Review and Evaluation of Microtox® Test for Freshwater Sediments

by

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GLOSSARY

Endpoints

Endpoint: An endpoint is a value that is calculated from the test data and demonstrates a specified effect. Several endpoints used in the Microtox® test are discussed below.

Effective Concentration 50% (EC_{50}): The EC_{50} is the sample concentration that reduces the reagent light output by 50%. The fact that an EC_{50} can be calculated presupposes a dose response relationship between sample and light production, but does not specify sample toxicity.

Effective Concentration 20% (EC_{20}): The EC_{20} is calculated similarly to the EC_{50} except that it uses a 20% reduction in reagent light output.

Percent Light Reduction: Percent reduction in light output of the highest concentration of a sample/extract as compared to that of a control or reference sample.

Other Terms

Control: A nontoxic reagent blank used to compensate for changes in test response.

Diluent: any solution used to dilute or reduce the sample concentration. Saline diluent refers to a toxicity-free, 2% NaCl solution prepared by Microbics and used to maintain osmotic balance.

Elutriate: Elutriate is a type of sediment extract. It is prepared by adding four parts saline diluent or distilled water to one part sediment, mixing and decanting. The Microtox® Manual uses the synonymous term, "eluate".

Gamma: the ratio of light lost to light remaining after the bacteria and reagent are challenged by a sample. The concentration that produces an EC_{50} has a gamma value of one.

Hit: A test result can be termed a "hit" when it is shown to be different from a reference or control sediment according to a preselected requirement. Sediment that produces a hit will most likely cause some form of significant biological impact.

Hormesis: a stimulatory effect caused by low levels of potentially toxic agents (Kwan and Dutka, 1990), and may produce a light output greater than the control. Samples showing hormesis are currently considered nontoxic. Microbics is developing software that can evaluate hormesis data. See Negative Gamma.

Inhibitory Concentration (IC): The term Inhibitory Concentration (IC) is sometimes used instead of Effective Concentration. For Microtox® purposes, the terms are synonymous.

Microtox® Reagent: a freeze-dried culture of the test organism, *Photobacterium phosphoreum*. Microtox® Reagent is reconstituted before use in the Microtox® Test.

MOAS: Microbics prepares nontoxic 22% Microtox® Osmotic Adjustment Solution (MOAS) to adjust sample salinity. MOAS is added to the sample in a ratio of 1:10.

Negative Gamma: A negative gamma occurs when the sample produces light output that is higher than that produced by a control. They may result from the stimulatory effects of certain chemicals at concentrations just below toxic levels. Negative gammas are not used in computations. See Hormesis.

Pore Water: the fluid surrounding sediment particles. Pore water contaminants are in dynamic equilibrium with contaminants associated with the solid phase (Giesy and Hoke, 1990). Contaminant concentrations in pore water are generally higher than those found in overlying water. Pore water can be separated from the sediment by centrifuging. The term is synonymous with interstitial water.

Reagent Solution: Reagent solution is produced by the reconstitution of Microtox® Reagent. The solution contains *P. phosphoreum* bacteria whose light production, which may vary according to contaminant level, gives a measurement of toxicity.

Reference Sediment: Sediment with characteristics similar to the test sediment (total organic carbon, grain size, etc.) collected from an area with low contaminant levels.

ABSTRACT

As part of a project to define sediment criteria, The Washington State Department of Ecology is evaluating several bioassays for use on freshwater sediments. This report reviews the several variations in methods of Microtox® sediment bioassay, the current uses of Microtox® sediment bioassays by different agencies, and recommendations for its use in testing freshwater sediments.

Microtox® is a relatively simple and inexpensive bioassay that indicates toxicity through a reduction in light output of the luminescent bacterium, *Photobacterium phosphoreum*. There are currently three Microtox® tests available for sediment testing. The Basic Test and the 100% Test detect contaminants that have been extracted into an aqueous phase. The Solid-Phase Test can detect both aqueous phase contaminants and those bound to sediment.

The Basic Test is the only test that is precise enough to use for the development of sediment criteria. The choice of which aqueous phase to use (pore water, saline elutriate, distilled water elutriate or organic extract), depends mainly on project goals and the contaminants of interest. The 100% Test can be used with aqueous samples of lower toxicity, while the Solid-Phase Test is useful for determining potential toxicity to burrowing organisms where the main route of contaminant exposure is through ingestion.

Microtox® data can be analyzed by calculating EC_{50} (Effective Concentration 50%) values based on dose response curves. For sediment criteria development purposes, the determination of a "hit" based on the Sediment Management Standards ("one-hit no adverse effects" and "two-hit minor adverse effects" approaches) and a 20% light reduction from reference, is preferred.

Microtox® can be used separately to screen many samples. However, it is most appropriate for inclusion in a "battery of tests" where sediment is evaluated based on the results of several different bioassays.

BACKGROUND

The Washington State Department of Ecology is evaluating bioassays as part of its Freshwater Sediment Criteria Development Project (FSCDP). The goal of this project is to derive thresholds or criteria values based on contaminant concentrations in sediments that will define the likelihood of biological harm to aquatic organisms. Bioassays are frequently used as the basis of criteria development. Most bioassays examined for freshwater sediment analysis are based on changes in arthropod growth or mortality. The FSCDP selected the bacteria-based bioassay, Microtox®, for further study because of its ease of use, low cost and potential high sensitivity (Bennett and Cubbage, 1992a). This test is based on measures of the inhibition of light production from luminescent bacteria challenged with sediment or extract from sediment.

The FSCDP has tested several bioassays including Microtox® at a variety of contaminated sites. Although potentially useful as one of several bioassays in a "battery of tests" (sediments are tested with several different bioassays), Microtox® tests have provided inconsistent results in recent marine bioassay comparisons in Puget Sound. At some sites with high concentrations of creosote, one Microtox® test showed a dose-response relationship, while a second Microtox® test showed no relationship (Bennett and Cubbage, 1992b). Microtox® tests of samples from Lake Union, an area with high concentrations of metals and organics, showed some toxicity throughout the lake. The arthropod tests (*Daphnia magna* and *Hyalella azteca*) showed toxicity at only two of 20 sites (Cubbage, 1992). Microtox® did not indicate toxicity in Steilacoom Lake sediments despite copper concentrations exceeding 1,000 ppm dry weight (Bennett and Cubbage, 1992c). EC_{50} values indicated sediment toxicity for two sites in Lake Roosevelt that had high cadmium concentrations (Johnson, 1991).

To help understand the advantages and disadvantages of the available Microtox® tests, we reviewed the different Microtox® tests and their interpretations and uses by other agencies. The major objectives of this review were as follows:

- Determine the different Microtox® tests available for sediment testing and review the advantages and disadvantages of each test.
- Review the current uses of Microtox® by agencies and universities in evaluating both marine and freshwater sediments for toxicity.
- Determine if a controlled experimental comparison among the different types of Microtox® tests could further evaluate the methods.
- If deemed useful, recommend the most appropriate Microtox® test methods for inclusion in the FSCDP.

