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Laboratory
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Research and Development

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# Project Summary

# IERL-RTP Procedures Manual: Level 1 Environmental Assessment, Biological Tests

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**This document is the second edition of the IERL-RTP Procedures Manual: Level 1 Environmental Assessment, Biological Tests for Pilot Studies. The first edition, prepared by Battelle-Columbus Laboratories, was published in 1977 (EPA-600/7-77-043).**

**This manual outlines the rationale and proposed methods for performing Level 1 health effects and ecological effects bioassays. The spectra of test methods contained within both the preceding categories were changed somewhat from the first edition. Tests such as the WI-38 cell toxicity assay were dropped and other tests (such as those for insect toxicity, plant root elongation, and bioaccumulation) were added. These changes were instituted to give a more comprehensive testing program.**

**In addition to those changes in recommended tests, the second edition includes new sections on sample collection and processing documentation, data evaluation and interpretation, and quality control/quality assurance procedures.**

***This Project Summary was developed by EPA's Industrial Environmental Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).***

## Introduction

This bioassay procedures manual is a guide for studies to be conducted by the Industrial Environmental Research Laboratory of the Environmental Protection Agency (EPA), Research Triangle Park, North Carolina. The manual is a revision of and supersedes the "IERL-RTP Procedures Manual: Level 1 Environmental Assessment, Biological Tests For Pilot Studies," published in April 1977[1].

The bioassay procedures in this manual complement the chemical and physical procedures of the Level 1 environmental assessment program and are an integral part of a comprehensive source assessment strategy. The biological testing manual complements the IERL-RTP Level 1 chemical and physical procedures manual[2]. The purpose of Level 1 is to obtain preliminary information, identify problem areas, and provide the basis for the ranking of streams for further consideration in the overall environmental assessment. The recommended biotests for testing the toxicity and mutagenicity of feed and waste streams of industrial processes are each described with a brief summary of procedures for collecting and preparing the samples to be tested.

Chapter 1 of the manual defines the goals and strategy employed in Level 1 testing and gives the background and philosophy of the phased approach to environmental assessment.

Chapter 2 discusses the Level 1 sampling activities and pretest-handling procedures that can be used for most industrial complexes. For each sample type, the discussion focuses on the general problem as well as specific

problems of preparation needed for sampling, the actual sampling procedures, and packaging of samples for shipment.

Chapters 3 through 5 specify the Level 1 health effects, aquatic, and terrestrial bioassay schemes. The schemes identify the methods of analyses, anticipated output and level of effort required for implementation, and the basic format for presenting the results of the tests.

Chapter 6 describes the data management, including data summary forms and an approach to consolidated toxicity assessment for multitest data for health and ecological effects.

Chapter 7 outlines the recommended quality control, quality assurance, and documentation procedures necessary to verify the quality of the assays.

Chapter 8 briefly discusses testing beyond that defined as Level 1.

## Definition of Level 1 Environmental Assessment Testing

Physical and chemical characterization of environmental emissions is critical to the definition of, need for, and design of control technology. However, the final objective of IERL-RTP's environment assessment is the control of industrial emissions to meet environmental goals that limit the release of substances that cause harmful human health or ecological effects. Consequently, the testing of industrial feed and waste streams for biological effects is needed as a complement to the physical and chemical data to ensure that the assessment is comprehensive. Biological testing can provide a direct measure of the toxicity and/or mutagenicity of substances to organisms that chemical analysis cannot. This is especially important when dealing with substances for which there is little available data on toxicity or when assessing complex mixtures where synergisms and antagonisms may alter the toxicity of the individual chemical constituents.

It should be stressed that the results of Level 1 tests are not intended for regulatory actions or recommendations, nor are they to be used as tests of acceptability or non-acceptability of emission release. The three-phased sampling and testing strategy was developed to focus available resources (both manpower and dollars) on industrial emissions which have a high potential for causing measurable health or ecological effects and for providing chemical and biological information on all sources of industrial emissions.

