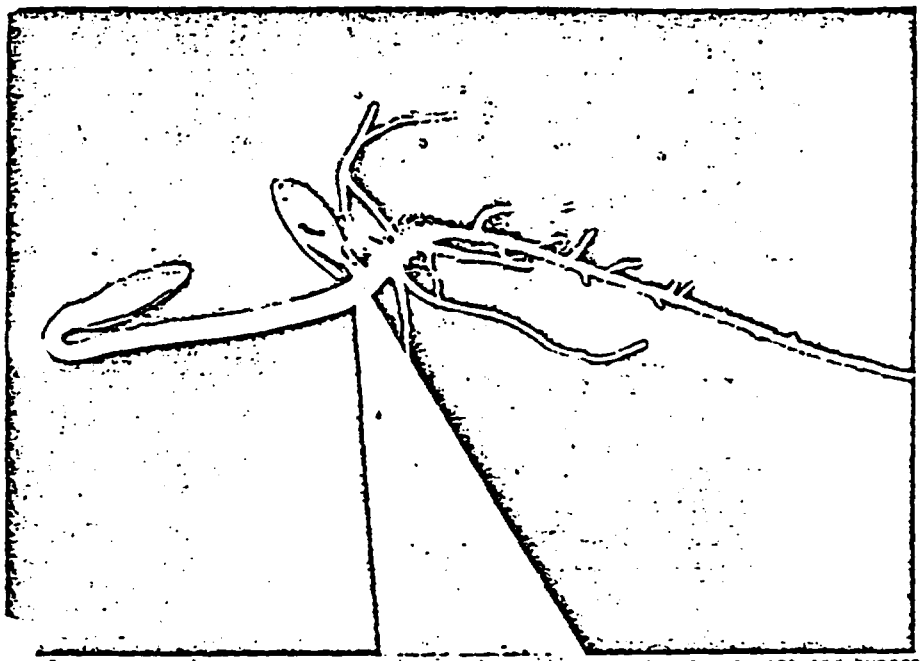
Measurement of root length is made at 115 hours from the start of dark incubation. It is important to measure each plate as nearly as possible to 115 hours (not to exceed ±30 minutes). To measure root length, remove a plate from the tank and place it on a flat surface. The lengths of all roots are measured to the nearest millimeter and entered on the data sheet. Measure from the transition point between hypocotyl and root to the tip of the root (Figure A-3). At the transition between the hypocotyl and the primary root the axis may be slightly swollen, contain a slight crook, or change noticeably in size (radish, lettuce, cucumber, red clover). In wheat, the single longest primary or seminal root is measured from the point of attachment to the root tip. For additional descriptions and photographs helpful in making root measurements, see References 28 and 29.

Range-finding Test

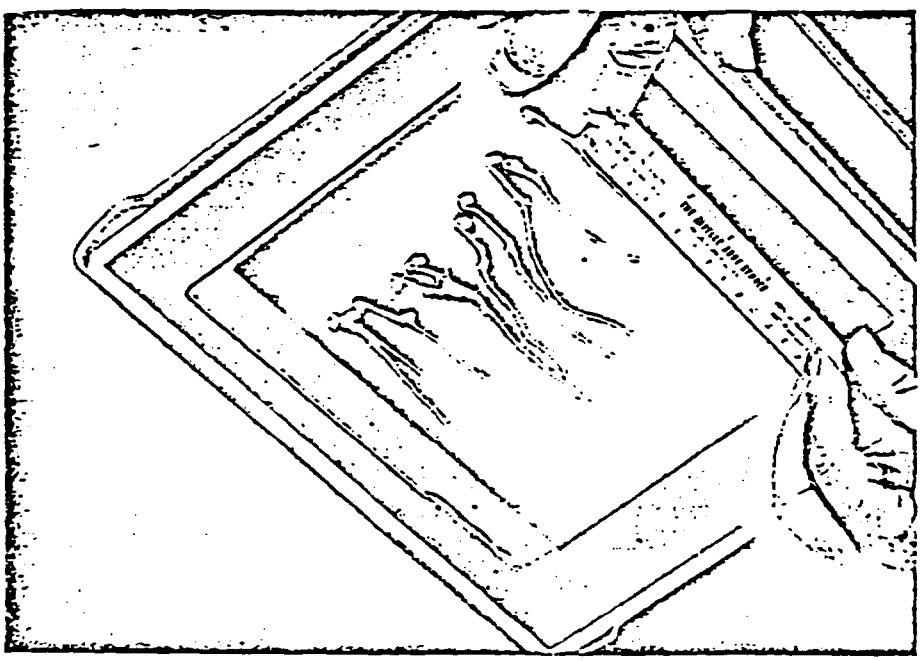
The purposes of the range-finding test are to determine if definitive testing is necessary, and to aid in the selection of concentrations to be used in the definitive test when needed. The range-finding test consists of two control tanks, two tanks of 100 percent effluent, and one tank each of 10, 1, 0.1, and 0.01 percent effluent.



