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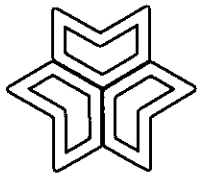

Environmental Engineering

Establishment of a Microtox
Laboratory and Presentation of
Several Case Studies
Using Microtox Data

Env.Eng.Report No. 77-83-8

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Technical Report

Establishment of a Microtox Laboratory and Presentation of Several Case Studies Using Microtox Data

by

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Division of Water Pollution Control
Anthony D. Cortese, Sc.D., Commissioner
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II. Executive Summary

The Microtox toxicity analyzer (Beckman Instruments, Inc; Carlsbad, CA) has been proposed as an alternative testing system to more conventional methods of assessing aquatic toxicity which use fish, invertebrates, or algae as test organisms. The Microtox system employs lyophilized marine bacteria, which, upon reconstitution, emit a constant level of light. When exposed to a toxicant, the level of bioluminescence is diminished in direct proportion to the toxicant concentration. The Microtox toxicity analyzer is equipped with a refrigerated reaction chamber, a precision photometer for measuring light output, and a digital display to monitor the instrument's functions. Relative toxicity is expressed as an EC50 value, or 'effective concentration' causing a 50 percent diminution in light output in a stated exposure period. Other criteria, such as an EC10 or EC25 may be used when a more conservative approach is desired.

The Microtox test has several advantages over conventional fish or daphnid acute toxicity tests, including: 1) usage of a statistically larger test population (more than 10^5 bacteria per test); 2) small sample requirements,:and 3) comparable precision and accuracy to other methods of measuring aqueous toxicity, at a fraction of the cost.

The type of sample collected for Microtox analysis is left to the discretion of the sampling program. Approximately one liter of sample should be collected in a clean, unused borosilicate glass container equipped with a teflon lined cap. All samples should be stored in a closed container at approximately $5^{\circ}C$ and analyzed as soon as possible, preferably within twenty-four hours.

The first step in the Microtox analysis is the reconstitution of a lyophilized bacterium (Photobacterium phosphoreum). These bioluminescent bacteria are then exposed to a range of toxicant concentrations. Light output is measured with a precision photometer after some predetermined exposure period, and compared with initial light output and reagent blanks to determine the toxicant concentration causing an EC50.

Microtox data can be analyzed with graphical methods similar to those utilized in other toxicity testing procedures. The manufacturer recommends the use of the gamma function, γ, which is defined as the ratio of the amount of light lost in a given exposure period to the amount of light remaining at the end of the test, to determine the EC50 value. The EC50 value corresponds to a gamma value of unity. This function reportedly produces a more linear plot than other techniques, and simplifies data analysis.

During its two year operation of a Microtox toxicity testing laboratory (1982-1984), the University of Massachusetts has analyzed

21 samples using the Microtox system. Several of these tests were in conjunction with fish, daphnid and algal bioassays. This report presents data for these 21 samples, four of which were analyzed concurrently using Microtox, fish, and daphnid bioassays. The Microtox system was the most sensitive test in all but one of the four multiple assays. The fish toxicity test was the least sensitive in all cases. In no case did the Microtox test fail to detect toxicity in samples showing a toxic response using fish or daphnids as test organisms.

In addition, several chemicals were investigated for their potential to exhibit a synergistic response with a few selected toxicants, in an attempt to increase the sensitivity of the Microtox test (Appendix B). The chemical components were tested singly, and in combinations of two, three and four chemicals. The toxic effects exerted by single solute systems were additive for all two component mixtures examined. The interactions within three and four chemical component systems were variable. None of the three compounds investigated (chloramphenicol, methylene blue, achromycin) enhanced the sensitivity of the Microtox test via synergistic reactions with the test compounds.

The Microtox test is considerably less expensive and quicker to conduct than fish, algal or daphnid bioassays. Approximately two hours and 15 minutes are required for an entire Microtox analysis as compared to a minimum of 48 and 96 hours for daphnid and fish toxicity tests, respectively. A single technician should be able to conduct about ten Microtox assays per week or 500 per year. The associated cost of establishing such a bioassay laboratory is $21,000 (1983 dollars) including the initial capital investment for the Microtox instrument and supply costs, but excluding personnel charges. Each additional year's worth of supplies for 500 samples costs about $11,000 (1983 dollars). If the direct costs of establishing a Microtox laboratory are distributed over one year (excluding interest), then the cost per test is $72, assuming only one technician, at a salary of $15,000/year, is needed to perform 500 analyses in that time. The cost per analysis, excluding the Microtox instrument capitol investment, is $52 (1983 dollars).

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VI. Introduction

The proliferation of synthetic chemicals resulting from our expanding industrialized economy has led to the entry of toxic compounds into the aquatic environment. The direct adverse effects of these contaminants on aquatic life include acute, sub-acute, and chronic toxicological hazards. Additionally, introduction of pollutants into the aquatic environment results in a decrease of aesthetic quality attributable to odor, color, and foaming, and stresses the system's self purifying capacity. Growing awareness of the deleterious effects of these contaminants on aquatic life has prompted state and federal agencies to develop technologies and methods to prevent, control, abate, and detect such pollution.

Toxicity is the ability of a chemical to adversely affect the life process. The minimum requirement for monitoring toxicity is a set of interdependent enzyme systems controlling measurable physiological patterns (Beckman, 1980). Toxicity tests determine the concentration of a chemical or percentage of some complex waste which causes either death, or some altered physiological process reflecting interference with the normal life cycle of the test organism. The established methods for detecting toxicants in water utilize fish, invertebrates, or algae as the test organisms. These toxicity tests can take many forms, which, depending upon the test organism, include: 1) acute; 2) chronic; 3) sub-chronic embryo-larval; 4) early juvenile; 5) avoidance; 6) respiratory activity; and 7) blood chemistry tests. There are many shortcomings inherent in these testing techniques. They are time and labor intensive (from 48 hours to 21 days to complete), and require large volumes of sample (up to 60 liters). Only a small number of organisms (ten per vessel) are usually tested resulting in a small test population and subsequently wide statistical confidence intervals. Fish and daphnids may additionally be subject to such variations as age, size, and level of stress.

Since light can be measured with a high degree of sensitivity and accuracy, a bioluminescent organism whose light is diminished in direct proportion to a toxicant exposure is an ideal test organism for assessing aquatic toxicity. Bioluminescence is the emission of light by organisms. Representatives of nearly every animal phylum and most plants, including photosynthetic organisms exhibit bioluminescence (Strehler, 1968). Some of the most thoroughly studied bioluminescent organisms include the firefly Photinus pyralis and the luminous bacteria.

The existence of luminescent bacteria has been known for over 300 years, first being reported in 1592 by Fabricus Aquapendente (Strehler, 1968). Luminous bacteria emit light through an oxidation of reduced flavin mononucleotide ($FMNH_2$) by molecular oxygen. This reaction, a branch of the electron transport chain, is catalyzed by

the enzyme luciferace, and is accompanied by the oxidation of a long-chain aliphatic aldehyde (Nealson and Hastings, 1979). Many species of luminescent bacteria emit light at a constant level under ideal conditions. In the presence of an antibacterial substance or toxicant, however, the amount of light emitted decreases an amount proportional to the concentration of the toxicant (Bulich and Greene, 1979). This phenomenon makes luminescent bacteria ideal candidates for the assessment and quantification of toxic substances.

For these reasons, bioluminescent bacteria have been suggested as an alternative test organism for the rapid and simple determination of toxicity in an aqueous sample. In recent years there has been extensive work in developing a bacterial bioluminescence test for detecting the presence of aqueous toxicants. In contrast to traditional methods of assessing aquatic toxicity, this analysis is reported to be rapid, reliable, inexpensive, and easy to perform. In addition, it requires a small volume of sample and in many cases is as, or more sensitive than conventional testing procedures (Bulich and Green, 1979; Bulich et al., 1979; Qureshi et al., 1980). The Microtox toxicity testing system, developed by Beckman Instruments Incorporated[1] in the late 1970's, represents the latest development in this technology.

The Microtox toxicity analyzer employs a lyopholized (freeze dried) marine bacterium (Photobacterium phosphoreum) which, upon reconstitution, emits a constant level of light. Upon exposure to a toxicant, the level of bioluminescence is diminished in direct proportion to the toxicant concentration. The lyopholized bacterial population represents several advantages, as a test population, over conventional fish and daphnid bioassays. These include: increased population size; uniform population characteristics; and greater reproducibility and reliability. It is additionally a very fast, simple, and sensitive technique.

The Beckman system includes a precision photometer equipped with a digital display and incubated reaction chamber, in addition to an output for an auxiliary strip chart recorder. Data are reported either as EC50 values (percent effluent or toxicant concentration causing a 50 percent reduction in light output for a

1. Beckman Instruments, Microbics Operations, 6200 El Camino Real, Carlsbad, California 92008; 619-438-9151)

stated time interval), or as any percent light diminution in a stated time period (10, 90, 99 percent, etc.). For example, a 30EC90 value would represent a 90 percent reduction in light output after 30 minutes of contact between the photobacteria and toxicant solution. EC values are comparable to LC (lethal concentration) values used in more conventional toxicity testing techniques. Additional methods for representing toxicity values are discussed later in this report.