## Classification of Streams For Sampling Purposes

Comprehensive assessments are organized around the five general types of samples found in industrial and energy-producing processes, rather than around the analytical procedures that are required to collect the samples.

The five sample types are:

(1) *Gas/Vapor* (Non-particulate laden) — These include samples from process streams, vents, and effluents. Samples contain inorganic and organic gaseous components.

(2) *Gaseous Streams* (Particulate or aerosol laden) — These involve sampling contained air or gas streams such as in ducts or stacks. Samples include particulates and higher molecular weight organics with boiling points higher than 100°C.

(3) *Liquid/Slurry Streams* — Liquid streams are defined as those containing less than 5 percent solids. Slurry streams are defined as those containing greater than 5 percent solids. Liquid or slurry streams are classified as aqueous or nonaqueous. A stream sample that contains more than 0.2 percent organics is considered nonaqueous.

(4) *Solids* — These include a broad range of material sizes (from large lumps to powders and dusts) as well as nonflowing wet pastes. Nonflowing wet pastes may be formed by wetting solids either with aqueous or nonaqueous liquids, or they may be highly viscous liquids such as some tars or oils. The distinction between solids and slurries can become blurred.

(5) *Fugitive Emissions* — These are transmitted to the environment without first passing through some stack, duct, pipe, or channel designed to direct or control their flow. They may be in any of the above physical forms and may result from nonducted gaseous, particulate, or liquid emissions from the overall plant or process units.

A flow diagram, showing the overall relationship of the samples to the Level 1 analysis scheme, is presented in Figure 1.

The types of samples obtained from the procedures outlined in Figure 1 are usually mixtures and present problems for biological test systems that have been developed and validated primarily with pure chemicals. Table 1 summarizes the characteristics of samples collected from various sources.

All Level 1 bioassays are not suitable for the entire range of sample types obtained from industrial sources. Certain tests, for example, provide reliable results with solid samples that are soluble in organic solvent carriers, but may not be reliable if used to evaluate a gas or slurry. In other situations, the amount of sample required for applicable bioassays is too large to permit the tests to be performed on the available sample (such as with SASS* samples).

Suitability of test systems for specific samples must be judged on an individual basis; but, as shown in Table 2, some generalizations and recommendations can be made with respect to bioassay/test sample compatibility.