MICROTOX® TEST DESCRIPTIONS

The Microtox® Test is based on changes in light production of the marine bioluminescent bacterium *Photobacterium phosphoreum*. Toxicity reduces enzyme activity and thus reduces light output. The test can be quite sensitive and can detect low concentrations of some toxicants. Three major Microtox® tests have been used for sediment testing. These are the Basic Test, the 100% Test and the Solid-Phase Test. All these versions follow the same general method of reconstituting preserved and dried bacteria, or reagent, in specially prepared distilled water (reconstitution solution). The combination of bacteria and reconstitution solution produces the reagent solution. Two percent saline diluent may be used to dilute the reagent solution and sample as needed. The bacteria are then exposed to sediment or sediment extracts depending upon the test version used, and the light output is measured on a photometer after five and 15 minutes from first contact with the test solution. Test solutions are osmotically balanced to match seawater.

Light output of serial dilutions of the test solutions form the basis for a dose-response curve. If a dose-response relationship exists between the concentration of test solution and light output, then the concentration that reduces the light output by 50% over the control is reported as the EC_{50} (Effective Concentration for 50% light reduction). The Basic Test and the 100% Test differ primarily in the concentration of the dilutions tested. The Solid-Phase Test is based on contact with the sediment instead of contact with elutriates or pore water like the Basic Test and the 100% Test. These tests are described in detail below.

Sample Preparation

Microtox® tests differ primarily in the way the sediment is prepared for contact with the organism. Sediment elutriates may be prepared through extraction with distilled water, saline water, or an organic solvent. In addition, the water within the sediment sample (termed pore water) can be centrifuged and tested without extraction. Preparation for the Solid-Phase Test is minimal and allows the Microtox®bacteria to contact the sediments directly. The different preparation methods and steps are shown in Figure 1.

Pore Water Preparation

Pore water is prepared by centrifuging the test sediment. If a sediment cannot produce enough pore water, then elutriate should be used. Sediment evaluation through pore water analysis is of value because contaminants have had time to reach equilibrium with the water. Studies indicate that pore water is the main route of contaminant exposure to aquatic organisms (Giesy *et al.*, 1990).

Elutriate Preparation, including Saline Extract

This procedure extracts one part sediment with four parts saline diluent or distilled water. The slurry is mixed end-over-end for 48 hours, and the heavier material allowed to settle

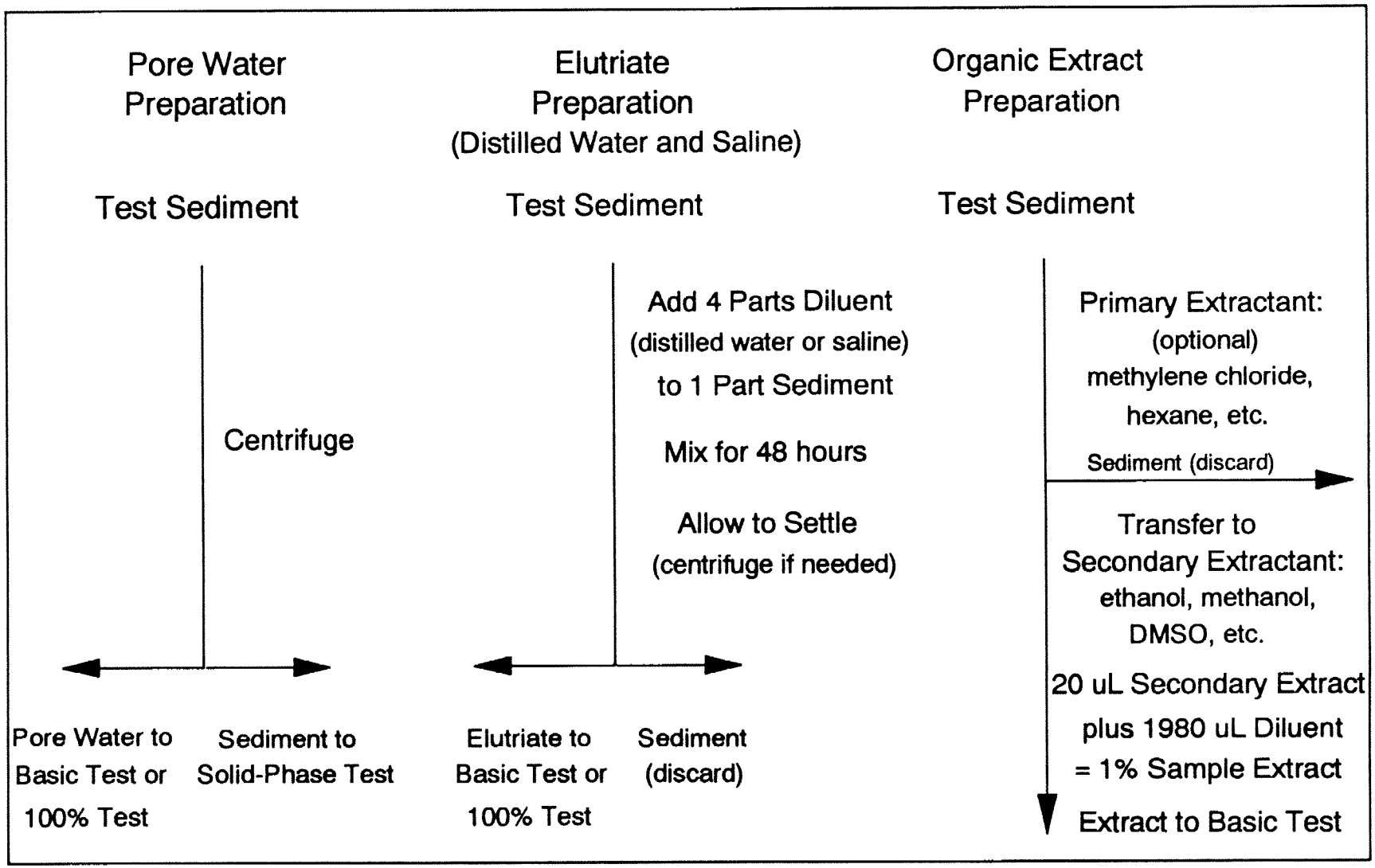

Figure 1. Microtox sample preparation.

out. Sediment is not dried prior to preparation, although it is recommended that the EC_{50} (or other endpoint) be adjusted to dry weight. (A separate aliquot of test sediment must be analyzed for percent solids.) It may be necessary to centrifuge the elutriate, measure and adjust pH, make water quality measurements, remove turbidity and make color corrections.

When the diluent used is the 2% NaCl solution prepared by Microbics, the resulting elutriate is called saline extract. For fresh water sediments the diluent is usually distilled water, and the elutriate must be osmotically adjusted to protect the *P. phosphoreum*. Elutriate testing is especially applicable for determining the level of water contamination resulting from the open water disposal of dredged or similar material.