Radish seedling with pointer showing transition point between root and hypocotyl.



Cucumber seedling with pointer showing transition point between root and hypocotyl.



Seedling showing method of root measurement using metric ruler.

Figure A-3. Examples of Measuring Root Elongations in the RE Test.

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A species need not be included in the definitive test if both tanks containing 100 percent effluent had mean root lengths of at least 65 percent of control and at least 10 of 15 seeds in one control, 8 of 15 seeds in the second control, and 8 of 15 seeds in both 100 percent effluent tanks germinated. Also, in this situation it is not necessary to examine the plates containing this species in the 10 to 0.01 percent tanks. If one or more of the species show mean root lengths less than 50 percent of the control at even the most dilute concentrations, it may be desirable to extend the range and repeat the range-finding test before proceeding to the definitive test.

Definitive Test

Estimation of an EC_{50} (concentration which reduces root elongation 50 percent) in this test will require two controls and at least six effluent concentrations chosen in a geometric series. The highest concentration in the definitive test should be the next concentration greater than the range-finding concentration which reduces mean root length to less than 50 percent of the control. For example, if the range-finding test shows that 1 percent leachate causes mean root lengths less than 50 percent of the control, then the definitive test would begin at 10 percent leachate. In a geometric series, the ratio of one concentration to the next is the same: for the above example, 10, 5, 2.5, 1.25, 0.615, and 0.312 percent. If more than six concentrations are used, not all species must be tested at all concentrations. However, each species must be tested with at least six concentrations and those concentrations must be in a geometric series.

Results and Data Interpretation

Assay Acceptance Criteria

To accurately estimate the EC_{50}, specific criteria must be met for each of the test species. For the definitive test, both criteria 1 and 2 (below) must be met and at least one of criteria 3, 4, and 5 must be met:

1. At least 10 of 15 seeds on one control plate and 8 of 15 seeds on the other control plate must germinate.

And

2. Each effluent concentration in a series must be at least 50 percent as strong as the next concentration, except for the controls.

And At Least One Of The Following

3. There must be at least one sample concentration ("low sample") for which mean root length was above 65 percent of the control and one concentration ("high sample") for which it was below 35 percent of the control; eight or more seeds must germinate in each of these concentrations. In addition, samples of greater dilution than the "low sample" must have a mean root length above 50 percent of the control and samples of lesser dilutions of the "high sample" must have a mean root length below 50 percent of the control.

Or

4. All conditions required in criterion 3 (preceding) must be satisfied with the sole exception that eight or more seeds need not germinate at the "high sample" concentration. However, there must also be one concentration stronger than

the "high sample" concentration for which fewer than 8 of 15 seeds germinated.

Or

5. There must be two concentrations which each have mean root lengths at least 65 percent of the control; at the lower concentration 10 or more seeds must have germinated and at the higher concentration 5 or fewer seeds must have germinated. In addition, there must be one concentration higher than the "5 or fewer" concentration for which 7 or fewer seeds germinate.

Since most effluents affect root elongation at lower concentrations than germination, criterion 3 will usually be used to satisfy the requirements of the definitive test in addition to criteria 1 and 2. However, in cases where germination is inhibited at lower concentrations than elongation, it may be necessary to use criterion 4 or 5 in place of 3. If a species fails to satisfy criteria 1, 2, and one of 3, 4, or 5, the definitive test must be repeated for that species.

Calculations and Reporting

Provided criteria 1, 2, and 3 are met in the definitive test, the EC_{50} is estimated in the following manner. For each species which satisfied these three criteria, plot on semi-log paper sample concentration on the logarithmic axis and percent control mean root length on the arithmetic axis. Draw a straight line between the two effluent concentrations used to satisfy criterion 3. Mean root length will be above 65 percent of control for one of these concentrations and will be below 35 percent of control for the other. The concentration at which this line crosses the 50 percent

point for root length is the EC_{50} for root elongation. If no effects were seen with 100 percent effluent, or if criterion 3 could not be met due to germination inhibition (criteria 4 or 5 instead), it is not possible to estimate an EC_{50} for root elongation.