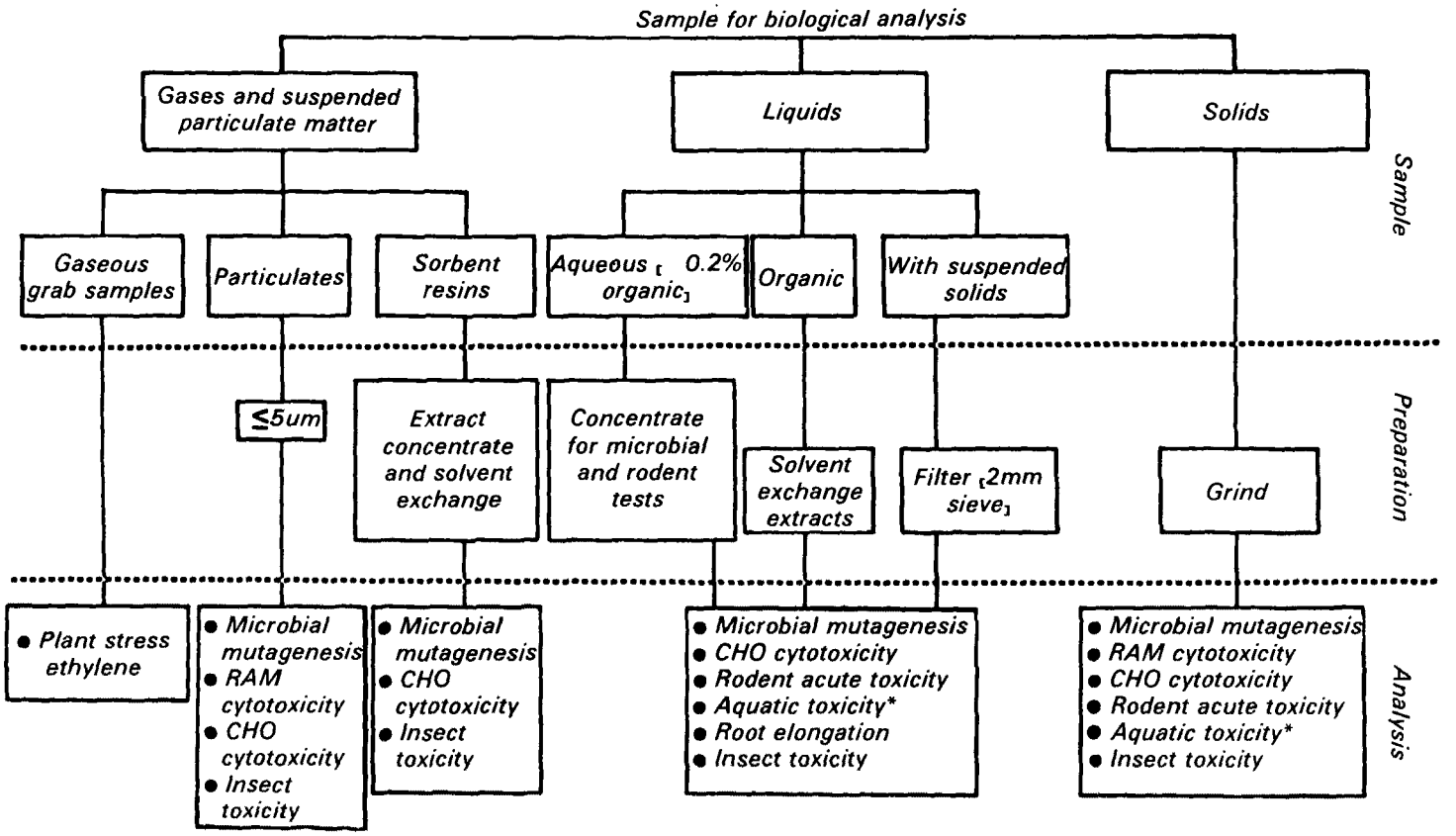
## Level 1 Health Effects Bioassays

The Level 1 health effects test include assays for determining toxicity and mutagenicity at several levels of organisms ranging in complexity from bacteria to mammalian cells in culture (both permanent cell lines and primary cells) to intact animals. Table 3 describes the biological characteristics of the target organisms in this group of tests. The tests are able to detect molecular changes such as DNA mutation (Ames test), acute cell toxicity (RAM and CHO tests), and complex toxicological responses in intact animals (WAT test).

Health effects bioassays are used to determine the concentration of test material that produces either a definite mutagenic or toxic effect on the test organisms in a short period of time. The Ames *Salmonella*/microsome mutagenesis assay (Ames) identifies the minimum effective concentration (MEC) of a test sample that produces significant mutagenesis in any of four tester strains of *Salmonella typhimurium* used.

The rabbit alveolar macrophage (RAM) assay measures four endpoints relating to cell death and metabolic impairment, following 20 hours

* Source Assessment Sampling System, developed by EPA (IERL-RTP) and manufactured by Research-Cottrell, Inc., Atherm Corporation, Mountain View, CA 94043




*Aquatic tests include freshwater or marine fish, invertebrate, and algal tests.

***Figure 1.*** *Biological analysis overview.*

continuous exposure. The effective concentration of toxicant that reduces each parameter to 50 percent of the control ($EC_{50}$) is calculated. The $EC_{50}$ is also estimated in the rodent cell (CHO) clonal toxicity assay based on the reduction in colony-forming ability of the cells following 24 hours of continuous exposure. Mortality and physiological observations are recorded in both the quantal and quantitative phases of the acute *in vivo* test in rodents (whole animal test, WAT). For samples exhibiting toxicity in the quantal phase, the dose lethal to 50 percent of the animals ($LD_{50}$) is calculated. Additional health effects endpoints may be measured in modified versions of the CHO toxicity test.