VII. Literature Review

Luminescent bacteria were first used for the detection of antibacterial substances in the early 1940's (Rake, et al., 1943; Kavanagh, 1947). In the mid-1960's, the use of bioluminescent bacteria was expanded as methods were developed for detecting the presence of toxic substances in the air using luminescent bacteria (Serat et al., 1969). The method was found to be easy to use and provided a sensitive, reliable indication of the presence of toxicants.

Several researchers have compared the Microtox bacterial bioluminescence toxicity test to other more conventional methods of assessing aquatic toxicity. Bacterial 5EC50's for 68 organic compounds were measured and compared statistically to 96 hour LC50's (96LC50) for fathead minnows by Curtis et al. (1982). They found the Microtox test to have precision equal to or greater than traditional fish toxicity tests, with a direct relationship between compound toxicity to bioluminescent bacteria and fish. Bulich et al. (1979) compared Microtox 5EC50 data for pure compounds with fish 96LC50 values found in the literature. In addition they simultaneously tested 50 complex waste samples with Microtox and fish. The data exhibited a good correlation between the two testing procedures. The authors also investigated the reproducibility of the Microtox assay, using sodium lauryl sulfate as a standard. The average 5EC50 after 81 determinations was equal to 1.57 mg/L with a standard deviation and coefficient of variation of 0.28 mg/L and 18.2 percent, respectively. Similar data, in terms of toxicant and sample size, are not available for fish and invertebrate toxicity tests. The United States EPA (1981), however, conducted multiple sets of laboratory tests consisting of static and dynamic aquatic bioassays with two species of fish and static tests with Daphnia magna tested in duplicate. The mean daphnid 48LC50 and coefficient of variation for replicate analyses of silver within a lab ranged from 0.525-47 µg/L and 4.21-27.9 percent, respectively. For fathead minnows, the mean static 96LC50 for silver and coefficient of variation ranged from 9.5-250 µg/L and 8.0-29.5 percent, respectively.

Vasseur et al. (1983) assayed 162 industrial wastewaters using Microtox, in many cases in conjunction with daphnid toxicity tests. Every sample which was toxic to Microtox (effluents which displayed ten minute EC50 values) was also toxic to daphnids (effluents which displayed a 24 hour LC50). Twelve percent of the samples which were non-toxic to Microtox displayed toxicity to daphnids. Microtox was found to be more sensitive than the daphnid test, especially in the case of organic compounds. The authors also tested the Microtox system for reproducibility with these effluents. With three replicates of each sample, the average coefficient of variation for Microtox was 27.6 percent. This is higher than the value calculated

by Bulich et al. (1979), but may be attributable to the smaller number of replicates utilized in the study.

Samak and Noiseux (1980) tested individual compounds and a complex petrochemical industrial wastewater using Microtox and zebra fish toxicity tests. The effluent was tested at various pH values to determine the sensitivity of Microtox to this parameter. The Microtrox response was stable between pH values of 5.5 and 8. The correlation coefficient between zebra fish 72LC50 values and Microtox 5EC50 values was 0.884.

Peltier and Weber, (1980) conducted numerous bioassays using the Microtox system as well as fish, and invertebrates as test organisms. They found that about 75 percent of the samples toxic to fish showed toxicity with the Microtox method. Fish were more sensitive than Microtox in about half of the tests where both methods indicated toxicity. Of the 48 samples found toxic to invertebrates, 30 were also toxic to Microtox. Invertebrates were more sensitive than Microtox in 70 percent of these 30 samples. Of the 18 samples missed by Microtox, only two were strongly toxic to invertebrates. The luminescent bacteria test was found to be an excellent screening test by Qureshi et al. (1980), but they noted that it did not perform as well for wastewaters containing certain specific compounds such as cyanide and ammonia.

Neiheisel, et al. (1982) conducted toxicity tests with fathead minnows, daphnids, and the Microtox bacterial toxicity assay on influent and effluent samples from two conventional activated sludge wastewater treatment plants. A mixture of 16 volatile priority pollutants was added to the influent of one plant while the second was operated as a control. They found that there was a significant reduction in toxicity in the secondary effluent of both systems compared to the influent and primary effluents. Fish, daphnid, and Microtox test values were similar for secondary effluents, indicating little or no toxicity. There was no difference in toxicity, with a few exceptions, between samples taken from the influent or primary effluent of the treatment systems. The Microtox test, however, was consistently more sensitive than the fish or daphnid tests for influent and primary effluent samples. The fathead minnow 96 hour and the daphnid 48 hour tests yielded similar toxicity values for comparable samples. The Microtox test was more sensitive in all cases, with lower 5EC50 values than the LC50 values achieved with the other tests.

Beckman Instruments, Incorporated (1983) presented comparative acute toxicity test data for Microtox, fish, and daphnid bioassays of complex effluents. Of 257 samples tested, 235 were assayed simultaneously with fathead minnows and Microtox and 155 were analyzed with both daphnid and Microtox toxicity tests. The Microtox and fish toxicity tests both detected toxicity (EC50 or LC50 $\leq$ 50) in 87 percent of the 235 samples jointly tested. The

daphnid and Microtox tests both detected toxicity in 75 percent of the 155 samples tested simultaneously. The toxicity values were within 2.5 orders of magnitude for 97.5 percent of the fish vs. Microtox results and 96.1 percent of the daphnid vs. Microtox results.

Lebsack et al. (1981) tested fossil fuel process waters with both the Microtox bacterial bioluminescence test and fish toxicity tests and observed the bacterial system to be more sensitive in three of nine cases. The obtained EC50 and LC50 values were similar in most cases, usually being within a factor of two of each other. In another study, Strosher et al. (1980) found that bioluminescent bacteria were more sensitive than fish to hydrocarbons such as diesel fuel, as well as more responsive to small changes in concentration of the toxicant. The authors suggested that this test could be very useful in studying the joint toxicities or synergistic effects of compounds.

Chang et al. (1981) tested a variety of compounds with the Microtox system and found it to have a correlation coefficient of 0.9 and 1.0 with rat and fish tests, respectively, for detecting toxicity. They found Microtox to have the advantage of a short test period and the statistical advantage of utilizing more than 10^5 test organisms per analysis. Dutka and Kwan (1981) compared Microtox to three other bacterial toxicity tests utilizing Spirillum volutans, Psuedomonas flourescens, and Aeromonas hydrophila. They found a great deal of variation in the sensitivity patterns of the four microbial tests, but Microtox was the most sensitive in a majority of cases. They concluded that the Microtox system was a sensitive toxicity assay procedure with its major benefit being quick turnaround time.

To determine the reproducibility of Microtox data, Beckman (1983) performed 30 separate assays of sodium pentachlorophenate using 30 separate vials of Microtox reagent. The mean 5EC50 and 15EC50 were 0.468 and 0.351 mg/L, respectively. The 5EC50 had a standard deviation of 0.052 mg/L with a coefficient of variation of 11 percent while the 15EC50 data showed a standard deviation of 0.041 and a coefficient of variation of 12 percent.

Additional research and discussion of the Microtox system was presented at the First International Symposium on Toxicity Testing Using Bacteria, held by the Canada Centre for Inland Waters (1983). Indorato, et al. (1983) analyzed 13 chemical compounds with the Microtox system and combined the results with the literature database to correlate fish LC50 and Microtox EC50 values. The data were found to compare favorably, showing the Microtox test to be a useful screening technique for determining the relative toxicity of new or untested chemicals. The authors also developed a mathematical correlation model to determine the need for performing

more complex and expensive fish tests. Mallak and Brunker (1983) compared the Microtox toxicity test to an *in vitro* enzyme assay by determining the toxicity of several metal working fluid preservatives. Overall, the Microtox system was more sensitive, and was found to have EC50 values within 25 percent of fish 96 hour LC50 values for most of the biocides tested.

In summary, the bacterial bioluminescence test represents the latest advance in the field of aquatic toxicity testing. The Microtox system is reported to be quick and easy to use, requiring only a few milliliters of sample and about 30 minutes to perform, and has precision and an ability to detect toxicity which compares with conventional testing methods at a fraction of the cost.

