1. MICROTOX® ACUTE TOXICITY TEST

B. THOMAS JOHNSON
Environmental Microbiology
Columbia Environmental Research Center
U. S. Geological Survey
4200 New Haven Road
Columbia, Missouri 65201 USA
btjohnson@usgs.gov

1. Objective, development, and scope

The Microtox Acute Toxicity Test[1], usually identified as Microtox, has played a leading and pivotal role in developing minimalistic microscale toxicity testing. "Speed, simplicity, reproducibility, precision, sensitivity, standardization, cost effectiveness, and convenience" (Isenberg, 1993) were features sought and developed in Microtox. This test uses a specific clonal strain of bioluminescent bacteria prepared in a unique lyophilized vial format. This approach is rapid, simple, cost-effective, and sensitive with large sample throughput capabilities. Microtox is a screening tool and provides an alternate to traditional, complex, and more costly whole animal testing with invertebrates and fish; the manufacturer's suggested applications are listed in Table 1. Microtox uses very few elements[2]: the *Reagent* (a specific bacterial strain of *Vibrio fischeri*), the test sample in compatible carrier solution, the *Diluent* test solutions, a duo-function *Analyzer* that includes an incubator and luminometer, a personal computer, and a data capturing and analyzing *MicrotoxOmni* software package.

"A simple rapid method for monitoring the toxicity of aquatic samples has been developed" (Bulich, 1979); thus in 1979, in this short statement, the bacterial toxicity bioassay known as Microtox® ushered in a new far-reaching revolution in bioassays and a paradigm shift in test organisms and, most importantly, introduced a new

[1] Use of specific products by USGS and its laboratories does not constitute an endorsement. Columbia Environmental Research Center (CERC) uses Microtox materials and equipment sold by Strategic Diagnostics Inc. (SDI) in Newark, DE, to preserve the Microtox protocol. SDI provides comprehensive instructive guides, manuals and computer software to operate the Microtox test at their Web site (www.azurenv.com). The Microtox protocol described here is a standard USGS SOP.

[2] The USGS as well as others (Environment Canada, 1992) adopted Microtox terminology to reduce confusion. Specific Microtox products are printed in italics with the initial letter in upper case.

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microscale biomonitoring tool in environmental toxicology. Over the last twenty-five years bacterial toxicity bioassays have emerged as important screening tools for toxicity assessments, for regulatory compliance, and for use in a battery of tests to rapidly monitor the health hazards and risks of chemicals that enter the nation's aquatic environment (Wells et al., 1998). This chapter describes Microtox, an ecotoxicological screening tool designed to detect aquatic toxicity, to detect changes in toxicity, and to predict expectations of other toxicity tests. The advantages, new and old applications, and limitations of Microtox are explored.

Table 1. Recommended applications for Microtox (SDI Web site, 2003).

➢ Wastewater treatment plant influent testing for protection of activated sludge.
➢ Wastewater treatment plant effluent testing for protection of receiving waters.
➢ Toxicity Reduction Evaluations (TREs) and Toxicity Identification Evaluations (TIEs).
➢ Surface water monitoring for identification of point source and non-point source pollution.
➢ Monitoring raw drinking water to detect contamination due to point source or non-point source pollution.
➢ Bioterrorism.
➢ Sediment and soil testing.
➢ Monitoring of remediation processes.
➢ Biocide monitoring of industrial processed waters.

Water by its very nature is a universal solvent, a natural repository, and a carrier of both biogenic and xenogenic chemicals. The magnitude of this problem is expressed in part in the U. S. Chemical Industry's *Statistical Handbook* (1998) that states the industry annually produces 70,000 chemical products in 12,000 plants. The broad ecological impact of these and other chemicals on the health and well being of aquatic communities presents a very complex problem of hazard and risk assessment for both ecotoxicologists and resource managers.

In the last century analytical chemists have made amazing strides in collecting, separating, and identifying waterborne chemicals at nano- and picogram concentrations (Manahan, 1989). However, ecotoxicologists have only begun to make similar strides in the detection and characterization of environmental toxicants (Wells et al. 1998; Ostrander, 1996; Rand et al., 1995). The unraveling of contaminants (chemicals "out of place") and toxicants (chemicals injurious to ecosystem health) centers on three basic questions: What is the toxicant (qualitative)? How toxic is it (quantitative)? And how does the toxicant move (bioavailability)?

The historical literature is most helpful (Gallo, 1995). Paracelsus[3] told us that all things are toxic and the dosage makes the "toxicant". In this context, a toxicant must be defined both qualitatively (identified) and quantitatively (how much); therefore toxicity is clearly dose-responsive. Following this logic a chemical in the environment may be a contaminant at one concentration and a toxicant at another concentration; dosage makes the difference. The bioassay or bioindicator test, predicated on the dose-response experimental design, has over the last fifty years become a critical element in defining the nature of environmental toxicants (Rand et al, 1995). Today, toxicological bioassays are based upon an experimental design of five elements: the sample, the biota, the duration, the endpoint, and the dose-response. The interaction of these five elements in Microtox (Fig. 1) is the thesis of this chapter.

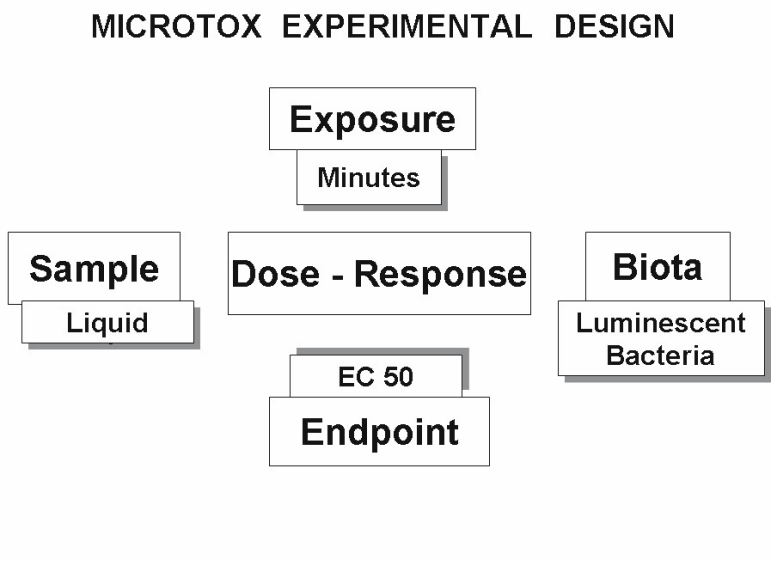


Figure 1. Experimental design: Microtox bioassay template.

2. Summary of test procedure (at a glance)

Microtox determines the acute toxicity of surface waters, ground waters, wastewaters, leachates, organic and aqueous sediment extracts, and passive sorptive device dialysates by measuring the changes of light produced naturally in samples exposed to bioluminescent bacteria under standard conditions. The Microtox

[3]Paracelsus (1493-1541) is often considered the father of modern toxicology. He brought empirical evidence into toxicology with his writings *"What is there that is not a poison? All things are poison and nothing without poison. Solely the dose determines that a thing is not a poison".*

Reagent bacteria, a selected strain of *V. fischeri* NRRL B-11177 (Fig. 2), are clonal cultures, which diminish possible genetic differences and ensure quality control of the tester strain and greater assay sensitivity and precision. The test bacteria are stored freeze-dried under vacuum in vials, which eliminates the tedium and cost of continuous culturing of a test organism. Most importantly, Microtox is available on demand because measurable light emission begins immediately after water activation of the lyophilized bacteria strain; bacteria require no preculturing. Aseptic technique is not required because of the short incubation period of the assay. All test media and glassware are pre-packaged, standardized, and disposable; the quantity is minimal, dramatically reducing both the material cost of the test and the disposal expense of toxic waste materials. The test requires minimal laboratory space and limited dedicated equipment: microliter pipetting devices, vortex mixer, incubator, and luminometer with computer assistance, and freezer storage. The test is well defined, computer assisted, and user friendly. Microtox is a unique bacterial bioluminescent inhibition assay.

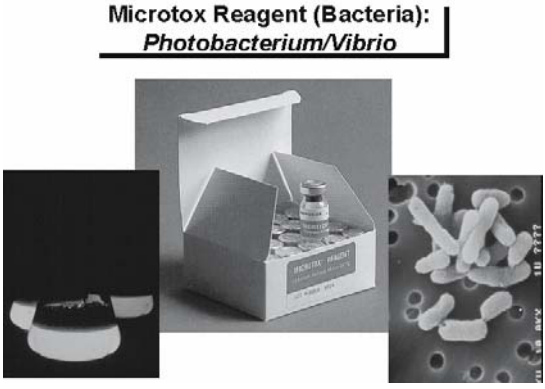


Figure 2. Microtox Reagent.

Microtox is microscale; all tests are conducted in microvolumes with microcuvettes. A single reaction cuvette contains *Reagent* bacteria, *Diluent*, and test chemical. Aqueous and organic samples are prepared in the basic dose-response design: 1 control and 4 concentrations in a 1:2 dilution series. Carrier solvents such as DMSO, acetone, and ethanol may be necessary to solubilize certain chemicals; osmotic correction with NaCl may also be necessary with freshwater samples. Freshly prepared glowing luminescent bacteria in stationary growth phase are added to the test sample and placed in a SDI *Model 500 Analyzer* (Fig. 3); readings are taken typically after either five or 15 minutes incubation. The endpoints of all tests are based on light emissions produced by bioluminescent bacteria. The amount of light remaining in the sample is used to determine the sample's relative toxicity, which can then be compared to the standard reference's toxicity. As the toxicant's concentration increases, bacterial light emissions decrease in a dose-dependent manner. Some samples may require an extended range protocol (eight to 10 dilutions

with two controls). The luminometer and supporting computer software (*MicrotoxOmni*® software) with a standard log-linear model are used to determine a 50 percent loss of light in the test bacteria, *i.e.*, the effective concentration (EC50) value. All EC50 values are expressed as weight or percent per mL with 95% confidence intervals and reported as the mean of three pseudoreplicates or true replicates; *replicates are a statistical measurement of the test's precision. The lower the EC50 value the greater the toxicity of the sample.* Manufacturer's suggested positive controls are phenol (organic) and zinc sulfate (inorganic). Typically tests are completed and data are available in < 30 min. This rapid response time meets the toxicologist's needs to conduct routine toxicity assays as well as to respond to emergencies such as wastewater effluents, chemical spills, and detection of unstable or transitory toxicants. Microtox protocol and rapid toxicological determination (< 30 min) make throughput capability of large samplings feasible both in the laboratory and in the field.