For <u>each</u> of the species either the concentration in (a) <u>or</u> (b) <u>or</u> the quantities in (c) must be calculated and reported.

(a) If the species satisfied criteria 1, 2, and 3, report an estimated EC_{50} for root elongation. Use graphical interpolation to estimate the EC_{50} and rank the test sample using evaluation criteria in Table A-1.

(b) If the species satisfied criteria 1 and 2 but not criterion 3 (criterion 4 or 5 used instead), report the lowest concentration for which fewer than 8 of 15 seeds germinated. The EC_{50} cannot be estimated for root elongation or inhibition of seed germination from data in this category. Currently, test samples are not ranked from data of this type.

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EARTHWORM TEST

Introduction and Rationale

Earthworms have been selected as an indicator species because they are representative of the terrestrial environment and are of considerable importance in improving soil aeration, drainage and fertility.

Earthworms differ from aquatic organisms in that they may be exposed to toxic chemicals in the aqueous phase via soil moisture, in the vapor phase, or by coming into contact with particulate matter on the surface of soil constituents. Moreover, they may be protected in soil because many chemicals become tightly adsorbed onto soil fractions, particularly organic matter, and the soil colloids making up the clay fraction.

Hence, a simple immersion test, which yielded consistent and reproducible results for relatively soluble chemicals or formulated pesticides, was rejected because it would not provide information on comparatively insoluble compounds which affect the worms only when they are in direct contact, or on compounds which affect the worms only as a vapor.

There are tests which involve the injection of test chemicals either into the pharynx or body of the worms and although these give reproducible results, they require considerable expertise and have the drawback that it is difficult to relate the results of such tests to field conditions.

The test method is proposed as a two-stage test. The first stage would be a relatively simple contact toxicity test involving exposure of the worms to leachates on filter paper to examine potential toxicity. Toxic samples would then be tested further using soils or applications of leachates in a precisely-defined soil medium. The contact test was chosen because the exposure of the worms in such a test more closely resembles the natural situation.

To provide a routine test for the protocol, a commonly used test species was selected. Eisenia foetida is not a common species in soil although it does occur in soils with considerable organic matter. It is common in sewage beds, particularly in trickling filters, where it is exposed to industrial chemicals. It is a species with a short life cycle, reaching maturity in seven to eight weeks at 15-20°C. it is prolific; a single worm produces 2-5 cocoons per week each of which will give several worms. It can be bred readily in a wide range of organic wastes. This means that laboratories could easily breed their own stock if supplied with cocoons from a central source, and a standard strain could be used. Therefore, E. foetida is the test organism.

Materials and Methods

Test Organisms

Test organisms should be adult Eisenia foetida (at least 2 months old with a clitellum) of weight 400 - 800 mg. All worms for a specific test should be from the same breeding box. Individual worms are used (1/vial) in the range-finding test; ten individual worms (about 4 to 8 g) should be

added to each test container for the definitive test.

Breeding of Test Organisms

Eisenia foetida can be bred in a wide range of animal wastes. The recommended breeding medium is a 50:50 mixture of horse manure and peat, but other animal wastes are also suitable. The medium should be of pH about 7.0, have low ionic conductivity (less than 6.0 mm mho/cm) and not be contaminated excessively with ammonia or animal urine. Wooden breeding boxes 500 x 500 x 15 cm with tightly fitting lids are ideal for large-scale breeding and should produce more than 1000 worms in six weeks. To produce sufficient worms, such a medium will support 1 kg worms in 20 kg waste and each worm will weigh up to 1 g. To obtain worms of standard age and weight it is best to start the culture with cocoons which take three weeks to hatch and seven weeks to become mature worms at 20°C.

Test Procedures

Range-Finding

Glass vials, 8 cm long x 3 cm diameter are recommended. The sides of these are lined with a strip of filter paper 9.5 x 6.7 cm (Whatman Grade 1). The leachate is applied in water as appropriate, to give a range of known concentrations. If solvents are used, one ml of solution is pipetted into each vial and evaporated to dryness under compressed air, rotating the vial horizontally as it dries. For certain chemicals, relatively insoluble in organic solvents, this may have to be repeated several times to achieve the greater deposits required. The control should be treated with organic solvent only, if used. One ml of water is then added to rewet the filter

paper.