Organic Solvent Extraction

The organic solvent extraction (solvent extraction) protocol is recommended for liquid samples that are insoluble in water. It is also recommended for sediment samples containing a large amount of oil or tar (Microbics, 1992). The protocol is designed to make available, to the test organism, the nonvolatile, neutral, nonionic organic compounds that are soluble in the organic solvent. Microbics makes no recommendations regarding the sediment to solvent ratio. Solvent extraction can expose the test organism to higher contaminant concentrations than would occur under natural conditions.

Organic solvents recommended by Microbics Corporation are 100% Ethanol (nondenatured), 100% Methanol, and 100% Di-methyl sulfoxide (DMSO). Giesy *et al.* (1988) mention one study that used a methanol-dichloromethane mixture as the primary extractant. The sediment extracts were then solvent-exchanged into ethanol. Kwan and Dutka (1990) found that methanol was more efficient than DMSO in extracting toxicants. It also produced the greatest number of "positive" results, thereby indicating the presence of toxicants. Sometimes hexane or methylene chloride are used as an initial extractant. The contaminants are then transferred into a less toxic solvent, such as ethanol, before testing (Casillas, 1992). Most solvents are potentially toxic to *P. phosphoreum*. Therefore, they should be diluted so that the solvent concentration does not exceed 1%. The selection of an appropriate extraction solvent depends primarily on its ability to solubilize the contaminants of interest.

Microtox® Test Methods

Each of the three different methods can be used to analyze light production. Figure 2 shows the overall steps in sample dilutions among the three methods. Once the dilutions are made, the Microtox® bacteria are added to each dilution, and the light output is measured. Curve fitting routines such as probit analysis or hand drawn curves are used to determine the EC_{50}.

Basic Test

The Basic Test is used to test water or sediment extracts having a high level of toxicity. Saline diluent and osmotic adjustment solutions are used for sample dilution and to maintain

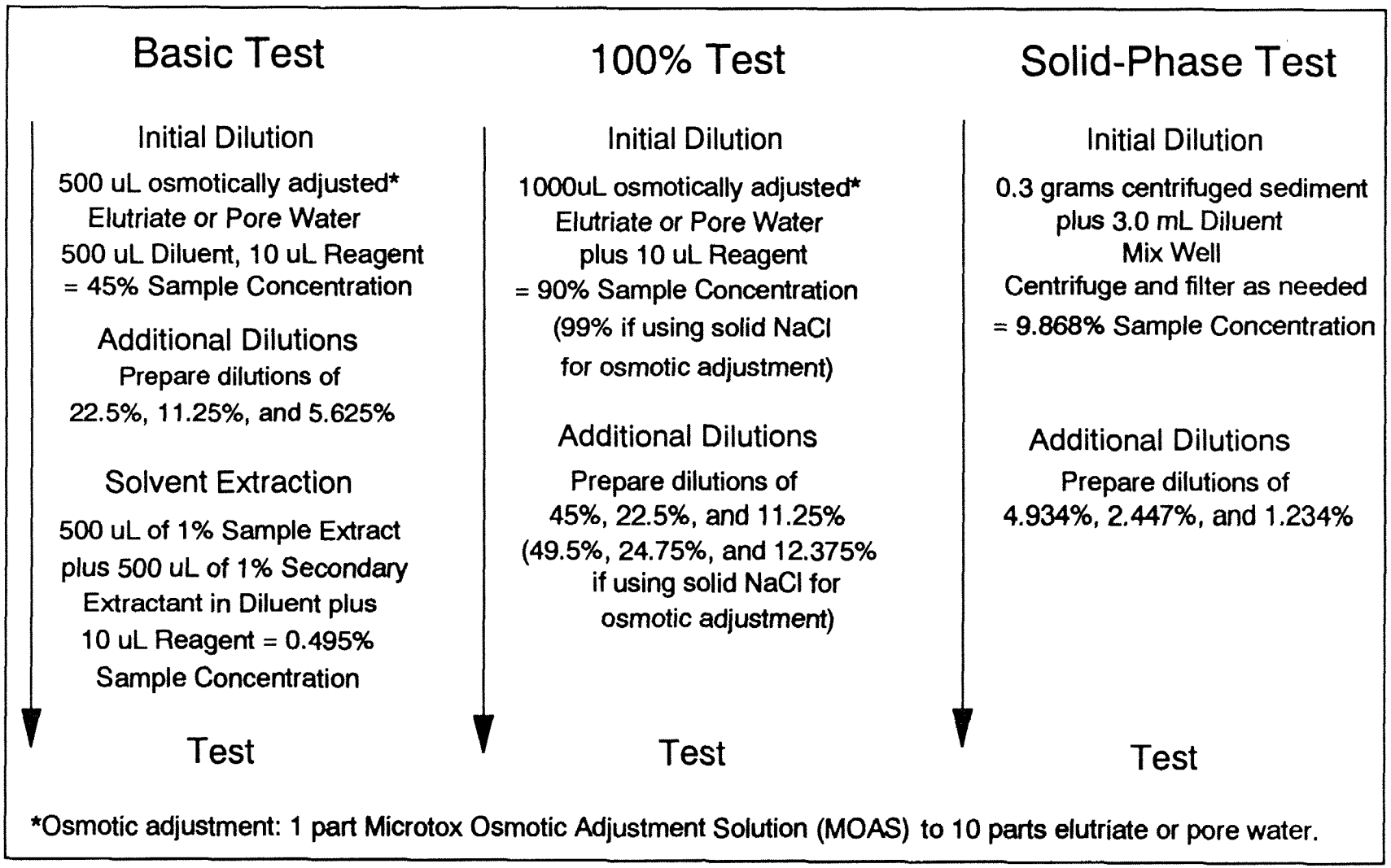

Figure 2. Microtox dilution preparation. Light output is measured after Microtox solution is added to each dilution.

osmotic balance. Osmotically adjusted sample and diluent are mixed in a 1:1 ratio. The maximum elutriate (or extract) concentration that can be tested is about 45% (Tarkpea and Hansson, 1989). The Basic Test can be used to evaluate pore water, elutriate and organic extract.

100% Test

The 100% Test is used for evaluating pore water and sediment extracts having a low or unknown level of toxicity. In this test, reagent solution is added directly to the pore water or extract. The maximum pore water/elutriate/extract concentration that can be tested is 90-99%, about double that of the Basic Test. The 100% Test is generally used as an environmental screening tool. It is more sensitive to operator technique, such as pipetting errors, than the Basic Test and precision may be lower. Certain variations in the 100% Test Protocols can be used to increase the confidence in the test data (Microbics, 1992).