Results from Level 1 health effects tests are interpreted by using evaluation criteria unique to each test. Test samples are ranked according to relative mutagenicity or toxicity using guidelines presented in the results and data interpretation section for each test.

## Level 1 Aquatic Ecological Effects Assays

Biological responses and bioaccumulation must be considered, as well as chemical and physical parameters, when assessing the potential impact of complex wastes on the aquatic environment. Biological testing for aquatic ecological effects usually consists of static acute toxicity tests on selected organisms representative of the various trophic levels. The bioaccumulation of components in complex mixtures is evaluated using a laboratory technique for simulating bioaccumulation phenommena.

Acute toxicity tests are used to determine the concentration of test material that produces an adverse effect on a specified percentage of the test organisms in a short period of time. Because mortality is normally an easily detected and an obviously important adverse effect, the most common acute toxicity test is the acute lethality test. The index most often used with fish is the 96-hour median lethal concentration (96-hour $LD_{50}$), and for macroinvertebrates, the 48-hour effective concentration (48-hour $EC_{50}$). The $LC_{50}$ is a statistically derived estimate of the concentration of toxicant in dilution water that is lethal to 50 percent of the test organisms during continuous exposure for a specified period of time, based on data from one experiment. This may be supplemented, in fish tests, with effects on behavior. The $EC_{50}$ for macroinvertebrates is an estimate of the concentration of test material that results in the immobilization of 50 percent of the test organisms during continuous exposure for a specified period of time in one experiment. Immobilization is defined as lack of movement except for minor activity of appendages. This measured effect is used because death is not always easily determined with some invertebrates.

In algal tests the principal criterion of toxicity is the effect on growth during

*Table 1. Level 1 Bioassay Sample Requirements*

| Source | Sample | Description | Characteristics |
|---|---|---|---|
| Air | | | |
| *Gas/Vapor (Non-particulate laden)* | *Grab* | *Gas* | *Organic, inorganic, or both. Sample limited by storage capacity.* |
| *Gaseous Streams (particulate/aerosol laden)* | *SASS Cyclone (10 μm + 3 μm)* | *Solids > 3 μm* | *May be inorganic, organic, or both. SASS samples may have limited size.* |
| | *SASS - Cyclone (1 μm)* | *Solids 1-3 μm* | *Same as above.* |
| | *SASS - Filter* | *Solids < 1 μm* | *On fiberglass mat. Combine with SASS 1-3 μm if possible.* |
| | *SASS XAD-2 Resin* | *XAD-2 extract* | *Organics in dichloromethane. Requires solvent exchange before bioassay.* |
| *Process Fugitive Emissions* | *High-Volume Sampler* | *Solids* | *Organic, inorganic, or both.* |
| *Fugitive Gases* | *High-Volume Sampler* | *XAD-2 extract* | *Same as SASS XAD-2 Resin.* |
| | *Grab* | *Gas* | *Same as Grab, above.* |
| Liquids | | | |
| *All Sources* | *Grab or Composite* | *Untreated* | *Aqueous, nonaqueous, or organic. Solution, suspension, or slurry. Unlimited sample except for fugitive runoff.* |
| Solids | | | |
| *Piles, Conveyors, Bins, etc.* | *Grab or Composite* | *Untreated solids* | *Coal, ash, residues, products; organi and inorganic, unlimited sample.* |

continuous exposure for a specified period of time. The exposure period for the freshwater algal bioassay is 120 hours; that for the marine algal bioassay is 96 hours. The 96- or 120-hour effective concentration, ($EC_{50}$), the concentration in which algal growth is inhibited by 50 percent as compared with growth in the control, is statistically estimated. For samples which stimulate algal growth, the stimulatory concentration ($SC_{20}$) is calculated. The 96- or 120-hour $SC_{20}$ is defined as the concentration causing a stimulation in growth of 20 percent relative to the control after 96 or 120 hours of exposure. Other related criteria which may be useful are the effects on rates of growth, on maximum standing crops, and on algal biomass at the end of the assay.