It is recommended that the toxic dose range be established in a preliminary test after which a more precise test may be made with a restricted dose range. The doses are calculated in terms of ml of leachate diluted with distilled water to give the following concentrations: 100 percent, 10 percent, 1 percent leachate.

For a more precise contact test, five doses in a geometric series (e.g. in the ratio 100, 50, 25, 12.5, 6.25) should be used. For each test, ten replicates per dose, of one worm per vial, would be the minimum requirement. Do not use more than one worm per vial.

In each test, a range of doses of leachate plus a positive control using 0.01 percent (W/V) copper sulfate and ten negative control vials should be used:

Vials should be laid on their sides for the duration of the test.

Test temperature = $20^{o} \pm 2^{o}C$.

Test in continuous dark.

Test duration = 48 hours.

Worms should be classed as dead when they do not respond to a gentle mechanical stimulus to the front end.

Discard vials after the test.

Definitive Test

In this test, worm survival is evaluated after 7 days in a mixture of an artificial soil and soil samples from the site. The amount of site soil to be used can be related to the range-finding test by assuming that

leachate represents a 25 percent dilution (4 parts water to extract 1 part soil). Thus, if the range-finding test provided an LC_{50} of 20 percent soil leachate (4 parts deionized water plus 1 part leachate) the dilution of soil would be equivalent to 1/20. The LC_{50} for the soil would be expected to be 1 part sample plus 19 parts of artificial soil. This would be the middle concentration of the definitive test and other concentrations in the geometric series would be selected accordingly. The ratios would correspond as follows: 1) For the geometric series - 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, negative control; 2) For a total of 20 parts, ratios of artificial soil to site soil (W/W) would be - 12+8, 16+4, 18+2, 19+1, 19.5+0.5, 19.75+0.75, 10.+0. The corresponding percentages of site soil per total soil would be: 40, 20, 10, 5, 2.5, 1.25, and 0 (negative control) percent. Because there may be a lack of correspondence between the range-finding and definitive test for a particular sample, it may be necessary to apply the soil test using range-finding concentrations.

Artificial Soil

Soil is a variable medium so for this test a carefully defined artificial loam soil is used. The medium for the definitive test should be based upon the three constituents listed in Table A-10; sieve and chemical analyses are not required. The peat is finely ground in a laboratory mill and the pH is adjusted to 7.0 by addition of appropriate amounts of calcium carbonate.

The dry constituents are blended in the correct proportions and thoroughly mixed mechanically in either a large-scale laboratory mixer or small electric cement mixer. Moisture content is then determined by drying a small sample at 80°C and reweighing. From these data, the amount of

TABLE A-10 COMPONENTS OF ARTIFICIAL SOIL

General Composition by Weight

70% Industrial Sand
20% Kaolinite Clay
10% Sphagnum Peat

Specific Composition

1. Industrial Sand

Diameter in Microns	Percent
45	1.7
45	9.3
63	29.0
90	34.3
125	20.8
180	4.0
250 & greater	0.8

2. Kaolinite Clay

Composition	Percent
SiO_2	58.5
TiO_2	1.3
Al_2O_3	28.0
Fe_2O_3	1.0
MgO	0.3
CaO	0.2
K_2O	2.0
Na_2O	0.3
loss on ignition	8.4

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deionized water required to achieve a moisture content of 25 percent of dry weight is calculated (25 g water per 100 g of dry soil). This is added and the medium remixed before use. This artificial soil mixes well and E. foetoda will survive in it for long periods. Its absorptive capacity is similar to that of a typical arable soil.

Preparing Site Soil Samples

Soil samples collected from the site must be prepared for mixing and "dilution" with the artificial soil. Soil is prepared by the procedures outlined in steps in Table A-11. Then, using a top loading balance, appropriate amounts of site soil and artificial soil are weighed to prepare the amount of soil needed for the appropriate tests.

Test Conditions

Test containers are 500 ml crystallizing dishes covered with plastic lids, petri dishes or plastic film. In each dish 400 g of the moist test medium is used. For each test dose, a 1600 g mixture of artificial soil and freshly sampled soil is prepared. For example, the test concentration desired is 50 percent. Therefore 800 g of moist artificial soil is added to 800 g of sample soil and thoroughly mixed. Then, four 400 g aliquots are weighed out and placed in each 500 ml crystallizing dish test container. For practical reasons, the sample soil concentration should never exceed 50 percent. Range finding test concentrations could be 0.5, 0.05, 0.005. Definitive test concentrations could be 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.016.