Tarkpea and Hansson (1989) compared the Basic Test and the 100% Test using assorted effluents. Confidence intervals for the 100% Test averaged 10.4 times broader than for the Basic Test. They concluded that an EC_{50} value determined by the 100% Test is not very exact, although it does indicate the magnitude of toxicity. Also, EC_{50} values (normalized to sample dry weight) from the Basic Test were generally lower (indicating a more toxic sample) than EC_{50} values from the 100% Test. Therefore, the Basic Test may be more sensitive.

Solid-Phase Test

The Solid-Phase Test allows Microtox® organisms to directly contact and interact with sediment-bound toxicants in an aqueous suspension of test sample. It therefore allows the detection of soluble and insoluble organic and inorganic toxic materials. The Solid-Phase Test provides an exposure route that is not always available with pore water and elutriate (Tung *et al.*, 1990).

In the Solid-Phase Test, the sediment is first centrifuged to separate the solids from the pore water. Solids are then mixed to restore homogeneity, diluent and reagent solution are added, and the sample is filtered and analyzed (Microbics, 1992). The color correction protocol adjusts for turbidity. Relative toxicity expressed as EC_{50} values can be compared to control sediments. Test procedures may require modification because of variations in site characteristics. Sediment toxicity can change with time, so samples should be processed as quickly as possible. Reference sediments may be used to produce baseline data against which test sediments are compared. During a project, aliquots are taken from the reference samples and analyzed concurrently with the test sediments. To maintain data quality, reference sediments that remain stable over extended periods of time should be selected (Brouwer *et al.*, 1990).

Tung *et al.* (1990) compared the Solid-Phase Test to the Basic Test, using both water and solvent extractions of spiked soil samples containing inorganic and organic components. The Solid-Phase Test produced lower EC_{50} values, demonstrating greater sensitivity than the Basic Test using sample extracts.

Brouwer *et al.* (1990) tested both the Solid-Phase Test and the Basic Test, using aqueous elutriates, on sediment spiked with either zinc chloride or polychlorinated biphenyl congener 194 (PCB-194). Light output for all tests was compared to the control. Both test methods gave virtually identical results for zinc chloride, with the light output decreasing to zero at a spiked-sediment concentration of approximately 0.024 mg $ZnCl_2$/g. For the PCB-194, light output in the Basic Test remained nearly unchanged even with 100% spiked sediment. In the Solid-Phase Test, the light output declined with increasing PCB-194 spiked sediment concentration. Brouwer *et al.* (1990) conclude that the Solid-Phase Test has the advantage of measuring the toxicity of the entire sediment. This is important if the sediment contains hydrophobic substances such as PCBs, polyaromatic hydrocarbons and other contaminants that have low water solubility. The Solid-Phase Test is apparently more sensitive to hydrophobic contaminants than a test using elutriates.

The Solid-Phase Test is best suited for ranking multiple samples and identifying toxicity hot spots (Microbics, 1992). The test protocol is being modified to emphasize sediment retrieval and handling, an increase in sample size (the current use of 0.3 g of sediment does not provide a homogeneous sample), and changes in data collection and analysis. With these modifications, the Solid-Phase Test will provide a more definitive quantification of toxicity, comparable to that of the Basic and the 100% Tests (Microbics, 1992). The revised protocol will be submitted to the American Society for Testing and Materials (ASTM) later this year as a generic method for sediment toxicity testing (Evereklian, 1992).

MICROTOX® APPLICATIONS

The following are reviews of Microtox® procedures used by different organizations and programs. They have been broken down into specific categories as follows:

Program Contact: Specific program, agency or entity that uses the test
Use: Purpose of using the Microtox® Test
Media Tested: Primarily marine or freshwater sediments
Contaminants: Chemicals for which the toxicity is being tested
Preparation Method: Main preparation method
Test Method: The Microtox® test used primarily
Endpoint: The endpoint used to report results
Hit: The definition of a significant toxic effect
Comments: Additional information on the test, how it is used, results, recommendations, etc.

Note that opinions and results in some reviews may differ from those in others. These discrepancies reflect the fact that Microtox® tests are used under many different conditions, that test procedures are often modified, results are subject to various interpretations, and toxicity testing with Microtox® is an inexact science.

The following regional, national and other programs use Microtox® as part of their sediment toxicity testing program.

Regional Programs

Puget Sound Estuary Program (PSEP) Protocols

Program Contact: Lead EPA Region X (multiagency group)
Use: Evaluate marine sediments
Media Tested: Marine sediment
Contaminants: Metals, organics
Preparation Method: Saline elutriate and organic extract
Test Method: Basic Test, which may be modified to give a higher sample concentration
Endpoint: Fifteen minute EC_{50} values are calculated for each test series and controls using linear regression analyses of the log gammas according to the Microbics statistical package. Ninety-five percent confidence intervals are calculated using a statistical procedure based on Fieller's Theorem.
Hit: Not defined
Comments: The organic extraction procedure is specific for neutral, nonionic organic compounds such as aromatic and chlorinated hydrocarbons. It does not efficiently extract metals or highly acidic and basic organics. The saline extract removes only the water-soluble fraction of sediment-adsorbed trace metals and organic pollutants from the sediments. It will not extract compounds like PCBs that have extremely low water solubility (PSEP, 1987).

Puget Sound Dredged Disposal Analysis (PSDDA)

Program Contact: United States Army Corps of Engineers - Seattle District
Use: Determining suitability of dredged sediments for open-water disposal
Media Tested: Puget Sound dredged materials
Contaminants Tested: Metals, PAH, organics
Preparation Method: Saline elutriate
Test Method: Basic Test
Endpoint: Same as WAC 173-204
Hit: Statistically significant difference between the test sediment and reference sediment responses that is greater than 20%
Comments: A decrease in the number of hits reported by Microtox® over the past three years, and the frequent occurrence of light enhancement, are phenomena under review by

the PSDDA agencies (Fox, 1992). Quality control data confirm that the problem is not the result of a decline in organism sensitivity.

Ecology Sediment Management Standards, Washington Administrative Code (WAC) 173-204

Program Contact: Washington State Department of Ecology - Sediment Management Unit
Use: Mandate Washington State Sediment Management Standards
Media Tested: Puget Sound marine sediments
Contaminants Tested: Metals, PAH, organics
Preparation Method: Saline elutriate
Test Method: Basic Test
Endpoint: Decreased luminescence from the bacterium *P. phosphoreum* after a 15-minute exposure at the highest concentration in the Basic Test
Hit: According to the Puget Sound Marine Sediment Quality Standards (no adverse effects) WAC 173-204-320(3)(e), a hit is when "Sediments are determined to have adverse effects on biological resources when ... the mean light output of the highest concentration of the test sediment is less than 80% of the mean light output of the reference sediment, and the two means are statistically different from each other (t-test, $p \leq 0.05$)."

WAC 173-204-420(3)(c) and 520(3)(d) define a minor adverse effects hit as being when "any two of the biological tests in any combination exceed the criteria of WAC 173-204-320(3)."