It may also be possible to establish the approximate concentration of test material which produces no observable deleterious effect by any of the criteria under study, which is the No Observed Effect Concentration (NOEC).

Since the reporting for each test is unique, evaluation criteria given for each test in the Level 1 manual are used to rank the relative toxicity of test samples in the various tests.

The recommended test organisms in freshwater tests are the alga *Selenastrum capricornutum,* 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 study, and 48 hours for the daphnid study. Thus, the principal finding obtained from an algal study is the 120-hour $EC_{50}$ or $SC_{20}$; from the fish study, the 96-hour $LC_{50}$; and from the daphnid study, the 48-hour $EC_{50}$.

The suggested test organisms in marine tests are the alga *Skeletonema costatum,* the juvenile sheepshead minnow *Cypinodon variegatus,* and the adult mysid *Mysidopsis bahia.* The primary parameters of toxicity obtained from a marine algal study are the 96-hour $EC_{50}$ or $SC_{20}$; from the marine fish study, the 96-hour $LC_{50}$; and from the mysid study, the 96-hour $EC_{50}$.

The aquatic tests described in this section are well suited for Level 1 environmental assessment testin because they develop useful informatio quickly and at low cost. The characte istics of the freshwater and marin aquatic ecological effects tests ar summarized in Table 4. The six recom mended aquatic bioassays have bee routinely used by EPA and others t monitor the biological impact of effluen on the environment. There alreac exists a body of published material ar technical expertise which can assist the application and interpretation Level 1 aquatic testing. These tes measure the effect of a test material c organisms that represent three succe sively more complex trophic leve characteristic of either fresh or mari waters. The freshwater or mari battery of tests is selected, based on t type of receiving water into which t effluent is discharged. Their princi limitations are (1) that they usually not closely simulate the characteristi of the receiving waters into which t test effluent is actually being discharg and (2) that the species tested may r be representative of the most sensit

**Table 2.** *Test Sample/Bioassay Compatibilities*

| Sample Type | Health Effects Bioassays[a] | | | | Ecological Effects Bioassays[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Aquatic | Terrestrial Tests | | |
| | Ames | RAM | CHO | WAT | Tests[b] | PSE | RE | IT |
| 1. Gas/Vapor (Non-particulate) | B[c] | NC | NC | NC | NC | R | NC | B |
| 2. Liquids (<5% Solids) | | | | | | | | |
| A. Aqueous | R | A | R | R | R | B | R | R |
| B. Nonaqueous[d] | R | A | R | A | A | B | A | R |
| 3. Solids and Slurries (>5% Solids) | | | | | | | | |
| A. Soluble | R | A | R | R | R | B | R | R |
| B. Insoluble | R | R | A | R | R | B | R | R |
| C. SASS particulates | R | R | A | NC | NC | NC | NC | A |

[a]*Standard test abbreviations are:*
*Ames: Ames* Salmonella*/microsome mutagenesis assay.*
*RAM: Rabbit alveolar macrophage cytotoxicity assay.*
*CHO: Rodent Cell clonal toxicity assay.*
*WAT: Acute* in vivo *test in rodents (whole animal test).*
*PSE: Plant stress ethylene test.*
*RS: Root elongation test.*
*IT: Insect toxicity assay.*
[b]*Aquatic tests include marine or freshwater fish, invertebrate, and algal bioassays.*
[c]*Identification of compatibility abbreviations:*
*R: Recommended for Level 1 environmental assessment testing.*
*NC: Sample not compatible with test methodology.*
*A: Compatible with bioassay with no modifications to protocol. Not recommended for routine Level 1 testing, but may provide additional information.*
*B: Compatible with bioassay with modification to protocol. Not recommended for Level 1 testing.*
[d]*Nonaqueous liquids include samples with greater than 0.2 percent organics, solvent exchange samples, and sorbent resin extracts. Extracts must be solvent exchanged to dimethylsulfoxide (DMSO) for testing.*