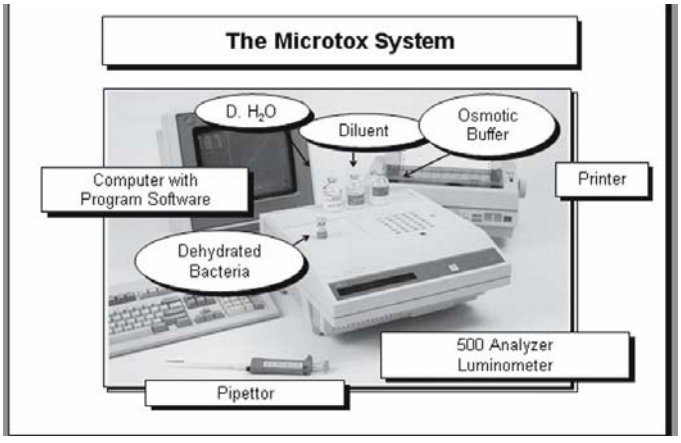


Figure 3. Microtox equipment and supplies.

Box 1. Required materials for testing.

The supplies required to implement the Microtox test are purchased from SDI.

Glassware

Each standard dose-response test (1 Control: 4 concentrations) requires ten disposable borosilicate glass cuvettes: two sizes 12 x 50 mm and 12 x 75 mm. To prevent spills and to make solution mixing easier larger 12 x 75 mm cuvettes can be substituted for the 12 x 50 mm.

Test organism

Microtox *Reagent*, the clonal bacterial isolate *V. fischeri* NRRL B-11177, is lyophilized and packed in 10 mL sealed vials. Each vial will test about 20 samples. Vials are shipped frozen and stored in the freezer compartment of a common refrigerator. Shelf life is 24 months.

Box 1 (continued). Required materials for testing.

Solutions

Microtox *Reconstitution Solution (Recon)* activates the *Reagent* for testing. The *Recon* is tightly sealed in the original container. Shelf life is 12 months.

Microtox Diluent is a 2% NaCl solution used to make dose-response dilutions. The sterile Diluent in sealed bottles is shipped and stored at room temperature. Shelf life is 12 months.

Microtox Osmotic Adjustment Solution is a 22% NaCl solution used to change the salinity of freshwater samples to the 2% required salinity of the assay. The solution is shipped and stored in tightly sealed bottles at room temperature. Shelf life is 12 months.

Box 2. Required equipment for testing.

Toxicity *Analyzer*

The SDI *Model 500 Analyzer* is a dual-purpose instrument serving both as an incubator and luminometer. The incubator is maintained at two temperatures: the thirty cuvette wells for test sample incubation at 15°C and a separate *Reagent Well* for storing one stock culture cuvette of luminous bacteria at 5°C. The luminometer contains a photomultiplier tube that measures the light emissions from bioluminescent bacteria. The *Analyzer* is interfaced with a PC containing the *MicrotoxOmni* software package for collecting, analyzing, and storing test data.

Pipettors

Rapid accurate and precise pipetting is essential for successfully dispensing multiple test solutions. Ergonomic pipettors are desirable because of the highly repetitive action of pipetting necessary for the dose-response experimental design.

Microtox uses several sizes of pipettors: two P-1000 Gilson Pipetman®, variable volume 100 µL – 1000 µL (or comparable); one P-100 Gilson Pipetman®, variable volume 10 µL – 100 µL; one EP-10 EDP-Plus® electronic pipettor, variable volume 1 mL – 10 mL (or comparable); and one EP-100 EDP-Plus® electronic pipettor, variable volume 10 µL – 100 µL.

Freezer

A freezer is essential for storage of the Microtox *Reagent* bacteria at –20°C. Self-defrosting units should be avoided.

Vortex mixer

A standard vortex mixer is used to stir liquids in test cuvettes. The mixer eliminates tedious mixing with pipettes and reduces ergonomic problems with repetitive hand movement. All vortex mixing should be brief measured in a few seconds. Prolonged mixing of test material and bacteria may affect the assay and should be avoided.

Computer

A standard personal computer (PC) with a *MicrotoxOmni* software program interfaced with the *Analyzer* is an essential element in Microtox testing; each step in the test protocol is recognized, controlled, analyzed, and recorded.

Printer

A printer interfaced with the PC makes data more accessible.

Box 3. Laboratory facilities.

Laboratory facilities for Microtox are consistent with modern Good Laboratory Practices (GLP) protocols. An organized, clean laboratory with limited traffic flow, good lighting and airflow, controlled heating-cooling, electrical outlets, and designated bench space will meet most needs. Because Microtox is a microscale test, laboratory space requirements are comparably small, instrumentation is limited and compact, glassware is microscale, and test solutions are microvolumes.

For health and safety purposes the laboratory must be considered a hazardous zone because the nature of the test substance(s) is usually an unknown and potentially toxic. The user should wear safety glasses, protective outerwear, and disposable gloves. To reduce cross contamination the use of disposable table coverings is recommended. A hooded bench area is useful, but certainly not necessary for all environmental sampling. Closed containers for spent test materials (both liquids and glassware) should be carefully labelled, stored, and monitored for GLP disposal. Although *V. fischeri* are saprophytic bacteria and not known as human pathogens, some laboratories destroy used culture material by heat or a disinfectant (APHA et al., 1998).

3. Overview of development and application of the Microtox toxicity test

An overview of the development and applications of Microtox reveals an intriguing tale of meeting an environmental challenge, of intellectual acuity, of entrepreneurism, and some good luck. In the early 1970s Beckman Instrument Co. (Carlsbad, CA) was asked by the petroleum industry in California to develop an acute toxicity assay, a substitute for the traditional fish and invertebrate tests, to monitor potentially toxic effluents from drilling operations. In formulating the task Isenberg in *The Microtox Toxicity Test: A Developer's Commentary* (1993) states the framework of the Microtox paradigm: "metaphorically …we needed to miniaturize fish, to teach them to talk, to report on their health, and to devise a way for them to be stored in suspended animation" in order to provide on demand availability and convenience. A toxicity bioassay needed "something alive" with "diverse, interdependent enzyme systems controlling a measurable physiological parameter" and an "appropriate measurement system". This toxicity test should be "fast, simple, reproducible, precise, … standardized, cost effective, convenient, and sensitive". The ambitious template for Microtox had been formulated. The question was could it be done?

A bit of serendipity or simply luck occurred when Beckman purchased the North American Rockwell collection of over 200 strains of luminescent bacteria. If luminescent bacteria could function as airborne biosensors of chemical warfare agents, scientists at Beckman (Isenberg, 1993) working on the Microtox Project wondered if these same bacteria could be used in an aquatic matrix. The attraction to luminous bacteria was tantalizing: rapid response time to a toxin and light emission from millions of cells that could be measured and reproduced with high precision. An "enzyme system controlling a measurable physiological parameter" had been found! The task was to find a strain of luminous bacteria with a sensitivity spectrum similar to traditional aquatic test animals.

Isenberg (1993) in reflecting on the Microtox Project years later stated that the work of Johnson et al. (1974) provided "an elaborate and compelling derivation of a general equation for the expression of (acute) toxicity" and formed the mechanistic model for the Microtox acute toxicity bioassay. Johnson et al. (1974) had published seminal work on a reaction rate theory that was based on isolated specific chemical processes and their relationship to complex biological reactions: significantly, the authors had used luminous bacteria to test their theories. Inventively, they expressed this physiological effect as a ratio of the activity lost to the activity remaining and termed this ratio gamma (Γ). Gamma proved to be a precise method when measuring light emissions from luminous bacteria. Gamma calculations permitted Microtox protocol to use simple regression statistics to compute toxicological endpoints: *i.e.*, EC50 values with confidence intervals.

Traditionally, bacteria are stored on agar-slants, frozen in liquid nitrogen or freeze-dried (lyophilized). For the Microtox scientists the obvious method of choice was the lyophilization process because bacteria freeze-dried under vacuum would remain viable and clonal and could be held for long periods of time with minimal care. However, the poor survival rate of bacteria following lyophilization, usually < 1%, was a serious problem. Essentially this meant that luminous bacteria from a freshly opened vial could not emit sufficient light for a bioassay. If bacteria had to be precultured to increase numbers, the "on demand" quality of a microscale bioassay was sacrificed and the clonal integrity of the bacteria would be questionable. This problem was solved when Beckman developed a proprietary technique for the lyophilization of luminous bacteria. This process improved the survival rate of bacteria with cells emitting high luminescence at the moment of reactivation with distilled water. Acceptable concentrations of physiologically active, light-producing bacteria were now available as a biosensor. The Microtox project now had a simple method of storing and shipping a clonal strain of bacteria to scientists around the world. These bacteria would survive, remain clonal, be sensitive, and be available for immediate use (*i.e.*, within minutes of demand). This achievement was pivotal in the development of a successful bioassay.

The next task that faced the Microtox's developers was integrating a device that controlled temperature with an instrument for photochemical measurements into a single laboratory unit. Beckman successfully produced an instrument with an incubator that could hold the test bacteria at optimum temperatures and a photometer to read luminous light emission of bacteria. Beckman's Director of Research, Richard Nesbitt, commented that the Microtox Project was the most complex problem the company had ever undertaken – "not just designing an instrument, but finding the right bugs (bacteria), growing them, preserving them in containers that would not poison them, and arranging to ship them thousands of kilometers, while they retained a product shelf-life of at least one year" (Isenberg, 1993). In 1979, Beckman introduced Microtox in the United States, Canada, and Europe. In 1985 the developers of Microtox formed Microbics Corporation in Carlsbad, CA. In the 1990s the corporation name was changed to AZUR Environmental. In 2000, Strategic Diagnostics Incorporated (SDI) in Newark, DE, purchased AZUR Environmental. SDI now sells all Microtox products. In the last ten years the frequency and volume of publications has nearly doubled, a good indicator of the growing global utilization and acceptance of the Microtox paradigm (Tab. 2).

Table 2. Interest in microscale toxicity testing applications[a] and time-related publications of Microtox[b].

1979-83[a]	1984-89[a]	1990-95[a]	1995-2003[b]	Total publications
29	201	279	891	1400

[a]Redrawn from a table by Wells et al. (1998); data included Mutatox®.
[b]Publications of Microtox Basic only; data derived from multiple Web sites.