Comments: Microtox® is used to establish a sediment quality standards level (*i.e.*, a goal based on a "no adverse biological effects" decision) and to confirm when concentrations exceed regulatory levels. Source control and cleanup sediment quality decisions are based on a "minor adverse biological effects" decision point. The Sediment Management Standards are federally approved water quality standards for Washington State. Therefore, National Pollutant Discharge and Elimination System (NPDES) permits, and Superfund cleanup levels, utilize Microtox® endpoints specified by the Sediment Management Standards.

Ecology/EPA Manchester Environmental Laboratory

Program Contact: Ecology/USEPA Region X
Use: Sediment evaluation
Media Tested: Marine and freshwater sediments
Contaminants Tested: Metals, organics, conventionals
Preparation Method: Saline elutriate for marine sediments, deionized water elutriate for freshwater sediments
Test Method: PSEP protocol for marine samples, modified PSEP protocol (use of deionized water) for freshwater samples
Endpoint: EC_{50} values calculated using the Microbics statistical package.
Hit: Not defined

Comments: In side-by-side tests, elutriate sometimes shows toxicity not indicated by pore water (Stinson, 1992). This comment agrees with the observation that "elutriates are often more toxic than pore waters" (Giesy and Hoke, 1989).

USEPA Region X - Aquatic Resources, Wetlands and Sediments Section

Program Contact: USEPA Region X
Use: Sediment evaluation
Media Tested: Marine and freshwater sediments
Contaminants Tested: Metals, organics, PAH
Preparation Method: Saline elutriate and organic extract
Test Method: Basic Test
Endpoint: Same as PSDDA
Hit: Same as PSDDA
Comments: EPA Region X uses PSEP protocols in general, and the PSEP protocols as amended by the PSDDA Program. For several years, marine sediment data have included negative gammas (light production increases when the bacteria are challenged), which are not currently interpreted in regulatory programs due to lack of interpretive criteria. Where there is light enhancement, sediment toxicity is sometimes indicated using other bioassays. EPA Region X, along with PSDDA agencies, is devoting resources to exploring continued use of the Microtox® Test and will also be considering the new Microtox® Solid-Phase bioassay procedures for use in the PSDDA Program.

Lake Roosevelt Project

Program Contact: U.S. Geological Survey, Tacoma, WA
Use: Investigate metal contamination in Lake Roosevelt
Media Tested: Lake sediments
Contaminants Tested: Metals
Preparation Method: Sediment, pore water
Test Method: Solid-Phase Test for sediments, either Basic Test or 100% Test for pore water
Endpoint: Not yet determined
Hit: Not yet determined.
Comments: The U.S. Geological Survey will be analyzing Lake Roosevelt sediments for metals and organics. Bioassays will include *Hyalella azteca*, *Ceriodaphnia dubia*, and Microtox®. The Solid-Phase Test will be used to analyze the sediment, and either the Basic Test or the 100% Test will be used to analyze pore water (Bortleson, 1992). The reason for using two tests is that sediments can be a primary source of contaminants for fish and other bottom feeders, whereas pore water may be the best way to indicate metals contamination. Microtox® was chosen because Johnson (1991) reported that EC_{50} values correlated with Lake Roosevelt sediment cadmium concentrations.

Commercial Laboratory

Program Contact: Parametrix, Inc. - Bellevue, WA
Use: Sediment analysis
Media Tested: Marine and freshwater sediments
Contaminants Tested: Metals, organics
Preparation Method: Pore water, saline and distilled water elutriate, organic extract
Test Method: Basic Test
Endpoint: Endpoint is project specific.
Hit: Hits are specified according to PSDDA and the Washington Sediment Management Standards.
Comments: Parametrix has used organic extractions for marine sediments and upland waste sites. The initial extractant was methylene chloride with a secondary extraction into 100% ethanol. Parametrix expressed concern that organic solvents could extract toxicants that are not readily bioavailable, the result being an indication of toxicity that is not environmentally relevant. They believe that enzymes may be more moderate extractants for organics. Parametrix has also considered using a selective extractant for metals such as dilute HCl. They suggest that mixing samples by tumbling is preferred to rapid shaking by machine, which might not mobilize the contents sufficiently.

National Programs and Others

Great Lakes National Program Office (GLNPO), USEPA Region V

Program Contact: Great Lakes Assessment and Remediation of Contaminated Sediments Program (ARCS)
Use: Biological assessment of contaminated sediments prior to remediation
Media Tested: Great Lakes sediment
Contaminants Tested: Non-specific contaminants
Preparation Method: Saline elutriate
Test Method: Basic Test and 100% Test
Endpoint: EC_{50} values calculated using the Microbics statistical package.
Hit: Not defined
Comments: Initial toxicity screening of sediment samples was done by testing elutriate with the Basic Test (for samples with high toxicity) or the 100% Test (for samples with lower toxicity). Elutriate worked well for indicating toxicity at highly contaminated sites, but was less successful at marginally contaminated sites. Experience at other sites shows that, for marginally contaminated sediments, pore water and the 100% Test provide a more sensitive test than elutriate because pore water is not diluted. Elutriate from sediments that had been stored for over a year showed no substantial change in toxicity (Ingersoll, 1992).

National Oceanic and Atmospheric Administration (NOAA)

Program Contact: National Marine Fisheries Service, Environmental Conservation Division
Use: Evaluate marine sediments
Media Tested: Marine sediments
Contaminants Tested: Metals, organics
Preparation Method: Saline elutriate and organic extract
Test Method: Basic Test
Endpoint: EC_{50} values calculated using the Microbics statistical package.
Hit: There is no specification for a hit.
Comments: Much of the Division's work has involved organic extraction techniques. The sediment sample is first extracted with methylene chloride. The contaminants are transferred into ethanol, which is then diluted to about 1% concentration before testing.

Organic extracts are more sensitive than saline extracts. A comparison of the two methods on 33 sediments produced an EC_{50} value for each solvent extracted sample, but only seven EC_{50} values resulted from the saline extract (Casillas, 1992).

The Division plans to use other sediment bioassays to calibrate the Microtox® tests. Microtox® tests will be run side-by-side with other tests, such as the amphipod bioassay, to try to establish a relationship between EC_{50} values and other bioassay responses. If a relationship can be established, they hope to use subsequent Microtox® data to predict the most probable toxicity response.

They have not yet tried the Solid-Phase Test. However, they believe it could be a very useful test and that both it, and solvent extraction, would produce the most valuable data. Microtox® is appropriate as one part of a battery of tests.

University of Minnesota

Program Contact: Minnesota Cooperative Fish and Wildlife Research Unit
Use: Screening sediment pore water samples of unknown toxicity
Media Tested: Freshwater system sediment associated pore water
Contaminants Tested: Metals, organics
Preparation Method: Centrifuged sediment pore water
Test Method: Prefer 100% Test. If samples show high toxicity, they use the Basic Test.
Endpoint: EC_{50} values calculated using the Microbics statistical package.
Hit: The Unit uses the following series of toxicity ranges based on five and 15 minute Microtox® EC_{50} values (Henry and Jaschke, 1992). Brouwer *et al.* (1990) report the use of a similar scale.