VIII. Procedure

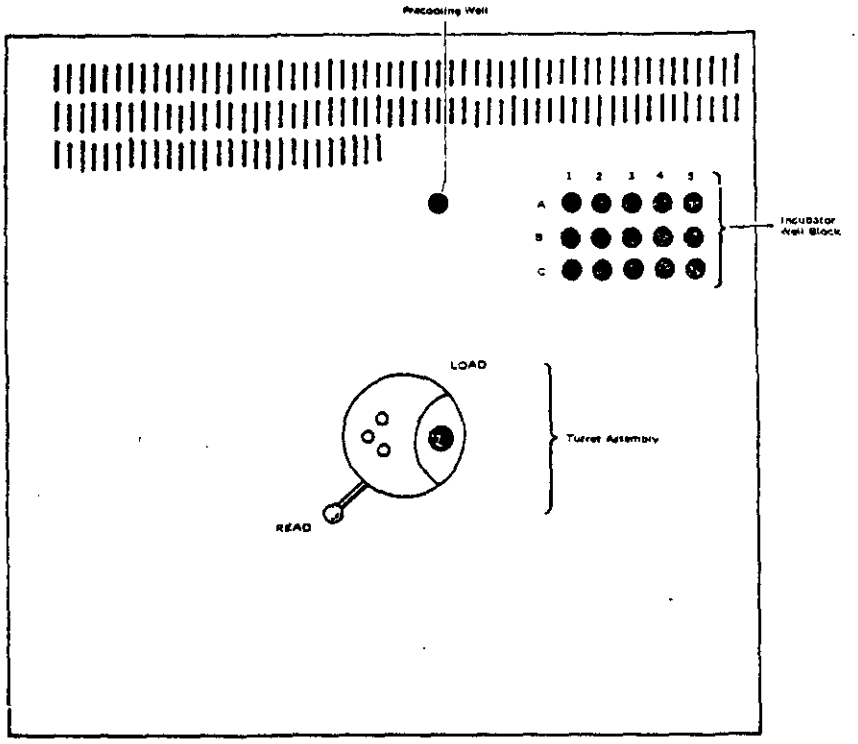
The basic components of the Beckman Microtox system are the lyophilized bacteria, Photobacterium phosphoreum (Reagent), a solution for reconstituting these organisms (Reconstitution Solution), and a precision photometer equipped with a refrigerated chamber in which the test is conducted and a digital display which monitors the functions of the instrument. In addition, an optional strip chart recorder is recommended to provide a permanent graphical display of the test results.

The degree of sample preparation for Microtox analysis depends upon the characteristics of the material being tested. Highly toxic aqueous samples may require dilution prior to analysis to bring light diminution into the 50 percent range. Microtox diluent (Diluent) is recommended for sample pre-dilution since this solution is used to prepare further serial dilutions later in the Microtox analysis. A rule of thumb used in other toxicity testing procedures is that the dissolved oxygen concentration should not fall below 5 mg/L. It has been the experience of this laboratory that adequate dissolved oxygen is introduced through sample preparation and dilution. Finally, the sample must be adjusted to the proper osmotic pressure for the marine bacterium used in the test, by the addition of osmotic adjusting solution (Osmotic Adjusting Solution).

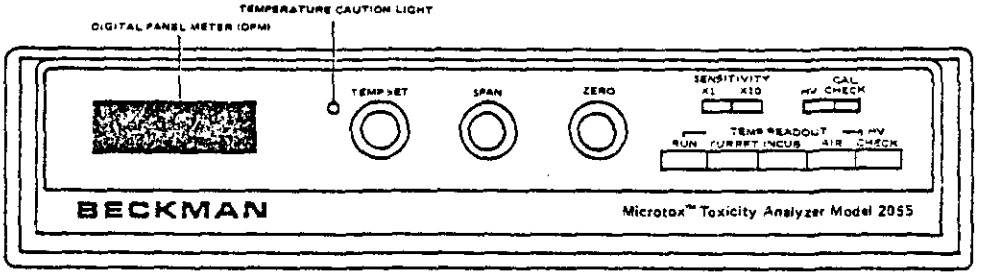
If the sample is highly colored it may affect the results of the analysis. Microtox has developed a special color correction procedure to compensate for these effects. Uncolored samples are analyzed with the standard procedure.

In order to obtain consistency in the bacterial inoculum utilized in the Microtox procedure, Beckman has developed a method of lyophilyzing (freeze drying) the bacterium Photobacterium phosphoreum. The first step in the analysis is the reconstitution of these bacteria to obtain a single batch, large population that possesses enhanced statistical properties over conventional organisms utilized in toxicity testing. Due to the volume of solutions used to reconstitute the bacteria and adjust the osmolality of the solution, the maximum percentage of an aqueous sample that can be tested with this procedure is 45.

Once the bacteria are reconstituted they emit a fairly constant level of light. The reconstituted bacteria are kept in the refrigerated incubator well block of the instrument which is maintained at $15^{o}C$. There are 15 wells in the block (Figure 1); A1-A5, B1-B5, and C1-C5. The B and C wells will ultimately contain equal dilutions of the reconstituted Reagent for testing, while the A wells will ultimately contain serial dilutions of the sample to be tested. Wells B1 and C1 will be used as controls while wells B2-B5 and C2-C5 will receive doses of sample transferred from wells A2-A5.



Controls and Features



Front Panel Controls and Indicators.

Figure 1. Schematic Diagram of the Microtox System (After Beckman, 1982b)

After a specified time period of exposure of the bacteria to the sample, light readings are taken by transferring the cuvettes from the incubator wells to the turret assembly which links the cuvette with the photomultiplier tube. The light output of the organisms is measured and compared to the blanks so that the light decrease attributable to the sample being tested can be determined.

The type and volume of sample collected for Microtox analysis is dependent on the water being sampled, and the information which is needed by the sampling program. In general, composite samples can yield general information about continuous effluents, but tend to mask or miss plugs of toxic substances. On the other hand, grab samples are only representative of the time of sampling. Ultimately, the sample type is left to the discretion of the investigator.

The Microtox test requires only three milliliters of sample per analysis, and the majority of the sample volume is therefore needed for other water quality analyses performed. Water quality parameters which should be determined on samples being analyzed by the Microtox test include: pH, alkalinity, hardness, conductivity, and dissolved oxygen. These parameters have been shown to affect sample toxicity, and should be reported along with toxicity test results. A one liter sample is sufficient to satisfy the requirements of these analyses.

Samples should be collected in clean, previously unused borosilicate glass containers with teflon lined caps, stored in a closed container at approximately $5^{o}C$, and if possible, analyzed within twenty-four hours.

The test can be completed in as little as five minutes, but may be extended several hours if desired. The duration of the test has been extended to three hours in the Environmental Engineering Laboratory with no apparent complications. Once the test is terminated, the light output data is analyzed to determine the EC50 for the sample. A decrease in light output of 50 percent is chosen by convention, and is not necessarily the best parameter. For some applications an EC10 or EC25 may be preferable if greater sensitivity is warranted. A modified version of the manufacturer's recommended procedures (Beckman, 1982b) as well as several procedural modifications developed in this laboratory are as follows:

A. Analyzer Preparation

1. Check turret and incubator temperatures.

2. Set controls and zero instrument.

3. Place new cuvettes in incubator wells.

4. Place a new cuvette in the precooling well and pipet 1.0 ml Microtox Reconsitution Solution into this cuvette.

5. For 2:1 serial dilutions pipet 2.5 ml Microtox Diluent into cuvettes A1 through A4. A1 is the non-toxic control while A2 through A4 will ultimately contain sample serial dilutions.

6. Pipet 0.5 ml Microtox Diluent into cuvettes B1 through B5 and C1 through C5. These cuvettes will ultimately contain equal dilutions of reconstituted Reagent for testing.

B. Sample Dilution Preparation

1. Adjust sample osmolality to two percent NaCl (by weight).

2. Make primary dilution of sample with Microtox Diluent if necessary.

3. Pipet 1.5 ml of sample into cuvettes A4 and A5. Cuvette A4 now contains 3.0 ml total.

4. Mix the contents of A4 by carefully aspirating and dispensing with the 500 µL pipet.

5. Transfer 1.5 ml from A4 to A3 and mix as in step 4.

6. Transfer 2.5 ml from A3 to A2 and mix as in step 4.

7. Aspirate 1.0 ml of the contents of A2 with a pipet and discard. The volume of A2 is now 2.0 ml.

8. Wait five minutes or more for thermal equilibrium.

C. Reconstitution of Microtox Reagent

1. Do not begin reconstitution until the Reconstitution Solution has been in the precooling well for at least five minutes.

2. Remove one vial of Microtox Reagent from the refrigerator.

3. To minimize warming, quickly remove cap and stopper and shake dry pellet to bottom of vial.

4. Pour the precooled Reconstitution Solution into the Reagent vial by rapidly inverting the cuvette. Mix by swirling for two to three seconds while holding the vial from the top to minimize warming.

5. Pour the Reconstituted Reagent back into the cuvette used to cool the Reconstitution Solution and replace cuvette in precooling well.

6. Immediately mix by aspirating and dispensing with the 500 µL pipet about 20 times.

D. Dilution of the Microtox Reagent

1. If a recorder is used, mark the start of this sequence.

2. Without removing the cuvette, aspirate 10 µL of Microtox Reagent.

3. Remove excess solution from pipet tip with a Kimwipe, being careful not to touch the opening.

4. Dispense the Reagent into cuvette B1. Transfer 10 µL Reagent into cuvettes B2 through B5 and C1 through C5 in the same manner using the same pipet tip.