4. Advantages of conducting the Microtox toxicity test

Toxicological risk assessments are a growing concern for aquatic resource managers. Increasingly they must address and answer these basic water resource issues: What is toxic? How toxic is it? Where is the toxin? Is it bioavailable? While many good, reliable toxicity bioassays are available to answer these pressing questions, Microtox is a leading choice for a number of reasons. First, and foremost, the protocol is completely standardized and the materials are globally available: 1) the bioluminescent bacteria are cloned, stored in a lyophilized state, and available on demand for immediate testing; no preculturing of test biota is needed; 2) all glassware and test solutions are prepacked and test ready; no premixing is necessary; 3) the *Analyzer* with programmed luminometer and incubator is wired for computer assistance; 4) the computer software package *MicrotoxOmni* directs, computes, stores, and displays data; 5) toxicological results are available in minutes, thus permitting rapid response time to address spills and urban stream monitoring in order to determine hot spots for focusing resources; and 6) technical and material support from the manufacturer is excellent and timely. Furthermore, this test reduces the costs of materials and disposables and minimizes dedicated laboratory space. Short exposure times and microscale supplies provide Microtox with large sampling throughput capabilities not generally possible with animal or other microscale toxicity tests. *Statistical power is predicated on numbers – numbers in terms of sampling sites, numbers in the frequencies of samplings at given site, and numbers of replicates produced for each sample.* Significantly, this sampling protocol and, as a result, the early recognition of areas of concern are attractive features that make Microtox a good environmental monitoring tool.

5. Test species

Marine luminous bacteria are a cosmopolitan group that occurs in planktonic, enteric, saprophytic, parasitic, and symbiotic (in light organs in some marine fish and invertebrates) forms. Using phenotypic and genotypic analyses contemporary bacterial taxonomists Bauman et al. (1983) grouped luminous bacteria into two genera: *Photobacterium* and *Vibrio*. The main components for bacterial bioluminescence have been identified as reduced flavin mononucleotide (FMN), a long chain aldehyde, oxygen, and the enzyme luciferase (McElroy, 1961). These findings suggest that luminous bacteria contain luciferase that catalyzes the oxidation of $FMNH_2$ and aldehyde by oxygen. Significantly, the bacterial luciferase system

appears to be coupled to cellular respiration via NADH and FMN. Treatises by Harvey (1952), McElroy (1961), and DeLuca and McElroy (1981) on bacterial bioluminescence offer comprehensive reviews of their findings and the biology of luminous bacteria.

Microtox is a prokaryotic microscale toxicity bioassay with luminescent, gram negative, saprophytic marine bacteria. These bacteria are ubiquitous in marine waters and are easily isolated and cultured from fish and seawater. Early studies (Bulich, 1979) suggested that specific isolates of *Vibrio* (originally taxonomically designated as *Photobacterium phosphoreum*) showed toxicological sensitivity to a broad spectrum of environmental contaminants. Additional investigations using these isolates under carefully standardized conditions revealed that an "on demand" toxicity test could be developed to measure a specific physiological parameter - bioluminescence - in real time. The prokaryotic cells used in Microtox are obtained exclusively from a cloned strain of a marine bacterium, *V. fischeri* NRRL B-11177, isolated, cultured and maintained by the manufacturer (currently SDI). This clonal strain is deposited by SDI at the Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL, USA.

6. Culture/maintenance of organism in the laboratory

6.1 PREPARATION OF REAGENTS AND CULTURE MEDIA

The Microtox *Reagent* requires no culturing. No specialized microbiological equipment is necessary. The Microtox *Reagent* bacteria*, V. fischeri* NRRL B-11177, are cultured, freeze-dried under vacuum (lyophilized), sealed in 10 mL vials, shipped in 10 vial lots by SDI, and stored frozen at –20^{o}C to ensure high-level light emissions. Self-defrosting freezers must be avoided. During power outages place the vials in an insulated box containing artificial ice and store in the freezer compartment. Bacterial *Reagent* in this container will remain frozen for several days. For quality assurance and quality control (QA/QC) each vial is dated with the manufacturer's suggested shelf life. The Microtox is an "on demand" acute toxicity bioassay. Biota are available immediately for use whether in the laboratory or in the field. Neither preculturing nor preincubation of cells is necessary.

6.2 WASHING OF GLASSWARE

Protocol for the Microtox assay requires that all cuvettes and pipette tips are disposables and never reused. Beakers for dispensing the *Diluent* are acid-washed and air-dried each day and used only for the *Diluent*. Stock bottles for control chemicals are acid washed and steam sterilized before use; all bottles are stoppered with teflon® liners.

7. Information regarding test samples prior to conducting bioassays

7.1 KNOWN SUBSTANCES

Manufacturer's Material Safety Data Sheets (MMSDS), the Merck Index, and reliable Web sites (Tab. 3) provide valuable information about the compound of interest: its chemical class identification, solvent solubility, hazard identification, stability, primary use(s), disposition, and possible toxicity to vertebrates.

Table 3. Web site generated database sources for Microtox.

Applied Science & Technology Index	General Science
Agricola	Geobase
Aqualine	Georef
Aquatic Sciences & Fisheries Abstracts	Medline
Basic Biosis	OCLC Article First
Biology Sciences	OCLC ECO
Biology Digest	SDI
Chemical Abstract Service	Toxline
Conference Papers Index Abstracts	Water Resources
Environmental Sciences & Pollution Management	

7.2 UNKNOWN SUBSTANCES

All environmental samples are collected in clean containers and held on ice. Prompt testing is most desirable and less likely to introduce experimental errors from microbial activity. If testing is delayed sediment samples for pore-water analyses, organic extractions, and passive membrane dialysates can be stored on ice or refrigerated (3^{o}C). Lipophilic test samples need to be dissolved in a solvent that will solubilize the material in the *Diluent* and also be compatible with the Microtox *Reagent*. Environmental samples are not collected in a complete vacuum of information; the geographical location (urban versus rural), source, season, etc, will provide the user important clues as to probable contaminants in the sample.

7.3 REFERENCE TOXICANT

Reference toxicants are essential elements in a good QA/QC program. The user monitors the relative sensitivity of the Microtox *Reagent* bacteria using reference toxicants under standard conditions in order to note the viability of the activated *Reagent* and to assess pipetting precision. Compound purity, stability, wide availability, aqueous solubility, dose-response profile, and low user hazard are essential components in selecting a good reference toxicant. SDI recommends phenol as an organic reference toxicant and zinc sulfate ($ZnSO_4$) as an inorganic reference toxicant. The 5-min EC50 values for phenol are typically in the 10-30 mg/L range

while the 15-min values for $ZnSO_4$ are between 1.5 and 3.0 mg/L (Fig. 4). For years the Microbics sales' force has used Listerine®, a commercial, globally available product, as a reference toxicant to avoid the problem of carrying chemicals aboard airplanes.

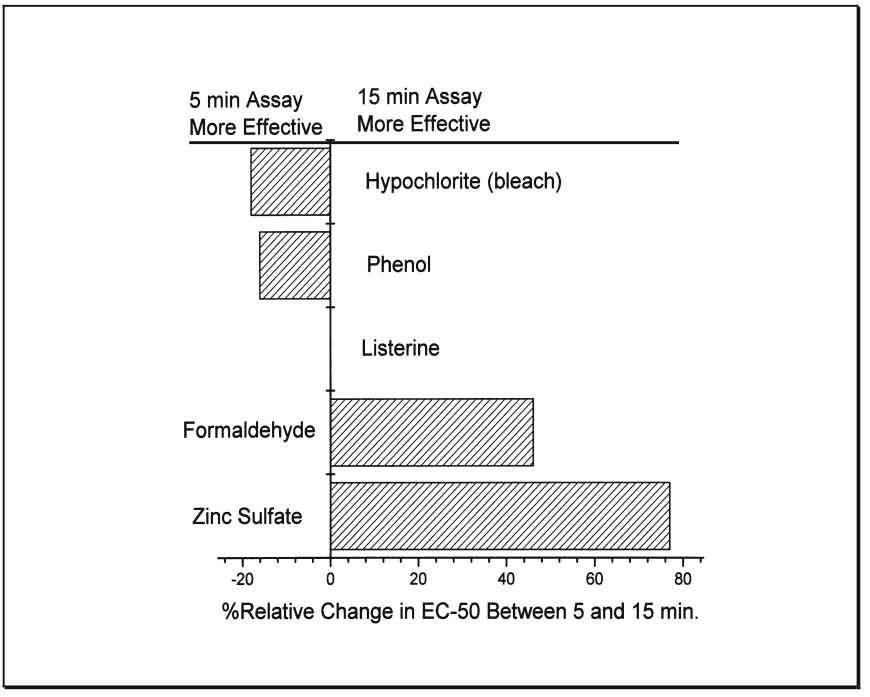


Figure 4. Influence of exposure times on EC50 values.

7.4 PREPARATION OF SAMPLE(S) FOR A TEST RUN

The sample to be tested must be in a liquid form and in an osmotically compatible solution, which may be water or a selected organic solvent. Lipophilic contaminants must be solublized in organic solvents. The compatibility of these solvents with Microtox should be investigated before extensive testing with unknown or pure compounds. Table 4 shows a list of common laboratory solvents and their compatibility with Microtox. Note that acetone, ethanol, and DMSO seemed the most compatible. At CERC we use a high purity grade of DMSO as our universal solvent for lipophilic chemicals; its very low toxicity, solubility range, low vapor pressure, and low freezing point makes it an attractive carrier solvent for Microtox.

Table 4. Influence of carrier solvents on Listerine® toxicity (EC50)[a].

Carrier solvent	*EC50[b]*	*CI[C]*
Control (Listerine®)	2.8	2.3 – 3.5
+ Dimethyl sulfoxide (DMSO)	2.5	2.2 – 2.9
+ Dichloromethane (DCM)	0.8	0.2 – 3.0
+ Hexane	0.1	0.1 – 7.1
+ Acetone	1.5	0.9 – 2.3
+ Methanol	3.6	2.6 – 4.7
+ 95% Ethanol	3.1	2.4 – 4.1
+ Isooctane	2.2	1.5 – 3.2

[a]The positive control Listerine® was exposed for 5 min with seven different solvents at concentrations not exceeding 5% of the total volume. Listerine® is a commercially available mouthwash with bactericidal properties. Range finding and definitive test for compound validation consisted of one control and four toxicant concentrations in a 1:2 dilution series.
[b]EC50 = µg/mL; [C] CI = confidence interval.

8. Equipment

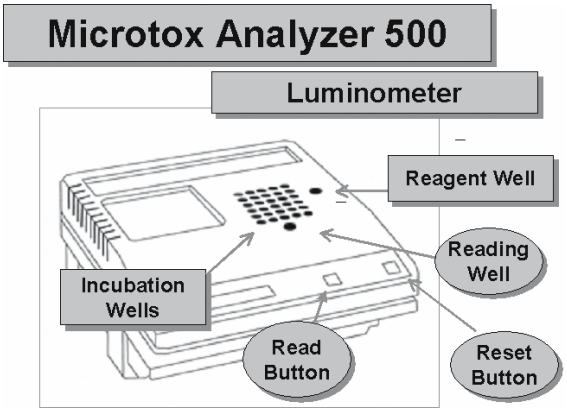


Figure 5. SDI Model 500 Analyzer.