Microtox® EC_{50}	Toxicity Level
0 - 19	Extremely Toxic
20 - 39	Very Toxic
40 - 59	Toxic
60 - 79	Moderately Toxic
80 - 99	Slightly Toxic
>100	Nontoxic

Comments: They report that Microtox® has shown greater sensitivity than either a *Chironomus tentans* 48-hour static sediment test or a *Daphnia magna* 48-hour static pore water test, both having percent mortality as endpoints. Analyses using the Solid-Phase Test have sometimes shown false positives because of interference from turbidity. This problem was most severe with sediment samples having a high clay content.

University of Texas and the U.S. Fish and Wildlife Service

Program Contact: Joint effort between the University of Texas School of Public Health and the U.S. Fish and Wildlife Service
Use: Sediment toxicity evaluation
Media Tested: Marine and estuarine sediments
Contaminants: Metals, organics, pesticides, PCBs, dioxins
Preparation Method: Sediment, pore water
Test Method: Solid-Phase Test and 100% Test
Endpoint: EC_{50} values calculated using the Microbics statistical package.
Hit: Not yet determined
Comments: Sediments were from marine and estuarine environments in Galveston Bay, Texas. Sediment analyses using the Solid-Phase Test have given EC_{50} values indicating a broad range of toxicity. Of approximately 40 sediments tested, an EC_{50} value could be determined for each sample using the Solid-Phase Test. No toxicity has been indicated with any of the pore water samples, implying a superior ability of the Solid-Phase Test at indicating the toxicity of sediment-bound contaminants. The Solid-Phase Test did not produce any negative gammas, although some were produced during the pore water analyses.

The test was difficult to apply to several samples with high levels of "gumbo" clay because the sediment could not be fully dispersed. EC_{50} values sometimes increase (indicating lower toxicity) directly with an increase in sediment grain size. This trend is reasonable since coarse sediments usually retain a proportionally smaller concentration of contaminants, by weight, than fine sediments. The work is being done by Mr. Sam Hoskin, a graduate student at the University.

U.S. Army Corps of Engineers, Waterways Experiment Station.

Comment: The Waterways Experiment Station at Vicksburg, Mississippi no longer uses Microtox®. They believe that the test lacks ecological relevance and is extremely sensitive to operator technique.

Wright State University

Program Contact: Department of Biological Sciences
Use: Sediment screening
Media Tested: Sediment samples
Contaminants Tested: Unspecified sediment contaminants
Preparation Method: Pore water is preferred
Test Method: Project dependent (Basic Test or 100% Test)
Endpoint: EC_{50} values calculated using the Microbics statistical package.
Hit: Sediments are considered toxic if three replicate EC_{50} test values are statistically different from the control at a 95% confidence interval using Dunnett's Test.
Comments: The lab operated by Dr. G. Allen Burton, Jr. uses all three Microtox® tests (Basic Test, 100% Test, and Solid-Phase Test). They prefer to avoid elutriates and dilutions if possible, and feel that pore water is a more sensitive indicator of toxicity. Initial results with the Solid-Phase Test show promise.

Microtox® is useful for screening large numbers of samples, although a negative test does not necessarily indicate the absence of contamination. The lab has found generally good correlation between Microtox® and whole organism bioassays (Burton, 1992).

EVALUATION OF MICROTOX®

Overall Sensitivity to Contaminants

The concentration of chemicals to which a bioassay shows an effect is an indication of sensitivity. The lower the concentration is that elicits an effect, the more sensitive is the bioassay. Bioassays differ in their sensitivity to various types of chemicals. Of 156 pollutants tested, 23 pollutants were most toxic to bacterial cells, 47 were most toxic to algae, and 43 were most toxic to protozoans (Giesy *et al.*, 1988). Microtox® is as sensitive to many compounds as most insect, crustacean, protozoan, mollusc, and fish bioassay species (Giesy and Hoke, 1989). For bioassays that test a single chemical, Microtox® is more sensitive than other microbial tests (Burton, 1991).

Metals

Bacteria are generally less sensitive to metals than plant and animal cells. *P. phosphoreum* is much less sensitive to mercury and cadmium than *D. magna*. The bacterium is very

sensitive to copper and some other metals (Giesy and Hoke, 1989). However, copper sensitivity was not apparent with the Steilacoom Lake sediments. Miller *et al.* (1985) found that Microtox® is sensitive to zinc. Microtox® values for lead and mercury should be based on a 15-minute reading, since response to these metals increases with time (Cusick, 1992). Table 1A gives 5-, 15-, and 30-minute EC_{50} values for copper, cadmium, and zinc.

Organics

Bacteria can be inhibited by crude oils. They are not very sensitive to some highly toxic organic compounds such as chlorinated organics (including PCBs), solvents, and various insecticides (Giesy and Hoke, 1989) which are highly toxic to other taxonomic groups. Microtox® sensitivity to pesticides is low, "less than that of *Daphnia*" (Cusick, 1992). Giesy *et al.* (1988) state that Microtox® is not very sensitive to extremely lipophilic organic compounds, although it is sensitive to solvent extracted aromatic and chlorinated hydrocarbons. Table 1B gives 5-minute EC_{50} values for 12 chemicals, demonstrating a wide range of response to organic compounds.

Conventionals: Ammonia, Sulfur, Water Hardness

Natural and anthropogenically derived ammonia may cause toxicity that might be attributed to other sources. Microtox® indicated little toxicity in pore water containing high concentrations of ammonia, which was the likely cause of toxicity to fathead minnows and *Ceriodaphnia dubia* (Ankley, 1990). Qureshi *et al.* (1982) also showed that Microtox® may be a poor indicator of ammonia toxicity. Osmotic pressure adjustment with sucrose can increase the sensitivity of the test to metals that may react with ammonia (Microbics, 1992).

Jacobs *et al.* (1992) report that elemental sulfur is highly toxic to *P. phosphoreum*. Sulfur is commonly found in anaerobic marine sediments, and is produced during the microbial oxidation of sulfide. Its presence in sediment extracts could result in erroneous estimations of the level of toxicity contributed from other contaminants.

Water hardness can influence aquatic toxicity. Microtox® is not as sensitive to hardness as the water-column organism *Daphnia magna* (Cusick, 1992).

Table 1A. Five-, 15-, and 30- minute EC50 values for copper, zinc, and cadmium. (Miller et al., 1985).

Metal	Five-Minute EC50, mg/L	Fifteen-Minute EC50, mg/L	Thirty-Minute EC50, mg/L
Copper	1.2	0.42	0.24
Zinc	12	1.6	0.7
Cadmium	106	25	14

Table 1B. Five-minute EC50 Microtox values for 12 chemicals. (Curtis et al., 1982).