5. Mix the contents of each cuvette by aspirating and dispensing with a 250 µL pipet five times.

E. Equilibration Period of the Diluted Microtox Reagent

1. Allow the cuvettes to equilibrate for at least 15 minutes.

F. Assay Procedure with Duplicate Determinations

1. After the equilibration period, depress X1 Sensitivity. The SPAN (100 percent ADJ) dial may be set to about four turns at this time if desired as a rough first estimate.

2. Transfer the cuvette from well B2 into the turret well and close the turret (read position).

3. Adjust the SPAN (100 percent ADJ) dial for a DPM reading of approximately 090 (90 percent on recorder scale).

4. Open the turret and replace cuvette B1 in its incubator well.

5. Place cuvette C1 in the turret well, close the turret, and record the light reading for approximately five seconds. Repeat this procedure for cuvette B2. If either C1 or B2 readings read less than 100 on the DPM, continue cycling the cuvettes in the order C2, B3, C3, B4, C4, B5, C5. If the C1 and B2 readings are both over 100 on the DPM, use the SPAN (100 percent ADJ) dial to adjust the B2 reading to 090 on the DPM and return to step 2.

6. Verify that the cuvettes in each column (1, 2, 3, etc.) contain at least one reading between 080 and 100 on the DPM. The cuvettes may be re-ordered and re-cycled if necessary.

7. Immediately pipet 500 µL Microtox Diluent from cuvette A1 to B1 and from A1 to C1, without removing cuvettes from wells. Mix each cuvette by aspirating and dispensing five times.

8. Using the procedure described in step 7 make the following sample dilution transfers:

   500 µL from: A2 to B2, A2 to C2

   A3 to B3, A3 to C3

   A4 to B4, A4 to C4

   A5 to B5, A5 to C5

   The same pipet tip can be used if the dilutions are made in the listed order. Perform all light readings within the same time frame required for transfer and mixing in steps 7 and 8.

9. At 5 and 15 minute intervals after starting step 7, take light readings following the procedure in step 5. Tabulate and reduce the initial data from step 3 and five minute data from step 9 while waiting for the next cycle. Verify that the blank ratios agree within 0.02. Tabulate the 15 minute data as soon as it is obtained.

G. Absorbance Correction Management For Highly Colored Aqueous Samples.

1. Pipet 1.5 ml Microtox Diluent into the outer chamber of a clean Absorbance Correction Cell (ACC) and place it in the turret well.

2. Pipet 1.0 ml Microtox Diluent into a standard cuvette and place it in incubator well A1.

3. Pipet 2.0 ml sample of chosen concentration, normally the highest assayed, into each of two standard cuvettes and place them in incubator wells C1 and C2.

4. Fill the other incubator wells with clean cuvettes.

5. Wait five minutes or longer for equilibration.

6. Pipet 50 µL of Reconstituted Reagent into cuvette A1. Mix the contents of A1 by aspirating and dispensing with the 500 µL pipet five times.

7. Lift the ACC out of the turret well long enough to transfer enough cell suspension from cuvette A1 into the inner chamber of the ACC to provide a liquid level approximately equal to that of the Diluent in the outer chamber. Immediately return the ACC to the turret well to minimize warming.

8. Close the turret (Read position). Set the SPAN (100 percent ADJ) dial for a reading of 90 percent and record the light level to establish a steady base line reading. Reset to 090 if the output drops below 070 and record for five more minutes.

9. Open the turret but do not remove the ACC, use a plastic aspirator to remove as much Diluent as possible from the outer chamber.

10. With the ACC still in the turret, transfer 0.5 ml to 1.5 ml of test sample from cuvette C1 into the outer chamber of the ACC.

11. Remove as much sample as possible with the aspirator.

12. With the ACC still in the turret, use a pipet to transfer 1.5 ml of test sample from cuvette C2 to the outer chamber of the ACC.

13. Close the turret (READ position) and record the light level for ten minutes or longer.

H. Precautions

1. A cuvette of Diluent should be kept in the precooling well at all times and incubator block should be either all full or all empty when power is on. This insures proper air purging and prevents moisture condensation.

2. Proper and reproducible pipet usage is essential to insure instrument precision.

I. Procedural Modifications

1. The time of the test may be extended, especially if it is suspected that the sample contains metals. There is often a significant decrease or recovery in light output after 30 minutes. This can be seen in Tables 1 and 2 which present data for samples analyzed with the Microtox system at the University of Massachusetts. In Table 1, it can be

Table 1
Sample Microtox Data
44 Percent Unfiltered Sanitary Landfill Leachate
Fitchburg, Massachusetts: July, 1982

Time (Minutes)	Light Diminution (Percent)
0	0
5	87.3
10	85.5
15	84.3
20	84.1
25	84.6
30	85.9
35	86.6
40	87.8
45	89.4
50	90.5
55	91.6
60	91.9

seen that the 5, 10 and 15 minute data give no indication of the curvature in the data plot apparent after 30 minutes. Prolonged exposure to this sample yielded a lower EC50. The data in Table 2 show a more pronounced example of increased toxicity with prolonged exposure, with the 60EC50 being approximately one-fifth of the 5EC50.

2. A more stable light output is reached about 20 minutes of reagent equilibration (E) after Reagent reconstitution rather than 15 minutes as suggested in the procedure. Table 3 illustrates this phenomenon. It can be seen from Tables 3a and 3b that a much smaller decrease in light output occurs after 20 minutes.

3. The Reconstituted Reagent is weakly buffered at pH 7. Below pH 5 and above pH 8, toxic effects may be due to pH rather than sample toxicity. If a sample is suspected to be strongly basic or acidic two sets of samples should be tested: one at the sample pH and one adjusted to pH 7. Toxic effects can be separated from pH effects in this manner.

4. Microtox Diluent should be used to dilute concentrated or highly toxic samples. Other diluents such as deionized or distilled water, phosphate buffer or MOPS buffer ($C_7H_{15}NO_4S$) have been shown to cause slight decreases in light output relative to the blank. Figure 2 compares various diluents to the standard Microtox diluent. Data for the phosphate buffer solution is not shown due to the erratic results obtained. At 1.8 percent and 45 percent it led to a decrease in light output of up to 15 percent while at 9.8 and 0.36 percent it stimulated light output as much as 110 percent of that obtained with Microtox Diluent.

Table 2

Sample Microtox Data
45 Percent Hollingsworth and Vose Industrial Effluent Sample,
Groton, Massachusetts: March, 1983

Time (Minutes)	Light Diminution (Percent)
0	0
5	14.9
15	37.8
30	61.0
45	75.0
60	84.5

Table 3a

Light Diminution of Microtox Reagent Following Reconstitution

Time (Minutes)	Percent Light Output Remaining Relative to 15 Minute Light Intensity: $I_t/I_{15} \times 100$*	
	Replicate 1	Replicate 2
15	100	100
20	88	88
25	84	81
30	80	77
45	77	71
60	75	67

*I_{15} = Initial light output 15 minutes after reagent reconstitution.

I_{20} = Initial light output 20 minutes after reagent reconstitution.

I_t = Light output at specified time after reagent reconstitution.

Table 3b

Light Diminution of Microtox Reagent Following Reconstitution

Time (Minutes)	Percent Light Output Remaining Relative to 20 Minute Light Intensity: $I_t/I_{20} \times 100$*	
	Replicate 1	Replicate 2
20	100	100
25	96	92
30	91	88
45	87	81
60	85	77

*I_{15} = Initial light output 15 minutes after reagent reconstitution.

I_{20} = Initial light output 20 minutes after reagent reconstitution.

I_t = Light output at specified time after reagent reconstitution.