Copper sulfate will be used as the positive control and should be included in both soil assays by adding a 0.1 percent solution with the deionized water to artificial soil to achieve proper soil moisture. The

TABLE A-11. PROCEDURE FOR HOMOGENIZING SOIL SAMPLES

1. Air dry soil to be tested. (Air drying is considered completed when an aliquot of soil has no more weight loss.)
2. Add 25 burumdum cylinders and ca. 2 quarts of air dried soil to a ball mill.
3. Mill to coffee ground size (ca. 5 minutes) then sieve through a 2mm mesh sieve.
4. Return larger particles back to the ball mill and repeat steps 2 through 4 until the sample is completely ground with the exception of rocks. Discard rocks.
5. Homogenize soil using a laboratory or small cement mixer thoroughly before use.
6. Clean the ball mill by adding 1 quart of silica sand and 10 burundum cylinders. Mill for 15 minutes, discard, and then brush out mill.

main purpose is to account for variability in the test organisms.

For each test, four replicates of 400 g each containing ten test worms should be used.

In each test, a positive and negative control (100 percent artificial soil), each with four containers, should be used. Mortality should be assessed by emptying the soil into a tray, sorting out the worms and testing their reaction to a mechanical anterior stimulus.

The average weight of test and control worms should be determined at the beginning and end of the test.

The test temperature = 20^{o} $\pm 1^{o}C$.

Test in continuous light.

Test duration = 7 days. An assessment of mortality at 7 days and continuation of test to 28 days is optional. If more than one mortality assessment is made it may be necessary to adjust the moisture content of the soil due to losses during sorting.

Results and Data Interpretation

The mortality/dose data should be plotted on log probit graph paper and the median lethal dose (LC_{50}) and its confidence limits estimated. If the LC_{50} cannot be established the LC_{0} and LC_{100} values should be given.

Mortality in negative controls should not exceed 10 percent. If there is some mortality a correction based on Abbott's formula can be made:

$$\text{Corrected mortality \%} = \frac{\text{Observed mortality \%} - \text{control mortality \%}}{100 - \text{control mortality \%}}$$

The LC_{50} values should be given as percent of sample soil (W/W).

SOIL RESPIRATION

Introduction and Rationale

Soil/litter microcosms can be used to define the impact of pollutants upon primary productivity in terrestrial ecosystems. The measurement of evolved CO_2 from microbial respiration in these microcosms can indicate the degree of pollutant stress within the system. Low CO_2 levels indicate high stress whereas no significant change or an increase in CO_2 can identify low pollutant stress or even stimulation of microbial activity.

A simple soil/litter microcosm toxicity test can be conducted within 21 days. Soil micro-organisms are exposed to various concentrations of the test materials (soil and/or soil leachates) at standard moisture and temperature conditions. Evolution of CO_2 is measured at predetermined time intervals throughout the test. Results are expressed as percent inhibition (EC_{50}) or stimulation (SC_{20}) between CO_2 evolved in control and amended microcosms at specified time intervals.

Materials and Methods

Required glassware includes one quart regular Mason jars with air tight lids and one ounce glass bottles with air tight lids. These should be washed according to GENERAL INSTRUCTIONS for the aquatic tests.

Triplicate microcosms for each treatment are prepared by placing 100 g of air-dried artificial soil (Table A-10) or combinations with test soil

(Table A-11), sieved to pass a 2mm screen into each of three one-quart Mason jars. Then, 20 ml of deionized water is added to bring the microcosms to the proper moisture. Finally, a one ounce glass bottle with CO_2 trapping solution is added and the air-tight Mason jar lid is sealed securely. A special blank must be used to correct for atmospheric CO_2 during titration. This consists of 3 clean Mason jars without soil and CO_2 trap but which are run concurrently with controls and test jars.

Equipment should include a constant-temperature room or incubator capable of providing temperature control of 20 $\pm 2^{o}C$. A ten liter capacity ball mill with 25 3.2 x 3.2cm (1-1/4") burundum cylinders and a 2mm mesh sieve. Standard laboratory equipment such as balances, pH meters, pipettors, magnetic mixers and bars, drying ovens, and appropriate glassware necessary to prepare reagents and perform the titration of CO_2 are also needed.

Test Procedure

The test material is introduced into the microcosm either as a soil or soil leachate. The soil sample may be added directly (100g) or by weight percentages as in the earthworm test (50, 25--etc.) added to the artificial soil. The aqueous test leachates are introduced into the artificial microcosms on a percent basis (V/W, ml/g), i.e. 100, 50, 25, 12.5, 6.25, 3.125, where 100 percent represents 25 ml of soil extract and further dilutions are made with deionized water (Table A-9). The extracts and acid traps are added after two days of incubation at $20^{o}C$ in the dark if leachate is to be studied.