Chemical	Five-Minute EC50, mg/L
Pentachlorophenol	0.08
Tetrachloroethane	8.6
Diazinon	9.8
Phenol	40.2
Trichloroethane	105
Cyclohexanol	115
1-Butanol	2,300
2-Butanone	5,050
Acetone	21,500
2-Propanol	35,000
Ethanol	44,000
Methanol	125,000

Microtox® Compared to Other Bioassays

Giesy and Hoke (1989) compared the Microtox® test to 14 other bioassays using the following ten criteria.

Criteria	Rating
Rapid	Excellent
Simple	Excellent
Replicable	Excellent
Inexpensive	Excellent
Standardized	Excellent
Discriminatory	Excellent
Sensitive	Good
Ecologically Relevant	Poor
Relatable to Field Effects	Poor
Relatable to Regulatory Standards	Poor

According to this rating, Microtox® is "excellent" for ease, economics, and related characteristics, and "good" for sensitivity. However, it is "poor" for ecological relevance, field effects, and regulatory standards. Except for one other microbial test, Microtox® was the only bioassay to receive the lowest score for each of these three categories. If Microtox® were expected to show high resolution, a "poor" rating in these three categories would be undesirable. However, this is probably not a serious problem where Microtox® is used primarily as a toxicant screen or as part of a test battery.

Pastorok and Becker (1989) review the ecological relevance of Microtox®. They point out that Microtox® is a bacterium representative of a group of organisms that form the lower levels of food webs. The indication of cellular metabolic state reflected by luminosity is probably very sensitive, but it is unknown what consequences these changes have on the survival of the organisms or how well they indicate changes in viability in other organisms.

Their report expresses concern that the Basic Test, using saline extract, is run on an extract of the sediment and not the sediment itself. Therefore, only water soluble contaminants are tested and results may not represent the full range of sediment contaminants. The ecological

relevance of using organic extract is in question because the process may remove contaminants that are not bioavailable, potentially overestimating a contaminant's biological effects.

Bulich *et al.* (1981) determined 5-minute EC_{50} Microtox® values for both pure compounds and complex industrial and municipal effluents. These values were generally similar to published 24 to 96-hour LC_{50} (Lethal Concentration 50%) fish bioassay data. Curtis *et al.* (1982) came to similar conclusions when they compared Microtox® EC_{50} data for pure chemicals to published LC_{50} values for *Pimephales promelas*. Qureshi *et al.* (1982) challenged Microtox®, rainbow trout, spirillum, and daphnids with 11 individual toxicants. Of the four bioassays evaluated, Microtox® ranked first and the third most sensitive in one test each, second most sensitive in five tests, and the least sensitive in four tests. These results indicate that Microtox® has a level of sensitivity comparable to that of three other well-established tests.

Microtox® Applicability

Table 2 compares the applicability of Microtox® tests and sample preparation procedures for sediment testing. The evaluations are based on the ability of the tests and procedures to give positive responses in the presence of the specified types of sediment contaminants. Selected criteria are defined as follows:

Sediment-bound (insoluble) metals: Metals in a chemical form that prevents them from dissolving in pore water or elutriate.

Soluble metals: Metals in a chemical form that allows them to dissolve in pore water and elutriate.

Hydrophobic organics: Organic compounds that are insoluble in water. They are usually adsorbed to organic matter and sediment particles, and do not appear in either pore water or elutriate.

Hydrophilic organics: Organic compounds that, because of their chemical structure, are at least slightly soluble in water. They may be found in pore water and elutriate.

Both the Basic Test and the 100% Test require that contaminants be extracted from the sediment before testing. No toxicity will be indicated for contaminants that cannot be extracted. However, these tests do provide information on water-soluble and solvent-soluble contaminants. Only the Solid-Phase Test can indicate toxicity from all types of contaminants.

Table 2 also compares the Microtox® tests and procedures for criteria that are important to test performance. The criteria are given a relative rating of High, Medium, Low or Not Applicable, and are defined as follows:

Table 2. Applicability and comparison of Microtox tests, characteristics and procedures for detecting sediment contaminants.

	Sample Preparation			Test		
Applicability to Sediment Testing	Elutriate	Pore Water	Solvent Extraction	Basic	100%	Solid-Phase
Sediment-bound (insoluble) metals	No	No	No	No	No	Yes
Soluble metals	Yes	Yes	No	Yes	Yes	Yes
Insoluble organics	No	No	Yes	No	No	Yes
Soluble organics	Yes	Yes	Yes	Yes	Yes	Yes
Comparison of Microtox Test Characteristics						
Reproducibility	Med	Med	Med	High	Med	Med
Contaminant sensitivity	NA	NA	NA	Med	Med	High
Error sensitivity	NA	NA	NA	Low	Med	Med
Interference sensitivity	NA	NA	NA	Low	Low	Med
Maximum percent sample concentration	20	99	0.5	45	99	10

NA - Not Applicable

Note: Evaluations of the Microtox tests and procedures are general and cannot be specifically applied to every test, under every condition, and with every contaminant. Microtox evaluation data are from sources in the text.

Reproducibility: The ability of a test to give similar results each time it is performed with the same sediment. High reproducibility is preferred.

Contaminant sensitivity: The ability of a test to detect a chemical effect such as toxicity. High sensitivity is preferred.

Error sensitivity: The sensitivity that a test has to errors in operator technique or other irregularities. Low sensitivity is preferred, which means that the test can compensate for procedural variations.

Interference sensitivity: The susceptibility of the test to interferences such as turbidity, color, pH, etc. Low sensitivity is preferred, meaning the test is not highly affected by these and related factors.

Maximum percent sample concentration: The maximum sample concentration that can be used in the test. High concentration is required for low toxicity samples, which otherwise may go undetected.

This comparison of Microtox® tests shows the relative merits of each method. Except f or the "maximum percent sample concentration," the evaluations are fairly general in nature and based on data summarized from various references. They do not necessarily determine a "best" method. Note that the Basic Test appears to be the least susceptible to error and is the most reproducible.

Advantages and Disadvantages of Microtox® Test and Preparation Procedures

The major advantages and disadvantages of each Microtox® test and preparation procedure are summarized below.