The SDI *Model 500 Analyzer* (Fig. 5) integrates an incubator with a luminometer. On top of the instrument are 30 temperature-controlled incubation wells (15ºC) identified as Rows A through F and Columns 1 through 5 and one temperature-controlled *Reagent Well* (5ºC). The experimental design (Fig. 6) for the standard configuration of Microtox is 1 control (A1): 4 test concentrations (A2 through A5) with a 1:2 dilution factor. A 1:14 design is the maximum that can be analyzed at one time (one control: A1 and 14 concentrations: A2 through A5, C1 through C5, and E1 through E5). The luminometer measures the light emission remaining after the

reagent has been exposed to the test sample. Following PC screen prompting each cuvette is removed from its incubation well and placed in the *Read Well.* The cuvette is depressed in the well, the luminometer reads the light emission from the bacteria, and the *MicrotoxOmni* software computes and records the data.

9. Microtox acute toxicity test: performing the test

First the SDI *Model 500 Analyzer* is turned on for 5 min to allow the incubator to achieve optimal temperatures: 15°C for the incubation wells and 5°C for the *Reagent* Well (Fig. 5).

Second the *Reagent* vial is removed from the freezer and opened. When the seal is broken, the dry culture material will produce a snowflake swirl effect indicating a vacuum was present. Simply adding 1 mL of *Reconstituted Solution* (*Recon)* to the freshly opened vial activates the *Reagent* bacteria; the contents of the *Reagent* vial are immediately transferred to a 12 x 75 mm cuvette and vigorously stirred on a vortex mixer (Fig. 7). The vial is placed in the *Reagent Well* and held for about 5 minutes to stabilize the culture's emission of light prior to testing. The activated *Reagent* normally remains "usable" for about 2 to 4 hrs. The *V. fischeri* are physiologically active and ready for testing. Aliquots of the *Reagent* are removed by micropipettor as needed for each toxicity assay. At CERC the half-life of the freshly activated culture is about two to four hours.

For a standard test with 1 control: 4 concentrations insert five cuvettes (12 x 50 mm) in Row A, five cuvettes (12 x 50 mm) in Row B (Fig. 6), and one 12 x 75 mm cuvette in the *Reagent Well.*

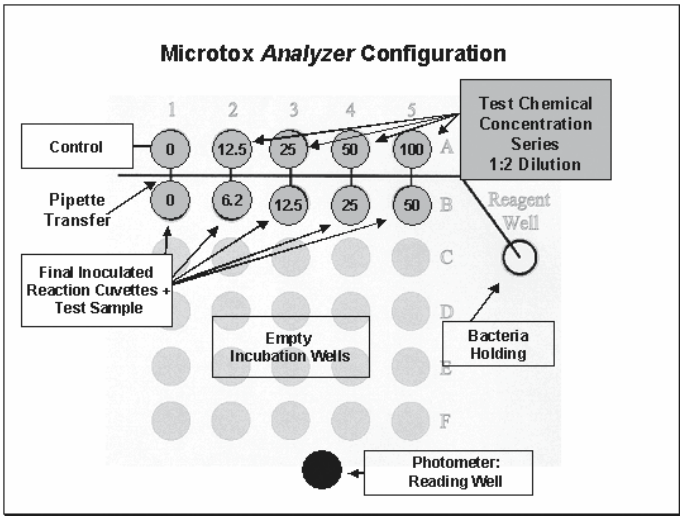


Figure 6. Microtox: dose-response design–1:4 (1 control: 4 test concentrations using 1: 2 dilution factor).

Pipette 2.0 mL of *Diluent* into cuvette A5*,* 1 mL into each of the remaining four cuvettes in row A and 0.5 mL of *Diluen*t into each of the 5 cuvettes in Row B.

Pipette the test sample into cuvette A5 and briefly use the vortex mixer to homogenate in the *Diluent*. Using a 1 mL-Pipetman transfer 1 mL from cuvette A5 into cuvette A4 and mix. Similarly transfer 1 mL from A4 to A3 and mix. Next transfer 1 mL from A3 to A2. After mixing discard 1 mL from A2 to bring its final volume to 1 mL. Cuvette A1 remains as a control. This process prepares 4 concentrations of the test sample. Now remove the vial from the *Reagent* well, mix for a few seconds, and load a 100 µL Pipetman with the bacteria from the vial. Dispense 10 µL of this bacterial inoculum into each cuvette in Row B. Place the tip of the pipette inside the cuvette just below the lip of the cuvette. Attempt to direct the inoculum into the *Diluent*, but do not submerge the tip in the *Diluent*. Briefly mix each cuvette to disperse the bacteria.

Figure 7. Vortex mixer.

Boot up the interfaced PC-*Analyzer*, activate the *MicrotoxOmni* program, and select a specific test protocol. Name the sample file and select desired test parameters as prompted: number of controls, number of dilutions, test duplication, initial concentration, units (% or weight per volume), osmotic adjustment, report form, and incubation time. Prompt the PC for desired exposure times - generally 5 or 15 min.

Now use the *Analyzer* to establish a base line reading of light emissions. Following the program's prompting, remove cuvette B5 and place it in the *Reading Well*. Press the *Read Button* and the luminometer will record light emissions. Continuing to follow PC prompting read zero time light levels of all the cuvettes in Row B. Verify that the light levels are reasonable, usually in the 90-100 % range.

Next activate the incubation timer by pressing the PC's space bar and introduce the test sample to the *Reagent* bacteria by transferring 0.5 mL from cuvette A5 to cuvette B5. Similarly, transfer 0.5 mL from cuvette A4 to B4, 0.5 mL from A3 to

B3, 0.5 mL from A2 to B2, and 0.5 mL from A1 to B1. For example, if 10 µg of test material were introduced into cuvette A5, after transferring 1mL to cuvette A4, cuvette A5 would now have only 5 µg of the test material. The transfer of this 0.5 mL from A5 to B5 would yield a final concentration in cuvette B5 of 2.5 µg of test material. Now again press the spare bar to begin a corrected incubation time (note that the software program corrects for the pipetting time).

At the end of the incubation period following PC prompting, place cuvettes from Row B in the *Read Well* and push the *Read Button*. The luminometer will make final light measurements of each cuvette and the *MicrotoxOmni* software will record, compute, and store the data (Fig. 8). The control cuvette is used to correct samples for the time-dependent drift in light output.

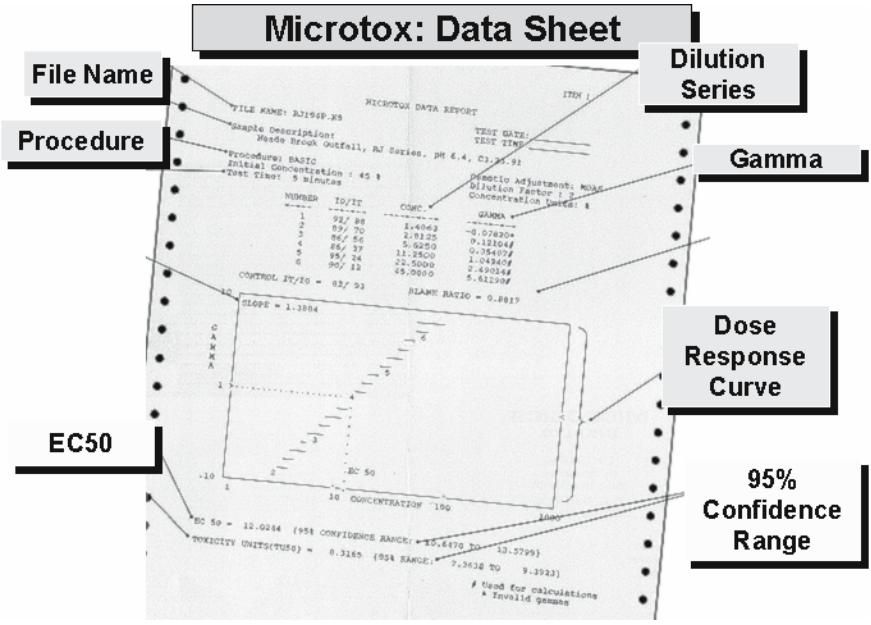


Figure 8. MicrotoxOmni *data sheet.*

The report should contain weekly EC50 data on recognized positive controls that are used as the laboratory's standard with predetermined coefficient of variance (CV) values (usually < 20%). The following questions concerning QA/QC criteria should be addressed: Was the protocol followed? Was the Microtox *Reagent* active with acceptable standard toxicant sensitivity limits for both positive and negative controls?

10. Test sample

10.1 CONCENTRATIONS

To determine the optimal test sample concentration for a definitive test, Microtox protocol suggests using a 1 control: 4 concentrations (1:4) design with a 1:2 dilution factor. The user should seek a concentration series in which the EC50 value is bracketed by at least one concentration on either side. The EC50 values are derived from a graph plotting the dose (the concentration of the test sample) against response (the effect on the test bacteria represented by gamma) on a log-log scale that requires at least three data points to plot a line. These EC50 values should have tight confidence intervals and replicate sampling tests should show coefficient of variance percentages below the manufacturers acceptable 20% CV (Fig. 9).