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Respiratory carbon dioxide is measured in the alkali traps twice weekly for the duration of the test which is typically 2 weeks.

Three replicate microcosms are required for each control and test (soil and/or soil leachate) concentration. Each microcosm is incubated at 20 $\pm 2^{o}C$ in the dark for 14 days. CO_2 evolution is measured twice weekly by titration. Reagents and titration procedures are outlined in Table A-12. If available, infra red gas analyzer methods can be used (3).

Results and Data Interpretation

The total CO_2 produced during the 14 day test is obtained by summing individual CO_2 measurements for each interval. Percent inhibition (I), or stimulation (S), is calculated after 14 days for each test concentration according to the following formulas:

$$\%I = \frac{C - T}{C} \times 100$$

$$\%S = \frac{T - C}{C} \times 100$$

where C is the mean CO_2 evolution in the control and T is the mean growth in the treated microcosm. Three endpoints are calculated from the percent response vs. concentration data. For samples which are inhibitory, an EC_{50} (defined as the lowest test concentration causing growth inhibition of 50 percent relative to control) is calculated. For samples which are

stimulatory, and SC_{20} (defined as the lowest concentration causing growth stimulation of 20 percent relative to control) is calculated. Also, the measurements of CO_2 made at other times can be used to evaluate anomalous results and to observe time trends of CO_2 production.

TABLE A-12. PROCEDURES OF TITRATING CO_2 IN TRAPS AND METHODS FOR PREPARING REAGENTS

A. CO_2 Titration Procedure

a. Replace the CO_2 traps at the designated intervals by opening the microcosm and removing the exposed CO_2 trap and replacing it with an unexposed one. (At the same time this step is being performed, insert an open vacuum line to aid in properly replenishing the air in the microcosm. Remove at least 3 times the volume of the air space.)

b. As quickly as practical, place an air tight cap on the exposed CO_2 trap; return the microcosms to the 20°C dark incubator.

c. Add five ml of 1.3N of $BaCl_2$ and a stir bar to each exposed CO_2 trap immediately prior to titration.

d. Titrate excess 0.6N NaOH remaining in the trap to pH 9.0 with a buret and pH meter (or autotitrator) using Trizma standardized 0.6N Cl to measure milligrams of CO_2 produced.

Formula for the Calculation of CO_2 Production:

mg of CO_2 = (Blank ml - Sample ml) x 22 mg of CO_2/ml/N x Normality of Acid

e.g., mg of CO_2 = (10.40 ml - 6.93 ml) x 22 mg of CO_2/ml/n x 0.6013 N

= 45.90 mg of CO_2 produced

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TABLE A-12 (Continued)

B. Preparation of Reagents

1. ~ 0.6 NaOH

a. Rinse 5 gal glass carboy with distilled H_2O.

b. Place on a large magnetic stir plate; add degassed distilled H_2O to the 18.9 liter mark.

c. Add 454 grams (1 lb) of NaOH pellets.

d. Stopper and stir overnight before use. (Maintain the NaOH stock solution in a CO_2 free atmosphere by using ascarite traps.

2. ~ 0.6N HCl

a. Rinse 5 gal glass carboy with distilled H_2O.

b. Add 1.0 liter of concentrated HCl.

c. Add distilled H_2O until the 20 liter mark.

d. Stopper and stir overnight.

e. Titrate 5 "tris" samples (0.5 to 0.9 grams of "tris" in 10.0 ml of distilled H_2O and 5 ml of 1.2N $BaCl_2$) to pH 5.0 with ca. 0.7N HCl; calculate mean and standard deviation ("s"). (If "s" is larger than 0.0015, do 5 more samples and combine results.)

$$\text{Normality of HCl} = \frac{\text{(Weight of tris in grams)}}{\text{(0.1211 g/meq) (ml of HCl used)}}$$

$$\text{e.g., Normality of HCl} = \frac{0.7089 \text{ grams}}{(0.1211 \text{ g/meq})\ (9.69 \text{ ml})} = 0.6041\text{N}$$

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3. 1.3N $BaCl_2$

   a. Weigh 317.56 grams $BaCl_2:2H_2O$.

   b. Dissolved in degassed distilled H_2O in a 1 liter volumetric flask.