Test Methods	Advantages	Disadvantages
Basic Test	Relatively insensitive to operator error. "Standard" test used widely. Most precise of the three Microtox® tests available.	Maximum elutriate/pore water concentration tested is 45%. Requires water or organic solvent extraction.
100% Test	Quicker and easier than Basic Test. Higher sample concentrations can be tested (99%) than with Basic Test.	More sensitive to technique errors and less precise than Basic Test. Requires water or organic solvent extraction.
Solid-Phase	Bacteria directly contact sediment, so extraction is not required. Tests toxicity from metals, also polar and nonpolar organics.	No ASTM standards. Low precision because of small sample size, interference from suspended clay, etc. Sediment is mixed during preparation, so test may not mimic *in situ* effects.
Preparation Methods		
Pore Water	Contaminants have sufficient time to equilibrate between water and sediment. Tests undiluted pore water, which best mimics *in situ* exposure for many organisms.	Sediment samples may not have adequate supply of pore water for analysis. Test may not indicate toxicity from insoluble contaminants.
Elutriate Extraction	Mixing may increase concentration of contaminant and make test more sensitive.	Dilution may reduce sensitivity. Aqueous extract will not dissolve nonpolar organic contaminants.
Solvent Extraction	Water insoluble contaminants can be extracted and tested, making the test more sensitive to these toxicants. May produce an EC_{50} value when other tests cannot.	Organic solvents alone may be toxic to bioassay organisms. Test may not correctly mimic *in situ* effects because it can extract unrealistically high concentrations of organics.

Significant Toxicity

Bioassay results need to be ascribed significance. Most bioassays show significant effect through statistical comparison with a laboratory control or a field reference of putative low contamination. Applications of Microtox® usually use this principle to assign significance to indications of toxicity. Either the EC_{50}s, or the actual light output of several replicates, can be compared with controls or reference sites using Dunnett's or another means comparison statistical tests. Because EC_{50} values are based on dose response and cannot always reliably be generated, the measurement of light output at the highest concentration offers some advantages. Some agencies have used a scale of EC_{50} values to ascribe significance. However, these ranges appear somewhat arbitrary and are not based on statistical comparison with controls.

Microtox® Inclusion in a "Battery of Tests"

Dutka *et al.* (1988) emphasize the need for a "battery of tests." They base their conclusion on tests with Saint John River Basin sediments using Microtox® and other toxicity tests. They state that "...individual ... screening tests do not provide a sufficient data base for realistic management decisions to be made ..." However, Giesy *et al.* (1988) showed that only two bioassays, Microtox® and *D. magna*, were needed to classify sediments from 30 Detroit River sites as either nontoxic, moderately toxic, or very toxic.

Microtox® is best suited for environmental toxicity screening and as one component of a "battery of tests." It is more likely to indicate the presence of toxicity if the sediment contains a variety of contaminants, rather than a single contaminant to which it may not be sensitive. It is less suited for use, by itself, as a rapid sediment assessment technique.

SUMMARY AND CONCLUSIONS

1) There are three types of Microtox® test: the Basic Test, the 100% Test, and the Solid-Phase Test. The Basic Test and the 100% Test are used to analyze pore water, elutriate (saline or distilled water), and organic solvent extract. Only the Solid-Phase Test allows contact of the test organisms, *Photobacterium phosphoreum*, with the sediment itself.

2) The appropriate Microtox® test to use for sediment analysis depends on the type of contamination present, its concentration, and the project goals. The decision-making process is summarized below:

    A) The Basic Test can detect contaminants that are soluble in water or an organic solvent. It is the only Microtox® test that produces results that are precise enough to be used for the development of sediment criteria. Since the maximum sample concentration is limited to 45%, the sample must have a fairly high level of toxicity

to be detected. The most appropriate aqueous phase to use with this test can be determined as follows:

1) Elutriate best represents potential water contamination caused by the open water dumping of dredged spoils or similar material. Saline or distilled water should be used for extraction of marine or freshwater sediments, respectively.

2) Pore water best represents the concentrations of water-soluble contaminants that a burrowing, aquatic organism would contact in undisturbed sediment under *in situ* conditions.

3) Organic solvent extract best represents the toxicity posed by sediment-bound, hydrophobic contaminants such as PAH, PCBs, etc. Care must be used in selecting a solvent that will both effectively dissolve the contaminants of interest, and will not be excessively toxic to the test organisms.

B) The 100% Test can be used when sample toxicity is unknown or is lower than that needed for the Basic Test. Results are not as precise as those produced by the Basic Test. The 100% Test is not presently usable for the development of sediment criteria.

C) The Solid-Phase Test is a highly sensitive indicator of sediment toxicity because it will detect both sediment-bound and water-soluble contaminants. It may indicate the presence of potentially harmful toxicity regardless of whether contaminants are ingested, adsorbed, etc. The test is not now usable for the development of sediment criteria because it has a low level of precision. This problem is currently being resolved through revisions in the test procedures. Pending acceptance of the revised procedures by ASTM, the test should be reconsidered for its applicability to sediment criteria development.

3) Microtox® responds to different classes of chemicals according to preparation methods and type of chemical tested. The test is useful as one test within a tiered or battery of bioassay tests and can indicate toxicity from a variety of chemicals. Though sometimes characterized as simple and easy, the test requires operator proficiency and it can be tedious.

4) The significance of toxic effects on Microtox® is measured in two ways. Both methods rely on paired comparisons with control or reference sediments with Dunnett's or a t-test. Some agencies compare the EC_{50}s between test and reference or control sediments. The Department of Ecology compares actual light output between test and reference sediments and ascribes significance at a greater than 20% reduction at $p < 0.05$. Advantages of this second method include no requirement to produce a dose response curve and the requirement that the difference be enough to reduce the effects of noise.

The ecological relevance of Microtox® results is contentious and its perceived lack of relevance has caused it to be rejected by the Army Corps of Engineers in Mississippi.

RECOMMENDATIONS

1) We recommend use of the Microtox® Basic Test to gather data in support of freshwater sediment criteria development. The test should be used with other bioassays in a tiered or "battery-of-tests" approach regardless of application. Prepare the sample as follows:

   A) Use the elutriate test (deionized water) to determine the level of potential water contamination that may result from disposal of dredged material. Elutriates should also be used if inadequate pore water is available.

   B) Use pore water to determine the toxicity of water soluble contaminants in sediments.

   C) Use organic solvent extract to determine sediment toxicity when sediments contain high levels of water-insoluble organic compounds.

2) The 100% Test is not recommended in support of criteria development because of its inherent low precision.

3) The Solid-Phase Test may be potentially useful because it best simulates the level of exposure experienced by organisms *in situ*. However, problems with inconsistent results caused by grain size variations, limited use due to its recent introduction, and lack of validation by ASTM keep us from recommending this test in support of criteria development at this time.

4) Microtox® tests should be organized to provide information about whether the toxicity results are significant. The Sediment Management Standards provide methods for determining a significant "hit" and we recommend the same methods. Briefly, significant effect is defined as a minimum of 20 percent light reduction in the 45% concentration at a $p < 0.05$ level when tested with a t-test. Because of the high precision of the Basic Test, only 2-5 replicates are needed. EC_{50}s should also be calculated, if dose response is achievable, to provide comparisons to other studies.

5) As part of future studies, we recommend that the performance of the Basic Test with pore water, the Basic Test with elutriate preparations, and the Solid-Phase Test be compared among replicate samples that are toxic to other bioassays. Precision and sensitivity could be compared.

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