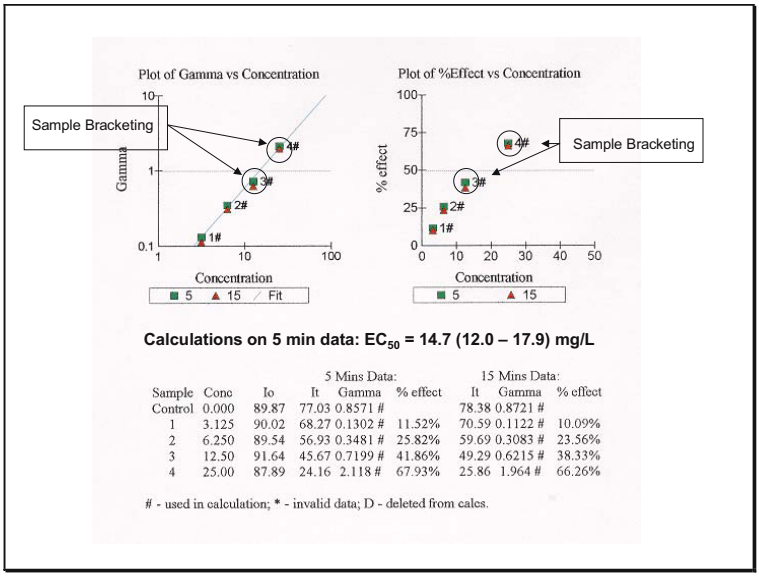


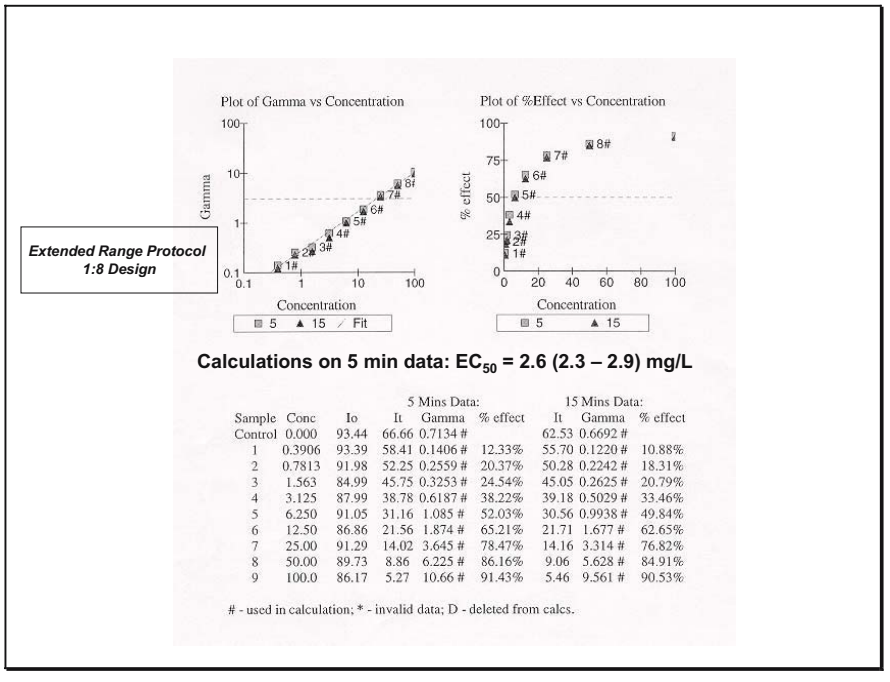
Figure 9. MicrotoxOmni *report on phenol: sample bracketing.*

If the trial range finding assay fails to generate an acceptable EC50 value with the 1:4 1:2 design, the user can probably solve the problem by simply re-testing the sample with the *Extended Range Protocol* (Microbics, 1992): dose concentrations are increased by one or two logs and the ratio of control: concentrations is changed to a 1:8 or even a 1:10 design with either a 1:2 or 1:10 dilution factor. With this expanded protocol a valid estimation of an EC50 value of even a very toxic substance can usually be determined. Figure 10 illustrates the use of the *Extended Range Protocol* to determine the EC50 value of 2,6 dinitrotoluene (2,6 DNT), a

munitions by-product of environmental concern. The initial trial assay showed that 2,6 DNT was more toxic than expected. Therefore, the design was expanded to a 1:8 concentration series with a 1:2 dilution factor and, as expected, Microtox produced acceptable, valid results with an EC50 value of 2.5 ± 0.5 mg/L (where n = 5).

10.2 EXPOSURE CONDITIONS

The Microtox software provides three standard exposure options: 5, 15 or 30 min. Figure 4 illustrates that exposure time does significantly influence the EC50 values of phenol, chlorine beach, Listerine, formaldehyde, and zinc sulfate. After a 15 min exposure period the EC50 values for $ZnSO_4$ (a commonly used inorganic positive control) and formaldehyde increased about 80% and 40 % respectively. These data suggest changes in absorption and metabolism of the test material during incubation. However, after a 15 min exposure period, the EC50 values for phenol (a commonly used organic positive control) and Clorox® (a household bleach) decreased about 20%. Interestingly, after a 15 min exposure period, the EC50 value for Listerine did not change (Fig. 4). Obviously, the exposure times must be considered when testing with unknown environmental compounds. For most screening exercises the exposure time is set initially at only 5 min.



Calculations on 5 min data: EC_{50} = 2.6 (2.3 – 2.9) mg/L

			5 Mins Data:			15 Mins Data:		
Sample	Conc	Io	It	Gamma	% effect	It	Gamma	% effect
Control	0.000	93.44	66.66	0.7134 #		62.53	0.6692 #	
1	0.3906	93.39	58.41	0.1406 #	12.33%	55.70	0.1220 #	10.88%
2	0.7813	91.98	52.25	0.2559 #	20.37%	50.28	0.2242 #	18.31%
3	1.563	84.99	45.75	0.3253 #	24.54%	45.05	0.2625 #	20.79%
4	3.125	87.99	38.78	0.6187 #	38.22%	39.18	0.5029 #	33.46%
5	6.250	91.05	31.16	1.085 #	52.03%	30.56	0.9938 #	49.84%
6	12.50	86.86	21.56	1.874 #	65.21%	21.71	1.677 #	62.65%
7	25.00	91.29	14.02	3.645 #	78.47%	14.16	3.314 #	76.82%
8	50.00	89.73	8.86	6.225 #	86.16%	9.06	5.628 #	84.91%
9	100.0	86.17	5.27	10.66 #	91.43%	5.46	9.561 #	90.53%

- used in calculation; * - invalid data; D - deleted from calcs.

Figure 10. MicrotoxOmni *report on 2,6-dinitrotoluene: extended range protocol.*

11. Post-exposure measurements and endpoint determinations

Light emitted from a bioluminescent culture represents an integrated response of millions of cells. Light lost by bacteria indicates a rate of biological activity as well as an indirect enumeration of organisms affected. Interestingly, light emission by luminous bacteria is a physiological endpoint of respiration (McElroy, 1961), and therefore reflects rapid changes in metabolism due to toxic inhibition; hence, the use of these bacteria makes Microtox a rapid (5 min exposure) response bioassay. The light production of bacteria during actual testing tends to gradually (and slowly) decline over time because the bacteria are stored at 5°C in a buffer and do not grow. The *MicrotoxOmni* software package corrects for these losses. Placing control cuvette B1 in the *Reading Well* and pressing the *Set* and *Read* buttons monitors luminescence in the *Reagent*. If the control cuvette emission reads less than 90%, the *Reagent* has failed and needs to be replaced. The *Reagent* has about a 2 to 4 h window of acceptable physiological activity.

The model for computation of light emissions where toxic effects are expressed as the ratio of activity lost to activity remaining was developed and named gamma (Γ) by Johnson et al. (1974) and adopted by Microtox. Gamma is computed by the formula:

$$\Gamma = I_0/I_t - 1 \tag{1}$$

where: I_0 = light emission of the test bacteria that is lost, and I_t = the final emission produced after exposure time. The concentration of the test chemical that causes Γ to equal 1, that is when the light lost equals the light remaining, is used to compute the EC50 value for the assay. The log transformation in the Γ approach permits simple regression analyses to compute EC50 values and confidence intervals. Although a simple straightforward measurement of light emission lost due to toxicity is feasible in this assay, a precise linear relationship is obtained by plotting the log of Γ against the log of concentration. Microtox software incorporated this feature for test endpoint calculations. With a PC and a *MicrotoxOmni* software package data sets are readily collected, computed, and reported in a clear, succinct format (Fig. 8).

Both negative and positive controls are an integral part of the Microtox protocol and are essential in monitoring the natural changes in light emission by bacteria. Positive and negative controls should be performed at least once for each *Reagent* vial. All EC50 values are recorded and compared as part of QA/QC records. Coefficient of variation deviations of positive controls greater than 20% should be re-evaluated immediately for cause. A laboratory that maintains a CV less than 20% is operating within an acceptable range (Microbics, 1992). Positive controls in Microtox indicate an acceptable performance of (1) the *Reagent* and *Diluent* (2) the *Analyzer* and PC-software, and (3) the test operator skills.

The endpoint of Microtox is the effective concentration value corresponding to the concentration of toxin that produces 50% inhibition of light emission from a specific strain of bioluminescent bacteria. Because Microtox bacteria are essentially a collection of enzymes, the biochemical nature of the toxicological response whether due to a lethal or stasis reaction is unknown; hence, the term effective replaces lethal as the test endpoint designation. The final Microtox report provides an EC50 value

and a 95% confidence range that indicates the quality of the data set. This endpoint is designated EC50 in the US and IC50 (Inhibitory concentration) in Europe and Canada.

12. Factors capable of influencing performance of Microtox testing results

As in all environmental toxicological tests, macroscale or microscale, a variety of confounding factors may interfere with an assay's normal functions and compromise its validity. When Microtox malfunctions, the most commonly occurring and expected problems tend to center around sampling, temperature, assay salinity and osmotic regulation, pH, color, turbidity, and organic carrier solvents. A pre-test cleanup of the sample with various chromatographic methods may advantageous. In addition, all organic carrier solvents - negative controls - should be assayed with Microtox before attempting to dissolve and test an environmental sample (see Fig. 9). Use only a high-grade sterile dimethyl sulfoxide (DMSO) that has been stored in tightly stopped dark bottles because this carrier solvent (DMSO) is easily compromised by air, light, etc., resulting in concomitant increases in acute toxicity. Monitoring the *Analyzer's* incubator temperatures can obviate temperature problems. Assay salinity problems are usually corrected with the use of the Microtox *Osmotic Adjustment Solution*. Aqueous samples should be checked to ensure that they are within the acceptable pH range of 6.0-8.5. Color, turbidity, and sampling problems are comprehensively addressed in the Microtox Handbook (Microbics, 1992).

13. Two different applications : toxicant potentiality and toxicant bioavailabilty

The first case study used Microtox as a screening tool to investigate the potential toxicological hazard of sediment contaminants in Pensacola Bay, an estuary that covers about 270 km^2 off the Gulf coast of Florida, USA. Samples for this extensive estuary investigation by USGS and the National Oceanic and Atmospheric Agency (Johnson and Long, 1998) were first concentrated by a standard organic sediment extraction procedure with dichloromethane (APHA et al., 1998), next evaporated, and then transferred to the compatible carrier solvent DMSO. Microtox analyses determined the EC50 values and, as a result, numerous sediment residues were identified as toxic (Tab. 5). While EC50 values determined what is toxic, a toxicity reference index was designed to identify how toxic the area was. Estuary regions were designated acutely toxic when the arbitrary toxicity reference index (TRI) numbers were greater than 1. For example, the Bayou Grande region had a TRI number of 14.1 indicating that the sediment was about 14-fold more toxic than the phenol-spiked reference sediment. (The EC50 value of the phenol-spiked reference sediment divided by the EC50 value of the test sample equals the toxicity reference index number: 5.2/0.37 =14.1). This Index identified areas of toxicological concern in the estuary. Microtox with extracted sediment samples and the TRI was an efficient economical screening tool for this study.