**Table 3.** *Characteristics of Level 1 Health Effects Bioassays*

| Characteristic | Salmonella | Cytotoxicity Assays | | |
|---|---|---|---|---|
| | Mutagenesis | RAM | CHO | WAT |
| Cell Type/Organ System | Prokaryotic Cell-Enteric Bacteria Species | Eukaryotic-Primary Rabbit Macrophage Cells | Eukaryotic-Hamster Cell Line | Integrated Organ and Tissue Systems |
| End Point(s) Measured | Point Mutation | Lethality and Metabolic Impairment | Cell Lethality | Lethality-Toxic Signs |
| Amenable to Sample Types | Solids, Liquids, Particulates | Solids, Liquids, Particulates | Solids, Liquids, Particulates | Solids, Liquids, Particulates |
| Data Expression | Positive or Negative | $EC_{50}$ (Viability, ATP) | $EC_{50}$ (Clonal) | $LD_{50}$ or Toxic Signs |
| Special Features | Requires In Vitro Activation System to Detect Active Metabolites | Especially Effective for Particulate Samples Because Cells are Phagocytic | Detects Effects on Reproductive Capacity of Cells. Same Cells May Be Used For SCE Assay | Can Detect Complex Toxicological Phenomena that are Dependent on Interactions of Several Organ Systems |

*Table 4. Characteristics of Level 1 Aquatic Ecological Effects Bioassays*

| *Characteristic* | *Static Acute Aquatic Bioassay* | *Algal Bioassay* |
|---|---|---|
| *Freshwater Species* | *Fish — Fathead Minnow, Invertebrate* — Daphnia | Selenastrum |
| *Marine Species* | *Fish — Sheepshead Minnow, Invertebrate* — Mysidopsis | Skeletonema |
| *End Point(s) Measured* | *Lethality* | *Cell Population Growth* |
| *Amenable to Sample Types* | *Liquids, Solids (leachates)* | *Liquids, Solids (leachates)* |
| *Data Expression* | $LC_{50}$ | $EC_{50}$, $SC_{20}$ |
| *Special Features* | *Can detect whole-animal effects on key aquatic ecological consumers* | *Effective measure of toxicity to aquatic producers* |

species native to those waters. They do, however, make it possible to rank municipal and/or industrial effluents in order of relative toxicity.

## Level 1 Terrestrial Ecological Effects Assays

The Level 1 terrestrial ecological effects tests include assays for determining toxicity of complex wastes in plant and insect test organisms. The tests are able to detect sublethal toxic response to stress in plants (PSE test), sublethal and lethal toxic responses in germinating seeds (RE test), and acute toxicity and reproductive impairment in insects (IT test).

These tests provide a range of terrestrial organisms for assessing the effect of effluent streams on the environment. Test organisms include maturing plants, germinating seeds, and insects. This group of tests offers testing capabilities for all sample types (including gases) with the advantages of low cost, reproducibility, and relatively rapid performance time. The characteristics of the terrestrial ecological effects bioassays are summarized in Table 5. A future goal for this manual is to include a test procedure for assessing the impact of effluent samples on soil microorganisms (decomposers).

Terrestrial ecological tests are used to determine the concentration of test material that produces a defined toxic effect on a specified percentage of the test organisms in a fixed amount of time. The plant-stress-ethylene (PSE) test is designed to assess and rank the toxic effects of gaseous effluents on plants by measuring the stress ethylene of plant response and by assessing relative foliar injury in exposed plants. The root-elongation (RE) test measures the inhibition of root elongation and seed germination. Although both parameters are observable toxic responses and are reported, root-elongation inhibition is the preferred end point. The concentration which inhibits root elongation by 50 percent of the control ($EC_{50}$) is estimated and used to rank effluent samples. The insect-toxicity assay measures the acute toxicity and reproductive capacity of fruit flies treated with environmental samples. The dose lethal to 50 percent of the flies ($LD_{50}$) compared to the control is calculated and used to rank test samples. In the optional fertility test, the effective concentration which reduces the fecundity of surviving dosed flies to 50 percent of control flies ($EC_{50}$) is calculated.