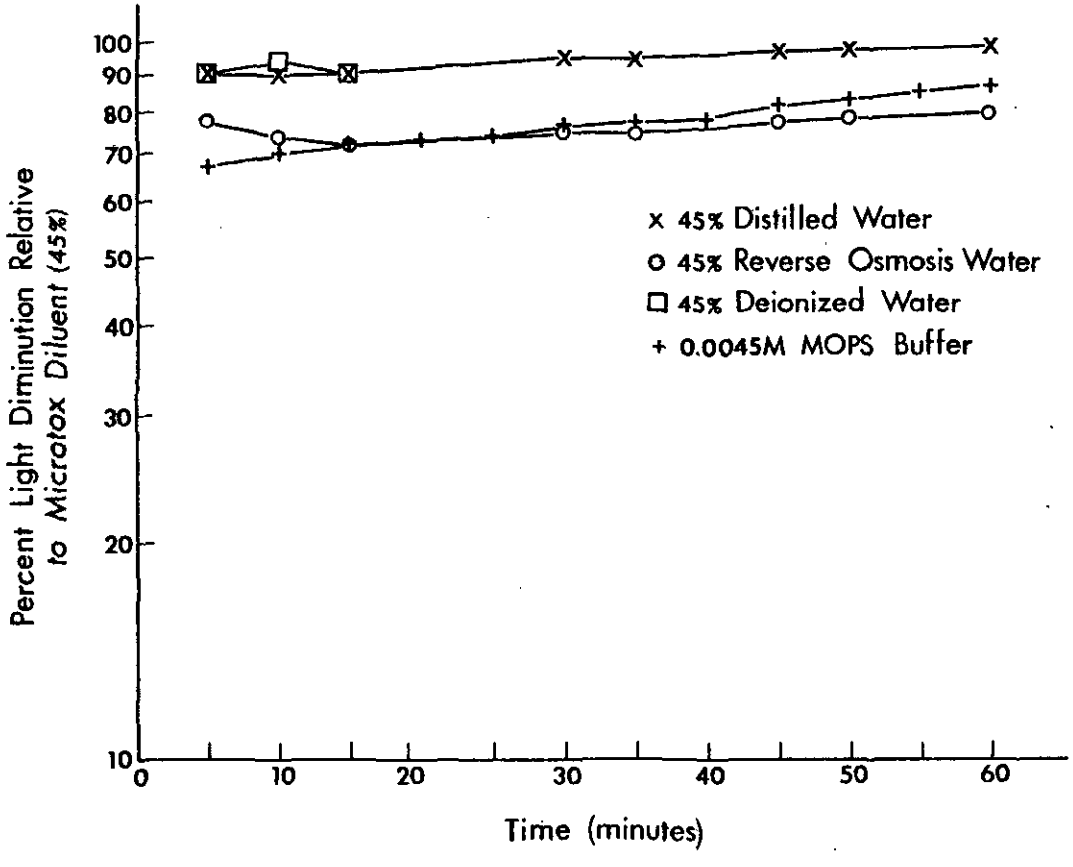


Figure 2. Comparison of Light Output Utilizing Various Diluents Relative to Microtox

IX. Methods of Data Presentation

Microtox data can be analyzed using graphical methods similar to conventional bioassay data reduction techniques discussed in earlier reports (Plotkin and Ram; 1983a, 1983b) such as log-linear plotting of concentration versus light diminution (percent decrease), light diminution versus time, or probit analysis. When several EC50's are observed after different test periods, it may be convenient to plot the concentration at each EC50 value against the time required to achieve the EC50. Beckman Instruments suggests that gamma, defined in Equation 1, be plotted against toxicant concentration after a specified exposure period to evaluate the EC50. This method is reported to result in a more linear plot and more precise data than other data reduction methods (Beckman, 1980). Gamma is the ratio of the amount of light lost during the test period to the amount of light remaining at the end of the test:

$$\gamma = \frac{I_i - I_t}{I_t} \qquad (1)$$

where γ is the gamma function, I_i is the corrected initial light intensity, and I_t is the final light intensity at the end of the test period, t. The use of this function reportedly simplifies the calculation of EC50 values since at the EC50, gamma equals unity. A semilog plot of gamma vs concentration is made, with gamma plotted on the log scale. The EC50 is easily found at gamma = 1 or log gamma = 0.

A graphical comparison of some of the data reduction methods is presented in Figures 3, 4, and 5. It is difficult to say which method is best. For a particular toxicant, one method may yield a better linear plot than another. In general, however, all three methods will give a good estimate of the EC50. A plot of percent light diminution versus concentration may be more easily understood on an intuitive level.

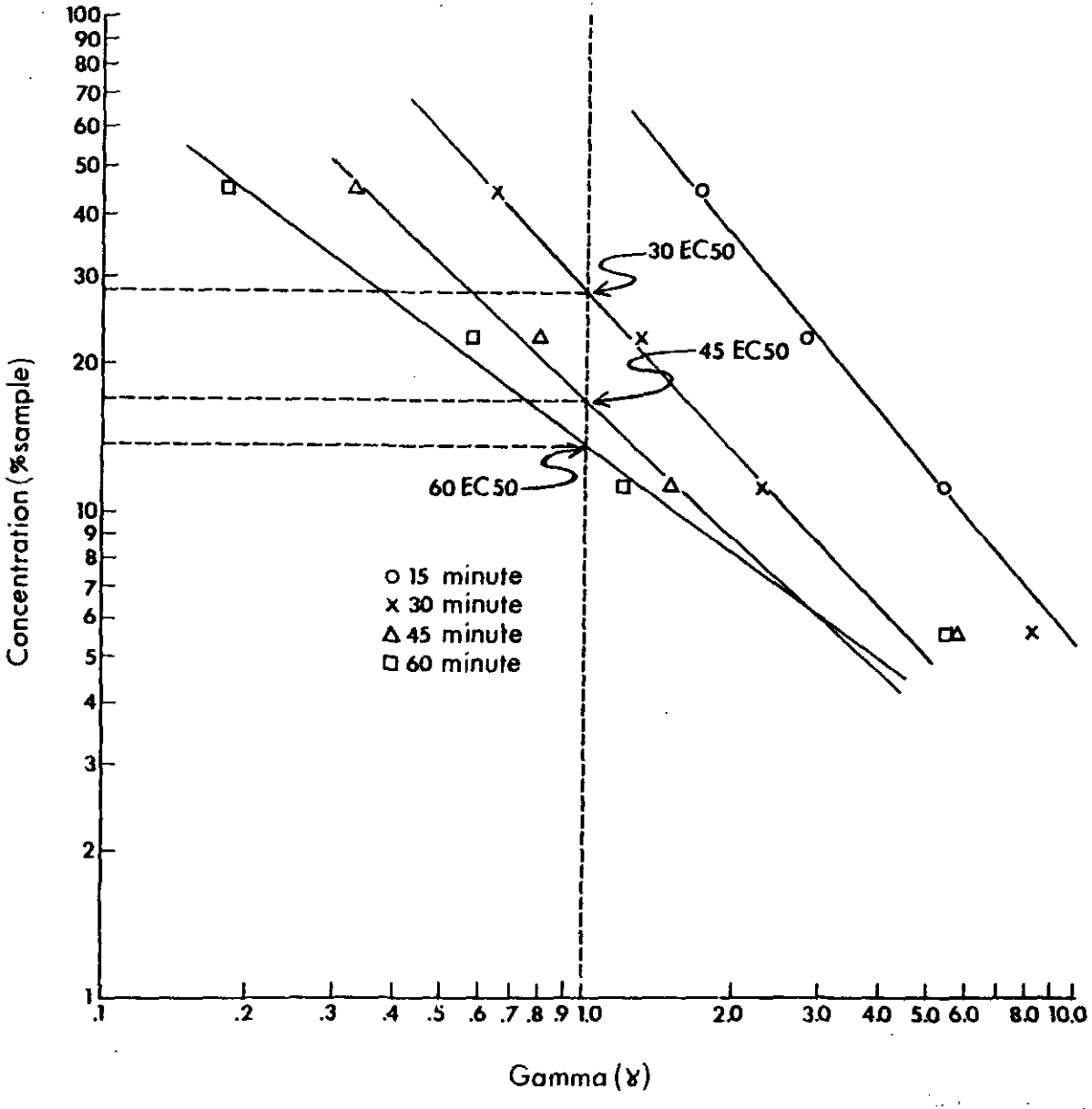


Figure 3. Data Reduction Example: Gamma vs. Concentration Using Raytheon Missile Systems Effluent Data, Lowell, Massachusetts.