4. Tris

   Aminomethane(hydroxymethyl)tris--Trizma Base (Sigma Chemical Company, St. Louis, Missouri).

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APPENDIX B

GUIDANCE ON SAFETY PROCEDURES

FOR WORKING WITH

SAMPLES FROM HAZARDOUS WASTE SITES

USING THE

BIOASSESSMENT PROTOCOL

GUIDELINES AND CONCEPTS OF SAFE PROCEDURES

The objective of these guidelines is to protect workers, the public, and the environment, and to insure that contamination does not occur and interfere with valid laboratory results. The major factor in providing for this protection is the common sense of the staff performing the bioassessments. The guidance presented herein is designed to complement this common sense. For example, it is extremely important that all staff follow good housekeeping procedures and maintain personal grooming and cleanliness within the confines of the laboratory area. There are safety courses that are available through OSHA and these should be taken wherever possible. Also there is access to experienced personnel within states, regions, or local communities. These should be drawn on prior to starting and whenever any possible hazards might occur that were not considered.

It is important to consider that which safety procedures are utilized, they must be commensurate with the hazard, and hazard depends on the concentration and types of materials that will cause exposure. Hopefully, explosive, ignitable, corrosive, or otherwise highly reactive samples will not be evaluated in the bioassessment protocol. Generally, soil samples and water samples will be utilized in the bioassessment protocol. The intent is to assess the toxicity of those samples. Sample size should be adequate for needs and not in great excess as disposal then becomes more of a problem.

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A key factor is the safety committee to oversee procedures written in advance of the setup of the laboratory facilities. This safety committee should include at least a chemist and a biologist and, whenever possible, these persons should have experience in hazardous wastes. In the absence of

such experience, a state or other local expert should be utilized.

The facilities are a key part in maintaining the integrity of the protective plans. Wherever possible, separate self-contained facilities should be utilized. For example, field equipment should be labeled for hazardous waste site use only. Disposable sample containers and protective gear and equipment should be used where possible. Solid and liquid waste handling procedures should be specified and materials should be placed in unbreakable containers that are easily transported. A separate storage area a separate preparation room, and a separate experimental area should be required. All of these facilities should be lockable and maintained under safe and secure conditions.

Air should be supplied using forced air fans and complete exchange of the air supply in the preparation area should occur on an average of once every five minutes at the minimum. Air supply in general should be commensurate with potential hazard and the cost of providing air exchange. Chemical fume hoods are an excellent means of providing this kind of safety for sample preparation.

Personnel are protected with respirators, gloves, and laboratory clothing which are disposable or, in the absence of severe exposures, washable. Laboratory services should be isolated with appropriate check valves and/or supply services. This includes air supply, water supply and gas supplies. Vacuum services should be entirely separate.

Finally, personnel involved should be clearly identified and their utilization of the laboratory facilities involved with hazardous waste materials should be controlled and recorded. Medical surveillance should be implemented where needed. In any case, a complete physical examination with chemical measurements of blood and urine samples should be implemented prior to any work in the laboratory area. One should avoid the perception as well as the reality of risk. Specific procedures are identified in the following paragraphs.

1. Work schedule and procedures. All work to be performed should be detailed in advance and written out for all personnel involved and for review by the safety committee. A responsible person should be designated the hazardous waste material disposal officer. Detailed procedures on handling of soil and water samples, dilution procedures in water and synthetic soils, disposable and unbreakable containers, storage access, analytical measurements, and pertinent information should be written out in advance of any experiment. Standard procedures should be followed to minimize the paper work involved. However, all personnel involved must sign a form stating that they have read and understood the instructions. A simple test designed to determine their understanding of the procedures can be maintained in the personnel file.

2. Sampling, handling and storage. Sampling should be done using careful procedures since actual sites will have more hazardous exposure than will the laboratory facilities. In some cases,

it will be wise to subcontract to a firm specializing in hazardous waste handling in order to collect samples. Designated, separate field sampling equipment should be utilized for collecting water and soil samples.

Samples should be stored in disposable, non-breakable sample containers. Metals should be placed in polyethylene containers. Organics should be collected and stored in disposable glass bottles and bottles packed in absorbent material that can account for the entire liquid in the sample. Labels should be affixed to all samples.

3. Personnel. Personnel who are allowed to have access to the sampling and laboratory facilities should be identified clearly. Personnel testing or monitoring should be performed and recorded. For each type of waste, the need for medical surveillance should be evaluated. Personnel should be medically tested whenever especially hazardous conditions occur.