Table 5. Sediment[a] toxicity profile of Pensacola Bay in Florida (adapted from Johnson and Long, 1998).

Location	*EC50[b]*	*TRI[c]*
Bayou Grande	0.4	14
Bayou Chico	0.5	11
Bayou Texar	0.7	8
Warrington	7.3	0.7
Bayou Channel	4.7	1
Inner Harbor	2	3
Harbor Channel	10.5	0.5
Lower Bay	10.4	0.5
Central Bay	1.8	3
East Bay	1.1	5
East Bay Extension	2.5	2
Blackwater Bay	3.3	2
Escambia Bay	4.7	1
I-70	1.5	4
River Delta	6.7	0.8
Floridatown	3.4	1
Toxicity Reference	5.2	1

[a]Dichloromethane extracts transferred to DMSO carrier solvent.
[b]Microtox EC50 = mg eq. sediment wet weight per mL.
[c]Toxicity Reference Index (TRI) = EC50 value of a phenol-spiked sediment divided by the EC50 value of the sample.

The second case study used Microtox in the SPMD-TOX paradigm (Box 4) to determine the toxicological hazards of bioavailable contaminants in Lake Tahoe and its tributaries, a large freshwater lake that covers about 500 km^2 in northern California, USA. As part of the USGS's National Water Quality Assessment (NAWQA) program, SPMD-TOX (Johnson et al., 2002), a new tandem microscale monitoring procedure, was employed to determine the effects of diverse and intensive land-use on aquatic communities. The SPMD is a semipermeable membrane device (SPMD) used to collect and concentrate waterborne bioavailable lipophilic chemicals (Huckins et al., 1996) and TOX refers to toxicity tests such as Microtox (Johnson et al., 2000). To assess the lake's potential acute toxicity, SPMD units (Fig. 13) were placed in 15 tributary streams for 30 days. The sequestered samples were recovered and dialyzed with hexane. The dialysates were transferred to DMSO for Microtox analyses. Data strongly suggested that acutely toxic substances were bioavailable in three areas: Incline Creek, North Truckee Drain, and Steamboat Creek (Tab. 6). In these studies EC50 values below 2.5 indicated sample toxicity. This Lake Tahoe study showed that SPMD-TOX was a sensitive, technically simple, and cost-effective assessment tool to monitor urban waterways for bioavailable chemical contaminants.

Box 4. The SPMD-TOX paradigm.

The tendency of organisms to accumulate and concentrate lipophilic chemical contaminants from the aquatic environment is well known (Spacie and Hamelink, 1985). To mimic this bioconcentration process Huckins et al. (1996) designed and patented the semipermeable membrane device (SPMD) as a passive abiotic integrative sampler of waterborne non-polar organic compounds. The SPMD monitors contaminant bioavailability and provides an assessment of organism exposure. The device is a low-density polyethylene lay-flat tube that contains a neutral lipid triolein to passively sample *in situ* bioavailable organic chemical contaminants from water and air (Fig. 11). The SPMD unit is typically mounted in a protective stainless steel container and shipped to and from the sampling site in a sealed metal container (Fig. 12). SPMD as an environmental contaminant-concentrating tool has many advantages: 1. SPMDs are abiotic which means they do not metabolize sequestered products but provide a true reflection of bioavailable contaminants in the environment; 2. They can "survive" in heavily polluted, toxic environments where living organisms may not survive; 3. They are not temperature specific; SPMDs can be used in both cold and warm water environments; 4. They are easily transported to sites of interest for sampling and to laboratories for processing; 5. Their retrieval and subsequent recovery of sequestered contaminants is simple; and, 6. Their use in large monitoring programs is cost-effective. The Microtox Assay with SPMDs as samplers was used in a risk assessment paradigm designated as SPMD-TOX (Fig. 13) by Johnson et al. (2000).

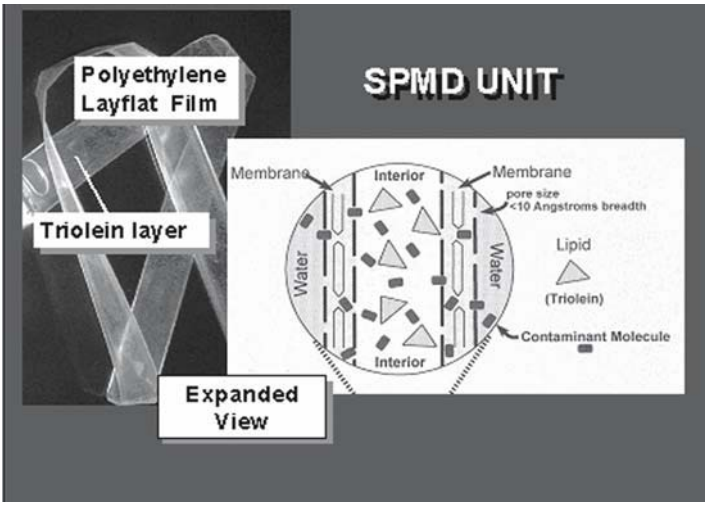


Figure 11. SPMD unit.

Table 6. Profile of SPMD-TOX dialysates[a] from tributaries of Lake Tahoe, California. EC50 values below 2.5 are designated areas of concern.

Sites	*Locations*	*EC50[b]*	*SD*
1	Glenbrook Creek at Glenbrook	8.7	2.4
2	Upper Truckee River	9.5	1
3	Taylor Creek	13.8	2.6
4	General Creek	14.1	1.6
5	Blackwood Creek at Hwy 89	15.9	1.3
6	Squaw Creek at Hwy 89	12.5	2.3
7	Incline Creek nr Crystal Bay	1	0.3
8	Truckee River blw Marble Bluff Dam	3.9	1.1
9	Truckee River at Wadsworth	3.9	0.6
10	Truckee River at Clark	4.8	0.6
11	Truckee River at Mogul	2.6	0.8
12	Truckee River nr Sparks	7.6	1
13	Truckee River at Lockwood	6.7	1.4
14	North Truckee Drain at Kleppe Ln	0.5	0.3
15	Steamboat Creek at Cleanwater Way	1.4	0.2
(Cs)	Control SPMD	>24	
(Cd)	Control DMSO	ND	
(Cb)	Control Blank	>20	
(Cp)	Control Phenol	15	2.1

[a]SPMD dialysates recovered in hexane and transferred to DMSO.
[b]Microtox EC50 = mg eq. SPMD/mL; n = 3, mean value ± SD.

These large field studies illustrate the use and the versatility of Microtox. Microtox, in both case studies, presented clear empirical evidence that identified pollutants in the sediment-water column. In the Pensacola Bay study, Microtox demonstrated the acute toxicity potentiality of the contaminant(s) and, in addition, the presence of these contaminants as sediment residue. In the Lake Tahoe study, Microtox again determined acute toxicity at the selected sites and, in addition, the bioavailability of these contaminants in the water column. Thus, this acute toxicity test provided additional information by the simple manipulation of a single element - the sample. Significantly, these simple modifications required minimal use of materials and financial resources. The organic extractions and SPMD dialysates of samples offered sensitive, technically simple, and cost-effective techniques to determine residual and bioavailable chemical contaminants. These two case studies demonstrate how to broaden the scope and breadth of information Microtox produces for environmental monitoring.

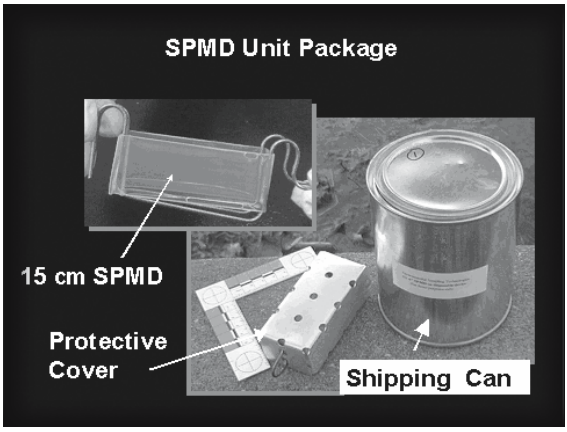


Figure 12. SPMD unit package.

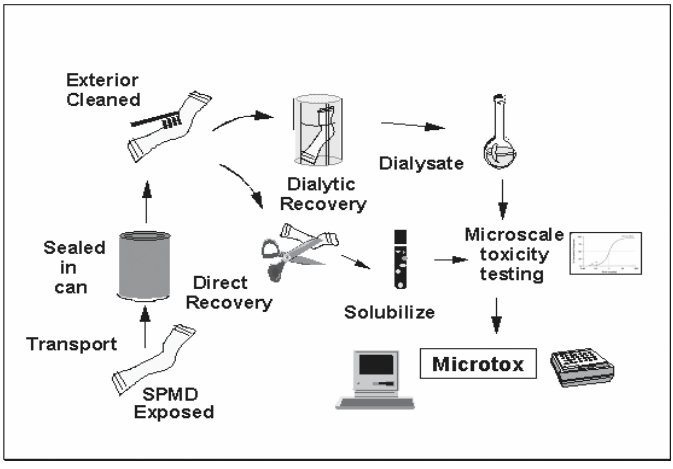


Figure 13. SPMD-TOX sample protocol.

14. Accessory/miscellaneous test information

14.1 LEVEL OF EXPERTISE IN MASTERING TECHNIQUE

Microtox is a user-friendly microscale bioassay to determine acute toxicity of aquatic samples. A modicum of intellectual curiosity, good hand-to-eye coordination, and the ability to read and follow precisely the Microtox protocols are good profiles for success. To guide the Microtox user through every conceivable aquatic test there are

well-written manuals, videos, software packages (Microbics, 1992 and *MicrotoxOmni*® software), and a web site (www.azurenv.com). The evolution from novice is, in most instances, for first-time users remarkably rapid. Using live microorganisms, reading bioluminescent emissions, testing unknown toxicants, making precise pipetting measurements and concentration dilutions, and manipulating computer software programs is simple. Academic credentials and/or laboratory experience are naturally helpful but not necessary. To interpret the data in the broad environmental picture however requires additional training and much experience.

14.2 MEDIA FOUND SUITABLE FOR TESTING

Citations covering the twenty-five year life of Microtox usage reflect its direction, depth, evolution, and diversity (Tab. 7). A national and international literature review covering the last ten years reveals nearly 900 peer-reviewed Microtox citations; over 50% of these citations were industrial-domestic wastes and leachate studies. Other studies used Microtox for toxicological assessments of industrial effluents; urban and agricultural storm waters; industrial and agricultural leachates; passive sorptive extracts; domestic and industrial wastewaters; groundwater, river, lake, and marine sediments; drilling mud and fluids; snowmelts, pesticides, oil spills, landfill leachates; soil exudates; and industrial and domestic inorganic and organic chemicals. A comprehensive topic oriented review of Microtox applications is available from the databases listed in Table 4.