The Level 1 terrestrial tests represent the state of the art for environmental assessment for terrestrial ecological effects. These tests have not been as thoroughly validated with complex environmental mixtures as have the health and aquatic ecological effects tests.

## Level 1 Data Formatting and Analysis

Procedures are described in the Level 1 manual so that data from all Level 1 bioassays can be organized into a uniform evaluation format to aid in the use and interpretation of the data. This format is structured so that data can be converted from the conventional bioassay output into four levels of response: Nondetectable (ND), Low (L); Moderate (M), and High (H).

To ensure uniform data recording and translation of raw data into the final summarized form, standard data recording and data transition forms have been developed [3,4]. Data transition forms are used in sequence for data summary and analysis. The critical data values determined for each assay (MEC, $EC_{50}$, $LD_{50}$, or $LC_{50}$) are recorded on critical data summary forms. Samples of health effects and aquatic ecological effects critical data summary forms are presented. Test materials are then ranked based on the critical data values reported in the summary forms. Evaluation criteria are presented for each test. The ranking of the level of response of a sample in each test is then recorded on the bioassay summary table presented in the manual.

## Level 1 Quality Control and Quality Assurance Requirements

If Level 1 assessments are to be used as a basis for decisions regarding further bioassay assessment, it will be necessary to ensure the quality of the test data. Quality control standardization and standardized data documentation will contribute significantly to ensurance of test quality and reproducibility.

A separate set of documents outlining recommended quality control and quality assurance procedures has been prepared and is available as a guide for laboratories conducting Level 1 bioassays. The procedures described are consistent with the intent and requirements of the U.S. Food and Drug Administration's (FDA's) Good Laboratory Practice (GLP) Regulations[5]. These guides outline the basic steps involved in the Level 1 procedures and provide sample quality control recording forms for sample data collecting. The quality control/quality assurance documents will be especially helpful to laboratories beginning to conduct Level 1 testing

completeness based on the suggestions in both the Level 1 manual and the quality control and quality assurance manual.

In order to ensure the quality of test results from biological laboratories involved in the environmental assessment program, audit samples have been made available, either as blind samples during analysis of assessment samples, or separately as coded unknown samples. Coded laboratory quality assurance samples have been prepared to submit to laboratories wishing to ensure Level 1 testing proficiency.

In addition to audit samples, the quality control and quality assurance manual is also designed to define the level of documentation required to comply with the proposed FDA GLP regulations. From time to time it will be necessary to review raw data and final bioassay reports for consistency and