5. Chemical information form. All available information on the chemicals that are potentially present at a site should be accumulated. This information will be invaluable in terms of analyzing potential environmental hazards that exist at a site for the protocol as well as protecting the personnel.

6. Identification of the potentially most hazardous operations. It is important that all operations be written down as in item 1 above. The most potentially hazardous operations should be identified in this section and described in detail. After identifying these operations it is important to explain what procedures should be followed during potential accidents and/or routine safety procedures. Each operation that may be included in this section should be carefully identified and discussed with appropriate clean-up and disposal procedures.

7. Accidental exposure and emergency treatment requirements and procedures. Based on the chemical list and the potentially most hazardous operations, appropriate procedures should be spelled out. Monitoring for potential health problems that might occur should be detailed in this section.

8. Accidental release information. Accidental releases from bioassessment procedures are probably not a critical factor. However, a chain of custody form (see Section 16) must be used for all sample handling so that storage, utilization, dilution, and ultimate disposal by the laboratory disposal officer will be recorded for future use.

9. Waste disposal procedures. All toxic materials, original samples and high dilutions of samples, must be packaged in unbreakable containers and deliverable to the disposal officer. For safety and public relations reasons it is important to dispose of all contaminated materials in a safe

manner to a hazardous waste site. Segregation of protective clothing and samples that are in the low response levels into one category and intermediate to high response dilutions and actual samples into a high level category will aid disposal operations. It is the responsibility of the disposal officer to dispose of these using approved state procedures or other applicable regulations.

10. Personnel protection. Protective clothing including laboratory coats, and shoes, covers, eye protection, gloves, and face protection should be specified where necessary. In addition, respirators and dust filters generally should be utilized in the preparation room and also where hazards suggest that it is necessary in the experimental area. Each individual should carefully wash in a secure area that is outside of both the preparation room and the laboratory experimental area. Soap and water are usually adequate for washing. However, it is important that the protective clothing be utilized in the preparation room and discarded at the door in safe containers before exiting to the wash area.

11. Signs. The laboratory facility should be isolated from general public contact. Authorized personnel should be the only persons allowed in the experimental area. Warning signs should be posted and controlled access should be maintained at all times.

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12. Work area identification and access control. Although signs are necessary for information reasons, it is important that all areas be locked and public access kept under surveillance and minimized. The storage area in particular should have double locking procedures with a signature form and chain of custody form for samples.

13. Work surface protection. Preparation rooms and laboratory facility areas should be covered with disposable plastic backed absorbent paper.

14. Contaminant devices. All samples should be stored in sealed containers in the locked storage area. Subsamples can be prepared in the preparation room and samples returned to the storage area. After an assay, all samples should be delivered in disposable containers to the disposal officer.

15. Storage. The storage of samples should be minimized where possible. Only enough sample to meet the needs of the bioassessment should be collected plus a minimal safety margin. After bioassessment and analysis and review of the results, the samples should be disposed of to prevent accumulation of old and unusable samples. The storage area should be double locked and only authorized personnel be permitted to utilize the locked storage area. Chain of custody forms should be used to follow all samples.

16. Laboratory transport. A chain of custody form detailing sources and dates of sampling, descriptions of sample materials, and potential hazards should follow all samples. This form should be easy to use and cross referenced to a permanent record of the sample. As the sample is transported from individual to individual, a signoff should occur. The chain of custody form is signed off finally by the disposal officer after final disposal and then the form returned to the file for permanent storage. All laboratory transport should follow a prescribed procedure from field, to transport, to laboratory storage, to preparation room where subsampling occurs and is recorded. The primary sample is returned to storage and the subsample is analyzed using bioassessment procedures. At successful termination of the bioassessment the waste material from the experimental assay and the primary sample should be transported to the disposal officer and then disposed of safely at a hazardous waste site.

17. Housekeeping. Good laboratory practices are the best guarantee of safety of personnel. Detailed procedures specifying handling, treatment, and disposal of samples and bioassay organisms should eliminate most potential problems. Prompt cleanup of all problems should occur to prevent more serious problems.

18. Laboratory facilities. Separate vacuum lines, water plumbing, and waste drainage must be provided. Careful labeling and isolation of facilities and maintenance equipment will

minimize problems.

19. Emergency personnel. Potential problems that might occur and require emergency personnel should be carefully reviewed. Samples that do not require emergency personnel should be handled separately from those that might. If emergency personnel might be required, it is important to check with them in advance of such requirements and it is the responsibility of the safety committee to insure that this process is followed.