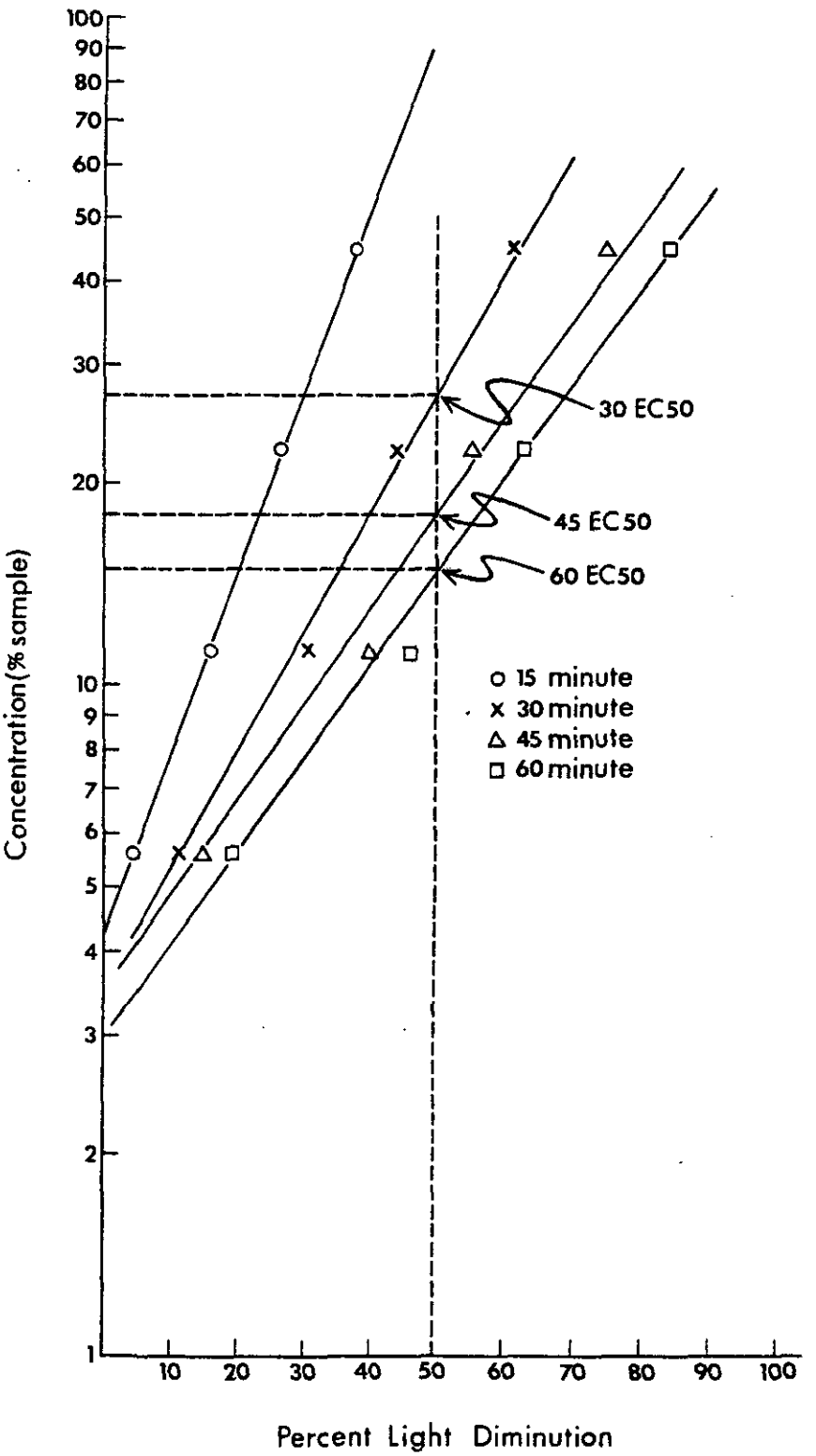


Figure 4. Data Reduction Example: Percent Light Diminution vs. Concentration Using Raytheon Missile Systems Effluent Data, Lowell, Massachusetts

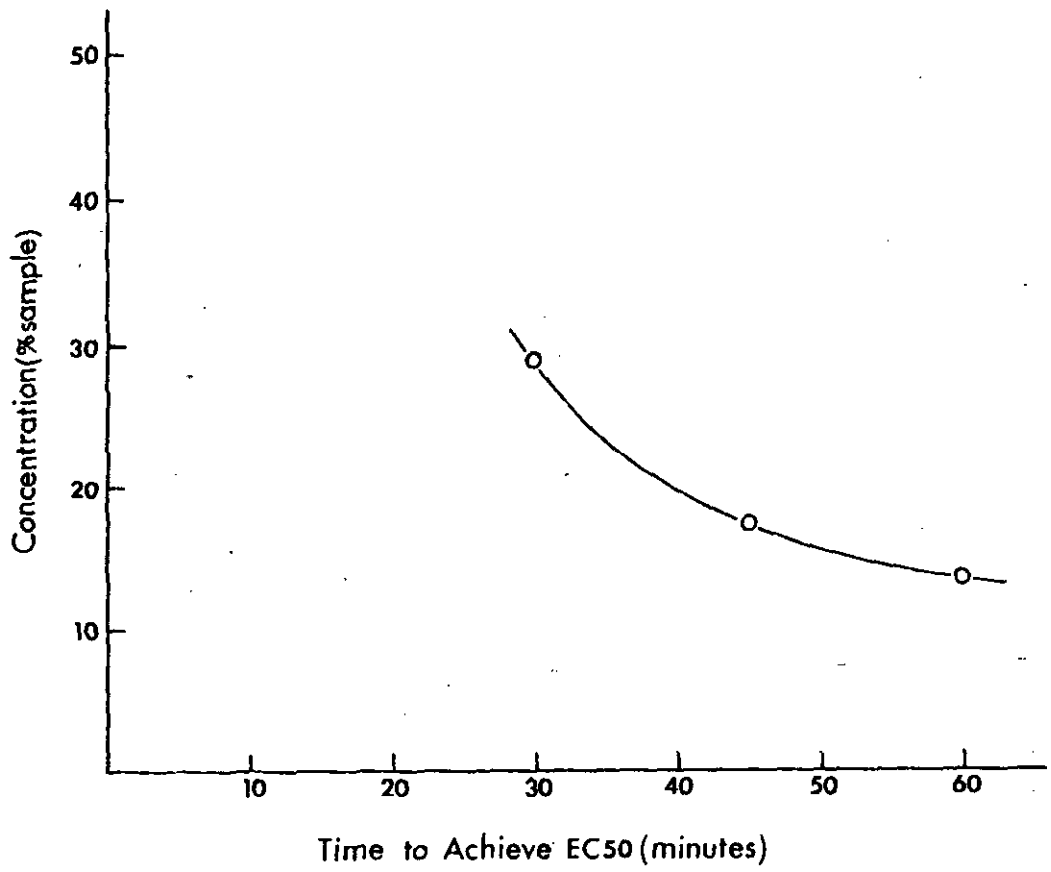


Figure 5. Data Reduction Example: Time to EC50 vs. Concentration Using Raytheon Missile Systems Effluent Data, Lowell, Massachusetts

X. Case Studies

Since its establishment, the Environmental Engineering Laboratory at the University of Massachusetts has analyzed 21 samples with the Microtox toxicity testing system. Several of these samples have been analyzed in conjunction with fish and invertebrate toxicity testing by this laboratory. Data for all 21 samples are presented here to illustrate the use of the Microtox toxicity analyzer in assessing the toxicity of aqueous samples. Water quality data for these samples are presented in Appendix A.

Miscellaneous Wastewater Samples

Seven municipal wastewater treatment plant (WWTP) and industrial wastewater influent and effluent samples provided by the Massachusetts Division of Water Pollution Control were analyzed in the fall of 1982: Arnold Print effluent, Adams WWTP effluent, Berkshire Tannery effluent, James River Paper effluent, Adams WWTP influent, Hoosic Water Quality District WWTP influent and Hoosic Water Quality District WWTP effluent. Microtox data for these samples are presented in Table 4. EC50 values were achieved for three of these samples: Arnold Print effluent, Adams WWTP influent, and Berkshire Tannery effluent. The remaining samples did not result in a 50 percent reduction in bioluminescence over the concentration range tested (0.36 to 45 percent sample).

Two of the samples which achieved EC50's, Arnold Print effluent and Berkshire Tannery effluent, were highly colored and required the use of the Microtox color correction procedure. The Arnold Print effluent was light brown in color and slightly turbid. The EC50 value, however, was not significantly changed by the color correction procedure. The Berkshire Tannery sample was black in color and fairly turbid. Use of the color correction method resulted in a slightly higher (10 percent) EC50 value relative to the uncorrected Berkshire Tannery sample.

Seven additional municipal and industrial wastewater effluents were tested for toxicity with the Microtox system in early 1983: Palmer WWTP, Omega Plating, Holyoke WWTP, Zero Manufacturing, Omega Plating, Palmer WWTP, and Holyoke WWTP. The Microtox data for these samples are also shown in Table 4. Of the seven samples tested, only two produced an EC50 value in the 30 minute test period; Omega Plating effluent and Palmer WWTP effluent sampled from 2/15-2/16/83. The Omega Plating sample of 2/15 exhibited a 30EC50 equal to 26 percent sample, while the Palmer WWTP sample taken from 2/15 to 2/16 showed a 5EC50 of 43 percent sample. The remaining samples did not result in a 50 percent reduction in bioluminescence over the concentration range tested.