14.3 TESTS TO DETERMINE THE SENSITIVITY OF MICROTOX

To assess the sensitivity of bioluminescent bacteria and validate the uses of Microtox for broad acute toxicity monitoring requires testing of many diverse chemical classes under standard protocol conditions in order to collect a valid estimation of a toxicological endpoint(s) – the EC50 value. The relative sensitivity of Microtox, to known and potential environmental contaminants both as pure chemicals and in complex mixtures as determined at CERC over many years, is shown in Tables 8 and 9. Kaiser and Palabrica (1991) provide an extensive compilation of Microtox data for over 1000 compounds. When feasible, specific chemical (or class) sensitivity should be determined before beginning a monitoring study. Intuitively, we know, or at least suspect, that Microtox may work well in some matrices and not so well in others. In many instances a simple pre-concentration step may solve a sensitivity range problem. For test validation, positive controls offer an obvious reference point for what is toxic while negative controls, such as carrier solvents, test for responsiveness and false readings. The bottom line is does the assay's range of sensitivity for potential chemical contaminants adequately meet the interest(s) and needs of the client.

Table 7. Selected literature citations of Microtox applications.

Literature citations	***Applications***
Bulich, 1979	Genesis: first paper introducing Microtox
Bulich et al., 1981	Toxicity assessment of complete effluents
Curtis et al., 1982	Predicting acute toxicity to fish
Yates & Porter, 1982	Agricultural: detection of mycotoxins
Casseri et al., 1983	Toxicity assessment of industrial waste waters
DeZwart & Sloof, 1983	Toxicity assessment of water pollutants
Plotkin & Ram, 1984	Assessment of land leachates
Bitton & Dutka, 1986	Microtox application review
Ribo & Kaiser, 1987	Test procedures and applications
Blaise et al., 1988	Trends in Canadian Environmental Protection
Kaiser & Ribo, 1988	EC50 data compilations
Mazidji et al., 1990	Waste water studies
Blaise, 1991	Microbiotest review
Munkittrick et al., 1991	Comparative species study
Kaiser & Palabrica, 1991	EC50 data compilations
Microbics Corporation, 1992	Comprehensive test protocols released
Ross, 1993	Progress review of Microtox
Isenberg, 1993	Developers commentary
Richardson, 1993	Ecotoxicology monitoring: comprehensive review
Kaiser, 1993	Comparative species study
Bengtsson & Triet, 1994	Wastewater assessments
Gailli et al., 1994	Soil assessments
Gaggi et al., l995	Battery of tests assessments
Ghosh et al., 1996	Phenol evaluation
Newman & McCloskey, 1996	Metal toxicity assessments
Qureshi et al., 1998	Current Microtox status from developers
Johnson, 1998	Microscale testing with Microtox test systems
Johnson & Long, 1998	Marine sediment toxicity assessments
Yim & Tam, 1999	Assessment of heavy metals effects on plants
Johnson et al., 2000	SPMD-TOX paradigm
Johnson et al., 2002	NAWQA urban river and stream risk assessments
Johnson et al., 2004	St. Lawrence River oil spill -SPMDs

Table 8. Microtox toxicological evaluation of polychlorinated biphenyls (PCBs), pesticides, and petroleum products (adapted from Johnson and Long, 1998).

Compound	*EC50*[a]	*95% CI*[a]
Insecticides: Organochlorine		
Aldrin	0.88	0.75 -1.05
Chlorodane (T)	1.3	1.1 - 1.5
DDE	0.97	0.8 - 1.2
DDT	1.25	1.04 - 1.52
Heptachlor	0.95	0.69 - 1.31
Hexachlorobenzene	0.63	0.54 - 0.73
Kepone	1.41	1.08 - 1.83
Lindane	1.56	1.22 - 1.99
Methoxychlor	0.86	0.78 - 0.94
Mirex	1.2	1.2 - 1.28
Pentachlorophenol	0.83	0.77 - 0.90
Toxaphene	4.9	2.6 - 9.5
Insecticides:Organophosphate		
Dyfonate	2.1	2.0 - 2.1
Malathion	0.85	0.64 - 1.1
Parathion	0.72	0.68 - 1.1
Insecticides: Carbamate		
Carbaryl	0.57	0.52 - 0.62
Carbofuran	0.91	0.74 - 1.11
Insecticides: Pyrethroid		
Permethrin	1.56	1.38 - 1.75
Herbicides: Triazine		
Atrazine	3.8	2.9 - 4.7
Simazine	4.4	3.3 - 5.8
Herbicides: Trifluralin		
Treflan	3.7	2.2 - 6.3
Herbicides: others		
Dacthal	1.3	1.0 - 1.6
Industrial: PCBs		
PCB 1248	0.55	0.51 - 0.59
PCB 1254	1.01	0.87 - 1.2
Industrial: others		
Dihexyl phthalate	82	42.2 - 159.4
Nonylphenol	0.44	0.29 - 0.65
Phenol	15.1	14.2 - 16.3

Table 8 (continued) . Microtox toxicological evaluation of polychlorinated biphenyls (PCBs), pesticides, and petroleum products (adapted from Johnson and Long, 1998).

Compound	***EC50[a]***	***95% CI[a]***
Petroleum products		
Fuel oil #2	0.06	0.04 - 0.10
Jet fuel JP4	0.12	0.10 - 0.13
Recycled motor oil	1	0.82 - 1.2
Gasoline	0.16	0.12 - 0.21
Crude oil	0.4	0.25 - 0.64

[a]5 min EC50 = µg/mL; CI = confidence interval; n= 3; DMSO carrier solvent.

Table 9. Microtox toxicological evaluation of complex mixtures containing polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), and pesticides (adapted from Johnson, 1998).

Complex mixtures	***EC50[a]***	***CI[a]***
PCBs:1242+1248+1254+1260	0.9	0.85 - 0.95
DDT+DDE+DDD	1.5	1.3 - 1.7
Kepone+Aldrin+Lindane+DDT+PCB1254	1.6	1.4 - 1.7
Phenanthrene+Chrysene+ Anthracene+Benzo(a)pyrene	0.6	0.56 - 0.59
Aminoanthracene+Benzo(a)pyrene+Aminofluorene+ 3-methylcholine	3	2.1 - 4.4
Aminoanthracene+Benzo(a)pyrene+Aldrin+DDT	1.8	1.6 - 2.0
Aldrin+DDT+Heptachlor+Endrin	1.6	1.1 - 2.2
Atrazine+DDT+Aldrin+PCB1254+ Pyrene	1.7	1.4 - 2.1
DDT+Benzo(a)pyrene+PCB1254+1260+Atrazine	2.2	1.6 - 2.9
Carbofuran+Carbaryl+Atrazine+Treflan	1.7	1.4 - 2.1
Carbofuran+Carbaryl+Atrazine+ Permethrin	1.2	0.94 - 1.5
Carbofuran+DDT+Atrazine+ Permethrin	1.6	1.5 - 1.6

[a]5 min EC50 = µg/mL; CI = 95% confidence interval; complex mixture = weight/weight; DMSO carrier solvent.

14.4 ALTERNATIVE CHOICES OF TEST SPECIES AND TEST METHODS

Toxicity testing of environmental samples may be undertaken with either macro or microscale assays. Whole animal testing with different fish and invertebrate species is usually possible if a sufficient test sample is available to support a traditional invertebrate and fish acute toxicity test. A number of investigators have compared the results obtained using Microtox with those obtained with different fish and invertebrate species. For example, when Munkittrick et al. (1991) reviewed hazard

assessments of various chemical groups, sediments, and complex effluents comparing the relative sensitivity of Microtox with tests using daphnid, rainbow trout, and fathead minnow tests, they found sample size, cost, availability, and sensitivity make Microtox the best available choice for rapid toxicological assessment of diverse environmental samples. Qureshi et al. (1998), in a recent comprehensive review of fourteen independent studies, compared the relative sensitivity of Microtox with three commonly used freshwater test species: rainbow trout, fathead minnows, and daphnids; the correlation coefficient values (a value of 1.0 equals perfect correlation) of the data sets for trout, minnows, and daphnids bioassays ranged from 0.74 to 0.89, 0.41 to 1.0, and 0.8 to 0.87 respectively, with an average of 0.85, giving an indication of the degree of similarity in data sets. These studies suggested that the predictive value of Microtox as a prescreening tool was 85% when compared with trout, minnows and daphnids.

A battery of tests could be applied to assay for suspected aquatic contaminants. The premise of this approach (Cairns 1984; Cairns et al., 1997) is that one cannot rely on a single bioassay of a "most sensitive species" to detect all aquatic hazards; different biota have different biological systems, and therefore conceivably different toxicant sensitivities. Ideally the battery would cover several trophic levels and yield no redundant data. For example, Ross (1998) explored this approach with 10 reference compounds (both organic and inorganic) using a battery of four microscale toxicity bioassays: Microtox, a bacterial bioluminescent test; *Selenastrum capricornutum,* an algal photosynthesis test; *Latuca sativa*, a lettuce root elongation test; and *Brachionus calyciflorus*, a freshwater rotifer survival test. Their study found that the four bioassays of this battery were complementary and enhanced sensitivity as well as increased both labor and material costs (argumentatively, do multiple tests really give enough additional information to warrant the increased time and costs?). A recent CERC literature review covering the last twenty years found less than 45 peer-reviewed citations that used a battery of tests for extensive toxicological biomonitoring; this suggests the jury is still out on the wide spread use and acceptance of this approach.

Other microscale acute toxicity tests are available: TOXKITs® (invertebrate on demand assays, Belgium), MetPlate® (a metal-detection test, USA; see Chapter 6, Vol. 2 of this book), ToxAlert® (a bioluminescent bacterial assay, Germany), and ToxScreen®, (a bioluminescent bacterial assay, Israel). In addition, enzyme inhibition tests (Obst et al., 1997) and immunoassays (Dankwardt et al., 1997) can be used to detect aquatic chemical contaminants. When considering an alternative test to monitor environmental toxins, the lack of commercial availability of specific assays, the absence of well-developed standard protocols, the unknown spectrum of sensitivity, the cost-effectiveness, and the absence of supportive literature should forewarn the user of possible problems.

14.5 ARE THERE ALTERNATIVE CHOICES FOR ENDPOINT DETERMINATIONS?

SDI recently introduced *Deltatox®,* a portable luminometer, with greater sensitivity to light emissions from luminescent bacteria than the *Analyzer 500*; however, the

Deltatox data is raw without gamma correction and the system lacks PC software for computation and reporting.