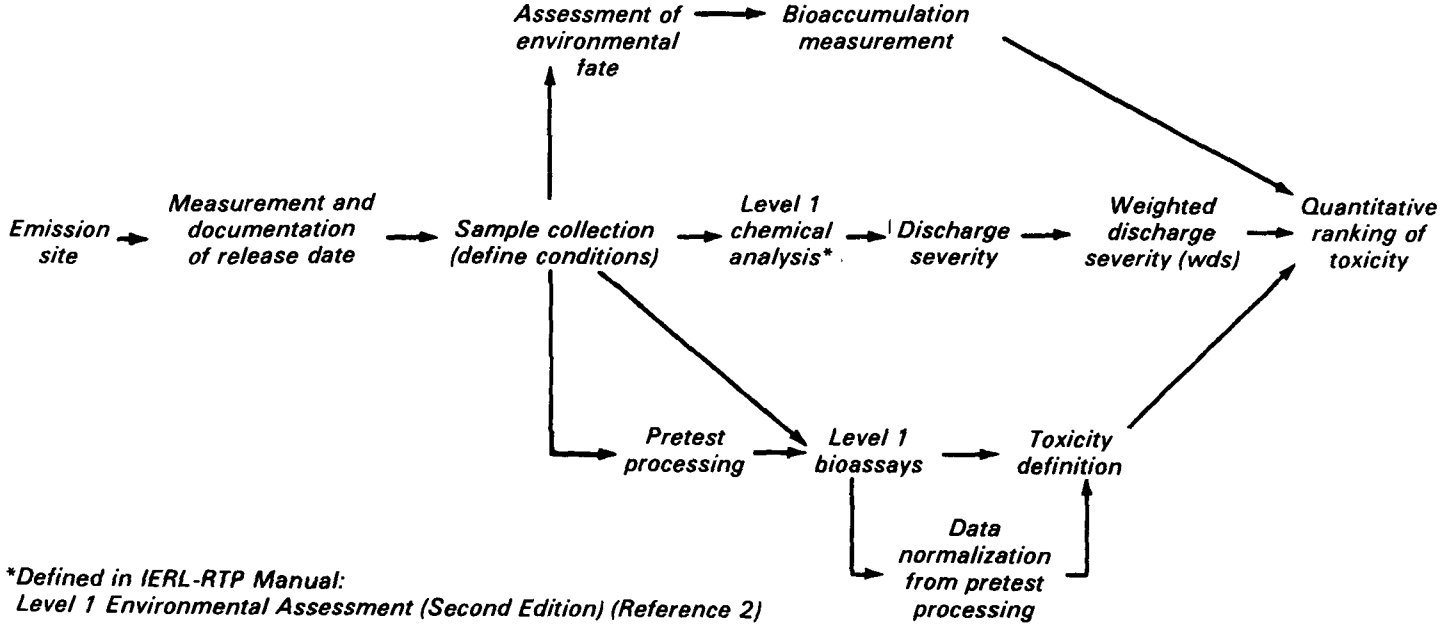
## Environmental Assessment Beyond Level 1

Level 1 environmental assessment should provide an accurate ranking of emissions of stationary sources with respect to their potential toxicity. Moreover, the ranking should ensure that the toxicity is from the emissions as released into the environment. Level 1 assessment should also generate information concerning rate of effluent discharge into the environment and environmental fate of the emission.

A composite of summarized bioassay and chemical analysis data will provide a measure of toxicity and the potential for damage to the environment. This concept is outlined in Figure 2.

***Table 5.*** *Characteristics of Level 1 Terrestrial Ecological Effects Bioassays*

| *Characteristic* | *Plant Stress Ethylene Test* | *Root Elongation Test* | *Insect Toxicity Test* |
|---|---|---|---|
| *Species* | *Bush Bean* | *Cucumber, Wheat, Red Clover, Radish, Lettuce* | Drosophila melanogaster |
| *End Point(s) Measured* | *Metabolic stress evidenced by ethylene production* | *Root length* | *Lethality, Reproductive capacity* |
| *Amenable to Sample Types* | *Gases, Liquids* | *Liquids, Solids (leachates)* | *Liquids, Solids* |
| *Data Expression* | *Positive or Negative* | $EC_{50}$ | $LD_{50}$ |
| *Special Features* | *Only validated Level 1 Bioassay for gases; sensitive* | *Detects toxicity to terrestrial producers; multiple species* | *Detects lethality to terrestrial consumer plus can provide data on fertility* |




*Defined in IERL-RTP Manual: Level 1 Environmental Assessment (Second Edition) (Reference 2)

***Figure 2.*** *Proposed scheme for a second stage evaluation of Level 1 results.*

## References


1. Duke, K.M., David, M.E., and Dennis, A.J. "IERL-RTP Procedures Manual: Level 1 Environmental Assessment, Biological Tests for Pilot Studies," EPA-600/7-77-043 (NTIS PB 268484), Battelle-Columbus Laboratories, Columbus, OH, April 1977,114 pp.
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5. DHEW Food and Drug Administration. "Nonclinical Laboratory Studies Good Laboratory Practice Regulations," Fed. Regist. Dec. 22, Part I 1978, pp. 59986-60020.



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*The complete report, entitled "IERL-RTP Procedures Manual: Level 1 Environmental Assessment, Biological Tests," (Order No. PB 82-228 966; Cost: $13.50, subject to change) will be available only from:*

*National Technical Information Service*
*5285 Port Royal Road*
*Springfield, VA 22161*
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