Table 4

Microtox Toxicity Test Results at Various Time Intervals (Minutes): Miscellaneous Wastewater Samples

Sample	Date	Sample Type[1]	Location	EC Value (% sample) at various exposure times, (minutes)
Arnold Print Effluent	8/31/82	G	Adams, MA	5 EC50 = 0.56 (0.56)[2] 15 EC50 = .50 (.50)[2]
Adams WWTP Influent	8/30/82	G	Adams, MA	5 EC50 = 11
Berkshire Tannery Effluent	8/31/82	G	Williamstown, MA	5 EC50 = 11 (10)[2]
James River Paper Effluent	8/31/82	G	Adams, MA	30 EC20 = 45
Adams WWTP Effluent	8/31/82	G	Adams, MA	30 EC15 = 45
Hoosic WQ District WWTP Influent	8/31/82	G	Williamstown, MA	30 EC35 = 45
Hoosic WQ District WWTP Effluent	8/31/82	G	Williamstown, MA	30 EC5 = 45
Palmer WWTP Effluent	2/14-2/15/83	C	Palmer, MA	5 EC25 = 45
Palmer WWTP Effluent	2/15-2/16/83	C	Palmer, MA	5 EC50 = 43
Omega Plating Effluent	2/15/83	G	Monson, MA	30 EC50 = 20
Omega Plating Effluent	2/15-2/16/83	C	Monson, MA	5 EC14 = 45
Holyoke WWTP Effluent	2/14-2/15/83	C	Holyoke, MA	30 EC21 = 45
Holyoke WWTP Effluent	2/14-2/16/83	C	Holyoke, MA	30 EC21 = 45
Zero Manufacturing Effluent	2/15-2/16/83	C	Monson, MA	30 EC35 = 45

1. G = Grab sample; C = Composite sample.
2. Values in parentheses were obtained with a non-color-corrected sample.

Fitchburg Sanitary Landfill Leachate

Fitchburg sanitary landfill leachate samples were tested for toxicity in the fall of 1982, utilizing fathead minnows (Pimephales promelas), invertebrates (Daphnia magna), green algae, (Selenastrum capricornutum), and luminescent bacteria (Photobacterium phosphoreum). The results of these tests are shown in Table 5. The leachate was shown to be highly toxic with Microtox, exhibiting a 5EC50 equal to 14 percent sample, moderately toxic to daphnids, with a 48LC50 of 62 to 66 percent sample, and slightly toxic to fathead minnows, exhibiting a 96LC50 equal to 100 percent sample. Algal cells were unable to grow in a solution containing ten percent leachate, but recovered when centrifuged and reinoculated into algal nutrient medium. All algal tests were terminated within fourteen to twenty-one days upon realization of the maximum standing crop (less than a five percent increase in chlorophyll a concentration in a twenty-four hour period).

Foxboro Metal Plating Wastewater

The effluent from the Foxboro metal plating plant, located in Foxborough, MA, was subjected to three bioassays in the winter of 1982, utilizing bioluminescent bacteria (Photobacterium phosphoreum), fish (Pimephales promelas) and invertebrates (Daphnia magna) as the test organisms. The results of these tests are shown in Table 6. The effluent displayed virtually no toxicity to fathead minnows but was highly toxic to D. magna and the photobacteria used in the Microtox system. The 48LC50 for daphnids was equal to 7.5 percent sample, and the 30EC50 determined with the Microtox system was 40 percent sample. Additional exposure to the sample resulted in a two hour EC50 of 13 percent sample.

Brockton, Massachusetts Wastewater Treatment WWTP Effluent

Several toxicity tests were conducted on wastewater effluent from Brockton, MA using daphnids, fathead minnows, and bioluminescent bacteria as the test organisms. The data for these analyses are presented in Table 7. The sample was not sufficiently toxic to kill 50 percent of the Daphnia pulex population during the 48 hour test exposure period and no mortality was observed for fathead minnows after 96 hours of exposure to 100 percent effluent. In addition, none of the concentrations tested achieved an EC50 at anytime during the Microtox test.

Bickford Pond Tributary

A grab sample from an unnamed tributary to Bickford Pond in Princeton, MA, was tested for toxicity utilizing bioluminescent bacteria (Photobacterium phosphoreum), fish (Pimephales promelas) and invertebrates (Daphnia pulex) as the test organisms. The results of these tests are presented in Table 8. The sample, which

Table 5

Fitchburg, Massachusetts Sanitary Landfill Leachate Toxicity Test Results

Test Organism	Toxicity Value (% sample)
Photobacterium phosphoreum	5 EC50 = 14
Selenastrum capricornutum[1]	1 < EC50 < 10
Daphnia magna	48 LC50 = 62-66 ILC50 = 37
Pimephales promelas	96 LC55 = 100

1. *Selenastrum capricornutum* is the green alga used in the algal assay bottle test. The EC50 reported is the percent sample resulting in 50 percent growth inhibition after 14-21 days incubation.

Table 6

Foxborough Metal Plating Toxicity Tests Results, Foxborough, Massachusetts

Test Organism	Toxicity Value (% sample)
Pimephales promelas	100 percent survival after 96 hours exposure to 100 percent effluent
Daphnia magna	48 LC50 = 7.5
Photobacterium phosphoreum	30 EC50 = 40
	2 hour EC50 = 13

Table 7

Brockton, Massachusetts WWTP Toxicity Tests Results

Test Organism	Toxicity Value (% sample)
Pimephales promelas	100 percent survival after 96 hours exposure to 100 percent effluent
Daphnia pulex	No 48 LC50 achieved 48 LC40 = 100 48 LC30 = 50
Photobacterium phosphoreum	No EC50 achieved 60 EC35 = 45

Table 8

Unnamed Tributary to Bickford Pond Toxicity Tests Results, Princeton, Massachusetts

Test Organism	pH	Toxicity Value (% sample)
Pimephales promelas	4.5 7	LC50 not achieved 100% survival after 96 hours exposure to 100% effluent
Daphnia pulex	4.4 7	Calculation not possible due to data scatter
Photobacterium phosphoreum	4.9	No EC50 achieved
	6.9	No EC50 achieved

was collected downstream from a wooded swamp, was believed to be free from conventional pollutants. In order to separate the toxic effects of the acidic pH of the sample from the effects of other possible toxicants contained in the sample, the Microtox, daphnid, and fish toxicity tests were conducted at both the in situ pH value as well as at neutral pH by adjustment with NaOH.

The results of these toxicity tests indicated that these waters were non-toxic to fathead minnows, and showed a minor toxicity with Microtox. It was not possible to interpret the results of the daphnid test due to data scatter.

Raytheon Missile Systems and Hollingsworth and Vose Effluents

Two aqueous samples were analyzed at the University of Massachusetts Environmental Engineering Laboratory with the Microtox toxicity testing system in March, 1983: Raytheon Missile Systems effluent, and Hollingsworth and Vose effluent. The results of these analyses are presented in Table 9.

Raytheon Missile Systems effluent displayed an EC50 equal to 28.7 percent effluent after 30 minutes of exposure. Additional exposure of the Raytheon Missile Systems effluent resulted in increased light diminution with EC50 values of 17.8 and 13.4 percent after 45 and 60 minutes exposure, respectively. Alternatively, the Hollingsworth and Vose effluent did not result in 50 percent light diminution at any time during the test period. Hollingsworth and Vose effluent resulted in a maximum of 27.7 percent light diminution after 45 minutes exposure, indicating a low toxicity to bioluminescent bacteria.

Oxford Pickle Company Effluent

In April, 1983, an aqueous effluent sample from the Oxford Pickle Company in South Deerfield, MA, was analyzed with the Microtox system. The sample was turbid with a slightly greenish color, but did not require the use of the Microtox color correction procedure. The low pH value of the sample (4.0), however, warranted Microtox analyses at both the in situ pH (4.0) and adjusted pH (7.0) values so that toxic effects attributable to acidity could be distinguished from the effects of possible chemical toxicants contained in the sample.

Microtox data for this sample are presented in Table 10. The effluent was highly toxic to bioluminescent bacteria at both the in situ pH (4.0) and at neutral pH (7.0) values with five minute EC50 values of 3.8 and 3.9 percent sample, respectively. At the pH value of seven, the bacteria appeared to recover slightly from the effects of the toxicant after 15 minutes. This recovery was not observed in the sample at a pH value of 4.0, which exhibited greater light diminution at 15 minutes. There was a slight recovery in the sample

Table 9

Microtox Toxicity Test Results at Various Time Intervals (Minutes): Raytheon Missile Systems Effluent, Lowell, Massachusetts and Hollingsworth and Vose Effluent, Groton, Massachusetts

Sample	EC Value (% Sample)
Raytheon Missile Systems Effluent	30 EC50 = 28.7 45 EC50 = 17.8 60 EC50 = 13.4
Hollingsworth and Vose Effluent	EC50 not achieved

Table 10

Microtox Toxicity Test Results At Various Time Intervals (Minutes): Oxford Pickle Effluent, South Deerfield, Massachusetts

pH	EC Value (% Sample)
4.0	5 EC50 = 3.8
7.0	5 EC50 = 3.9
4.0	15 EC50 = 2.4
7.0	15 EC50 = 5.1
4.0	30 EC50 = 3.6
7.0	30 EC50 = 4.8

at the pH value of four after 30 minutes exposure, with an EC50 value of 3.6 percent sample. The sample at neutral pH appeared to have stabilized at 30 minutes with an EC50 value of 4.8 percent sample.

These results indicate that this sample was highly toxic to bioluminescent bacteria (Photobacterium phosphoreum) utilized in the Microtox toxicity test. In addition, data from analyses at both the in situ pH value (4.0) and adjusted pH value (7.0) suggest that toxicity was attributable to a chemical constituent within the effluent rather than the acidic quality of the sample.