14.6 MICROTOX AUTOMATION POTENTIAL

During the late 1990s in Europe, first with Compagnie Generale des Eaux and later with Siemens Environmental and Yorkshire Water, the *Microtox-OS* On-line System was developed, tested, and implemented. Toxicity samplings of drinking water sources, influents and effluents from water, and sewage treatment plants were made automatically at 15-min intervals. The *Microtox-OS* On-line System had technical problems, little commercial success, and did not remain on the market long. National events and security interests will undoubtedly be a strong catalytic force in developing automated systems to protect the Nation's domestic water resources.

14.7 TEST SAMPLE THROUGHPUT

Microtox can generate large numbers of data points in a day because set up, dilution, exposure, and data reports are completed in < 30 min. One person using the 1:4 Microtox protocol can routinely test about 18 SPMD dialysates (in DMSO carrier) with one *Reagent* vial in about a half day. An individual rarely performs Microtox for a full day due to the tedium of repetition with concomitant error problems. Data analysis requires additional time. The statistical power of toxicity data is based in part on numbers: numbers in terms of sampling sites, numbers in the frequency of samplings at given site, and numbers of replicates of each sample. Resource managers often need large sample numbers to make valid environmental decisions. An attractive feature of Microtox as an environmental biomonitoring tool, yet often overlooked, is the rapid and large test sample throughput.

14.8 RELATIVE COST OF TESTING

Is the Microtox assay "cost-effective", the term frequently used in the literature to describe Microtox? Numbers are necessary for environmental monitoring. Multiple samplings increase data precision, which in turn pinpoint troublesome areas that may need immediate attention. The ecotoxicologist using Microtox can perform more intensive samplings at specific sites and between sites than is possible with other animal or plant toxicity assays. Microtox can be used universally, even in developing countries; its standardized protocol, test sample throughput, its simple technique, its prepackaged supplies, and its reliable equipment provide the numbers needed for data analyses. If a Microtox user tested 18 samples a day for 100 days in a year, 18,000 samples would have been tested in ten years. Numbers make Microtox a cost-effective assay and a simple biomonitoring tool for water resources.

This accounting exercise examines the cost of an environmental sample using a typical 1:4 dose-response series with three replicates; this 1:4 sampling design requires 30 test observations. The Microtox user needs consumables: *i.e., Reagent, Diluen*t, *Recon*, cuvettes, and pipette tips and non-consumables: *i.e.,* a SDI *Analyzer*, pipettors and a vortex mixer. The literature frequently lists the cost of consumables for a Microtox sample generally in the $50 to $100 US range (Ross, 1993). The SDI

Model 500 Analyzer, high quality and durable pipettors, and a vortex mixer are long-term investments. Over the last ten years, my Environmental Microbiology Laboratory has used two *Analyzers,* pipettors, and one vortex mixer and found the quality of the equipment both reliable and durable; the *Analyzers* malfunctioned only twice, one electrical problem and one mechanical, the pipettors needed only minor inexpensive QA/QC care, and the vortex mixer required no attention. The price - $150 - for a Microtox sample analysis includes my costs for Microtox products, equipment, overhead expenses, and labor. The number, collection, volume, transportation, and storage of environmental samples prior to testing will vary with the resource manager's needs, problems, priority and economic resources and directly influence the final cost.

14.9 DEGREE OF ATTAINED TEST STANDARDIZATION

Standardization and validation of toxicological tests are always tedious and time-consuming exercises for both the sponsoring organization and the applicant. Final acceptance and recognition by the national and international scientific communities that Microtox was a valid, reliable assay for environmental risk assessment involved a complex matrix of evaluations: experimental design, sample handling and disposal, sensitivity spectrum determinations, positive-negative control selections, QA/QC incorporations, and interlaboratory round-robin testing. A key element for Microtox occurred in 1984 when the Organization for Economic Cooperation and Development (OECD) accepted Microtox as part of a combined bioassay-chemical paradigm to assess the biohazards of industrial chemical contaminants in aquatic ecosystems (OECD, 1984). L'Association Francaise de Normalisation (AFNOR), American Society for Testing and Materials (ASTM), Deutsches Institut Normung (DIN), International Standards Organization (ISO), and the Organization for Economic Cooperation and Development (OECD) have disseminated Microtox protocols and led, promoted, and contributed to multiple environmental uses of Microtox for toxicological assessments (Tab. 10). Over the last twenty-five years Microtox has drawn both national and international attention as a multifaceted toxicological monitoring tool because of its broad range of sensitivity to known environmental contaminants, its microscale protocol, simplicity, and cost-effectiveness per unit test, its successful use for screening and ranking environmental samples, its support of regulatory compliance, and its ability to predict the outcome of other environmental bioassays.

Table 10. Status of Microtox: regulations and standards (adapted from Qureshi et al., 1998).

Organizations	***Applications***	***Status***
Energy Resources Conservation Board, Canada	Drilling waste	Guide
Inter-government Aquatic Toxicity Group, Canada	Effluent	Final
International Standards Organization, France	Effluent	Process
L'Association Francaise de Normalisation, France	Effluent	Standard
Deutsches Institut fur Normung , Germany	Effluent	Standard
National Government Lab. & Research Institute, Italy	Effluent	Process
Netherlands Normalization Institute , The Netherlands	Effluent	Final
Environmental Protection Agency, Mexico	Wastewater	Standard
Environmental Protection Agency, Spain	Leachate	Standard
Environmental Protection Agency, Sweden	Effluent	Issued
Environment Agency, United Kingdom	Effluent	Process
American Society for Testing and Materials, USA	Wastewater	Issued
United States Public Health Service, USA	Wastewater	Issued

Table 11. Microtox® toxicity test system (adapted from Johnson, 1998).

Microtox	*Basic*	*Solid-phase*	*Chronic*	*Mutatox*
Toxicity test	Acute	Acute	Chronic	Genotoxic
Vibrio fisheri	NRRL B-11177	NRRL B-11177	NRRL B-11177	Dark M169
Sample type	Liquid[a]	Solid[b]	Liquid[a]	Liquid[a]
Test medium	Buffer	Buffer	Nutrients	Nutrients[c]
Growth phase	Stationary	Stationary	Log	Log
Design	Dose-response	Dose-response	Dose-response	Dose-response
Test duration	30 min	30 min	< 24 h	< 24 h
Test endpoint	< Light	< Light	< Light	> Light
Tox designation	EC 50[d]	EC 50[d]	LOEC[e]	Genotoxic[f]
Software	Yes	Yes	Developmental	Yes
Development	In common use	In common use	Introductory	Experimental
Sensitivity	Broad spectrum	Broad spectrum[g]	Experimental	Experimental
Data base	Broad	Expanding	Experimental	Experimental

[a]Wastewater, porewater, dialysates, compatible organic solvent extracts.
[b]Soil or sediment samples.
[c]Rat hepatic S9 fractions added for metabolic activation phase.
[d]EC50 = effective concentration with 50% loss of light.
[e]LOEC = Lowest Observable Effect Concentration.
[f]Genotoxic = two or more positive responses in a dilution series.
[g]Clay and turbidity questions.

14.10 ADDITIONAL USES AND ENDPOINT DETERMINATIONS

Microtox has expanded and the Microtox® Test System (Tab. 11) today includes four toxicity tests: Microtox® Acute Toxicity Test (described here), Microtox® Solid-Phase Toxicity Test (see Chapter 2 of this volume)), Microtox® Chronic Toxicity Test, and Mutatox® Genotoxicity Test. The four bioassays are all based upon the measurement of luminescent bacteria light emissions but differ in the strain selection (wild type versus dark mutant), the sample presentation (liquid versus solid), the growth cycle (stationary versus log), the duration (minutes versus hours), the changes in bioluminescent emissions (decrease versus increase), and the toxicological endpoints (lethality versus genotoxicity) (Johnson, 1998). The Microtox® Test System can be considered a battery of tests, all used as rapid screening assays, to detect the presence of toxic substances in the biosphere - water, soil, sediment, and air.

15. Conclusions

Microtox, a widely used biomonitoring tool for aquatic contaminants, is an acute toxicity test, a screening tool, and a stand-alone bioassay worthy of emulation. Microtox is an on demand test that is simple, rapid, and cost-effective with readily available biota, a sensitivity spectrum clearly defined, a standardized method and comprehensive tutorial protocol software. The Microtox assay is user friendly and easy to run, tabulate, and report data in a timely manner. Monitoring and screening tests such as Microtox are not surrogates; they cannot replace the more expensive bioassays that use native species of interest. However, this biomonitoring test can be viewed as a microscale biosensor expressly designed and used to detect a broad spectrum of environmental chemical contaminants. While the value of environmental relevance and the spectrum of sensitivity favor the macroscale test with native fish and invertebrate species of concern, the microscale test provides large sample capacity, speed, and cost-effectiveness.

Microtox has been accepted as a toxicological biomonitoring tool with multiple applications as reflected in the nearly 1000 published peer-reviewed reports in the last 10 years. Isenberg (1993) and his founding colleagues did “metaphorically speaking” develop a bioassay in which the biota “could speak” and “could be placed in suspended animation” for “on demand” availability. The success of Microtox ushered in a new far-reaching revolution in microscale bioassays, produced a paradigm shift in test organisms, and, most importantly, introduced a new biomonitoring tool in environmental toxicology. The future uses and directions of Microtox and the microscale concept of toxicity monitoring are essentially a function of the user’s creativity.

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Abbreviations

AFNOR	Association Française de Normalisation
APHA	American Public Health Association
ASTM	American Society for Testing and Materials
CAS	Chemical Abstracts Service
CERC	Columbia Environmental Research Center
CI	Confidence Interval
CV	Coefficient of Variation
DCM	dichloromethane
DDD	dichlorodiphenyldichloroethane
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DIN	Deutsches Institut Normung
DMSO	Dimethyl sulfoxide
2,6,DNT	2,6, dinitrotoluene
FMN	Flavin mononucleotide
GLP	Good Laboratory Practices
ISO	International Standards Organization
MMSDS	Manufacturer's Material Safety Data Sheets
NADP	Nicotinamide adenine dinucleotide phosphate
NAWQA	National Water Quality Assessment
PAHs	Poly Aromatic Hydrocarbons
PC	Personal computer
PCBs	PolyChlorinated Biphenyls
OECD	Organization for Economic Cooperation and Development
QA/QC	Quality Assurance and Quality Control
SDI	Strategic Diagnostics Inc.
SOP	Standard Operating Procedure
SPMD	Semipermeable membrane device

TIEs	Toxicity Identification Evaluations
TREs	Toxicity Reduction Evaluations
USGS	U.S Geological Survey