The Environmental Engineering Laboratory has examined the reproducibility of the Microtox system after 45 minutes exposure to 5.60 mg/L cadmium. This data is presented in Table 12. Four sets of replicate analyses by the same technician on four separate days yielded a mean gamma value of 0.9501 with a standard deviation and coefficient of variation of 0.0975 and 10.27 percent respectively.

Summary of Case Studies

The results of the toxicity tests conducted at the University of Massachusetts Environmental Engineering Laboratory, since its establishment, are presented in Table 11.

In all but one case where multiple toxicity tests were performed utilizing daphnids, fish, and Microtox, the Microtox system was the most sensitive method. In the case of the Foxborough Plating sample, the daphnid toxicity test was more sensitive than Microtox, with fathead minnows showing the least sensitivity. Fathead minnows were the most tolerant test organism in all cases. In no case did the Microtox test fail to detect toxicity in a sample that showed toxicity with other testing methods.

The Microtox test is rapid and simple to perform, and requires only a small amount of sample. It also appears to offer equal or superior sensitivity to other techniques of determining aqueous toxicity for the samples tested to date. The Microtox test has been shown to have good reproducibility, with a coefficient of variation of 11 to 12 percent for 30 identical samples analyzed by one technician on the same instrument (Beckman, 1983). Although a much smaller sample size was used, similar variation has been seen in fish and daphnid toxicity tests (USEPA, 1981).

Observation of the varying responses of the different testing techniques suggests that EC50 values determined with the Microtox test cannot be correlated with specific LC50 values found using other test organisms. However, the Microtox system's ability to sensitivity detect toxicity rapidly make it an ideal screening tool for testing aqueous samples.

Table 11

Toxicity Data Summary

Sample	Date	Toxicity Value (Percent Sample) Algae[1]	Fish (Hours)	Daphnid (Hours)	Microtox (Minutes)
Arnold Print Effluent	9/82	-	- -	- -	5EC50 = 0.56 15EC50 = 0.50
Adams WWTP Effluent	9/82	-	-	-	5EC50 = 11
Berkshire Tannery Efflulent	9/82	-	-	-	5EC50 = 11(10)[2]
James River Paper Effluent	9/82	-	-	-	30EC20 = 45
Adams WWTP Influent	9/82	-	-	-	30EC15 = 45
Hoosic WQ District WWTP Influent	9/82	-	-		30EC35 = 45
Hoosic WQ District WWPT Effluent	9/82	-	-		30EC5 = 45
Foxborough Plating Effluent	11/10/82	-	100% survival after 96 hours	48 LC50 = 7.5	30EC50 = 40
Fitchburg Leachate	7/7/82	1<EC50<10	96 LC55 = 100	48 LC50 = 62-66	5EC50 = 14
Brockton WWTP Effluent	6/21-6/22/83	-	100% survival after 96 hours	48 LC40 = 100	60EC35 = 45

Table 11, Continued

Sample	Date	Toxicity Value (Percent Sample) Algae[1]	Fish (Hours)	Daphnid (Hours)	Microtox (Minutes)
Palmer WWTP Effluent	2/14-2/15/83	-	-	-	5EC25 = 45
Palmer WWTP Effluent	2/15-2/16/83	-	-	-	5EC50 = 43
Omega Plating Effluent	2/15/83	-	-	-	30EC50 = 20
Omega Plating Effluent	2/15-2/16/83	-	-	-	5EC14 = 45
Holyoke WWTP Effluent	2/14-2/15/83	-	-	-	30EC21 = 45
Holyoke WWTP Effluent	2/15-2/16/83	-	-	-	30EC21 = 45
Zero Manufacturing	2/15-2/16/83	-	-	-	30EC35 = 45
Unnamed Tributtary to Bickford Pond	3/16/83	-	LC50 not achieved at pH = 4.5 100% survival after 96 hours at pH = 7	Calculation not possible due to data scatter at pH = 4.4 LC50 not achieved at pH = 7	EC50 not achieved at either pH
Raytheon Missile Systems Effluent	3/23/83	-	-	-	30EC50 = 28.7 45EC50 = 17.8

Table 11, Continued

Sample	Date	Toxicity Value (Percent Sample) Algae[1]	Fish (Hours)	Daphnid (Hours)	Microtox (Minutes)
Hollingsworth and Vose Effluent	3/23/83	-	-	-	60EC50 = 13.4 EC50 not achieved
Oxford Pickle[3] Effluent	4/28/83	-	-	-	5EC50 = 3.9 15EC50 = 2.4 30EC50 = 3.6
Oxford Pickle[4] Effluent	4/28/83	-	-	- - -	5EC50 = 3.9 15EC50 = 5.1 30EC50 = 4.8

1. Algal toxicity test conducted over three week time period.
2. Value in parentheses was obtained with a non-color-corrected sample.
3. pH = 4
4. pH = 7

Table 12

Microtox Reproducibility Data after 45 Minutes Exposure to 5.60 mg/L Cadmium

Date	% Light Remaining after 45 Minutes		Y	
	Run 1	Run 2	Run 1	Run 2
7/20/83	46.55	51.39	1.1482	0.9458
7/21/83	50.77	50.84	0.9698	0.9670
7/24/83	50.69	51.25	0.9728	0.9513
7/26/83	56.10	53.67	0.7825	0.8631

For Y n = 8

$\bar{X} = 0.9501$

$\sigma = 0.0975$

Coeffient of variation = $\frac{\sigma}{\bar{X}}$ x 100 = 10.27 percent

XI. Conclusions

The research efforts at the University of Massachusetts' Environmental Engineering Laboratory, as well as those of previously mentioned authors (Curtis et al., 1982; Bulich et al., 1979; Peltier and Weber, 1980; Neiheisel et al., 1982; Beckman, 1982a; Lebask et al., 1981; Strosher et al., 1980; Chang, et al., 1981), make possible a comparison between the Microtox toxicity testing system and more conventional methods for determining aquatic toxicity. This comparison indicates that there are differences between the fish, invertebrate, and Microtox data. It should not be surprising that 100 percent correlation was not observed between the Microtox toxicity test and other test organisms. Responses are known to vary between fish and invertebrates and even, for that matter, between different species of the same test organism. In many cases, Microtox shows greater sensitivity than other toxicity testing methods. This is beneficial and would result in increased protection of aquatic systems. The major concern is the group of compounds shown to cause lethality to fish and invertebrates, but not to the bioluminescent bacteria utilized in the Microtox test.

XII. Equipment, Supply and Time Requirements

The equipment, supply, and time requirements for Microtox analysis are shown in Tables 13 and 14. The Microtox toxicity test is considerably less expensive and quicker to conduct than other methods of assessing aquatic toxicity currently in use. Approximately two hours and 15 minutes are required for an entire Microtox analysis. This figure excludes sampling time which is dependent on site location. A single technician, then, should be capable of processing about ten samples per week, or 500 samples per year inclusive of data analysis.

Table 15 details the direct costs, in 1983 dollars, of a Microtox Laboratory. Approximately $21,000 are required to establish a laboratory and furnish supplies for one year of Microtox analyses (500 tests). This figure includes the initial capital cost of the Microtox Toxicity Analyzer ($9,135). The cost for each additional year's worth of supplies is about $11,000. If the capitol cost of the Microtox instrument is distributed over the first year without considering interest, then cost per analysis is $72 assuming one technician performs 500 analyses in this period. The cost per analysis, excluding the Microtox instrument capitol investment, is $52.

Table 13

Equipment and Supply Requirements

Item	Quantity	Cost[1]	Notes
Equipment			
Beckman Microtox 2055 Toxicity Analyzer	1	$ 9,135.00	
Startup package	1	808.00	Includes reagent and solution for 40 tests as well as ancillary accessories and supplies
10 inch chart recorder	1	600.00	
Microtox micropipettes			
10 µL	1	69.00	
250 µL	1	69.00	
500 µL	1	69.00	
EQUIPMENT SUB-TOTAL		10,750.00	
Supplies			Enough for 40 additional tests
Recorder paper	6 rolls	24.60	
Recorder pen	4 pack	8.40	

Table 13, Continued

Item	Quantity	Cost[1]	Notes
Microtox Reagent and Reconstitution Solution	40 ml	560.00	
Diluent	2 x 500 ml	70.50	
Osmotic Adjustment Solution	50 ml	25.00	
Cuvettes	2 x 360	72.00	
Color Correction Cuvettes	4	50.00	
Pipette tips			
1-200 µL 1000		42.00	
250-500 µL 1000		42.00	
SUPPLIES SUB-TOTAL		894.50	
EQUIPMENT AND SUPPLIES TOTAL		$11,644.50	

1. 1983 dollars.

Table 14

Time Requirements

Item		Time	Notes
Sampling		Variable, depending on site and type	
Chemical Analyses	DO	10 min	Dissolved oxygen meter
	pH	10 min	pH meter
	Salinity	10 min	Conductivity meteer
Sample Pretreatment		15 min	Dilution and Osmolality adjustment
Instrument and Reagent Preparation		30 min	
Sample Analysis		30 min	May vary with sample
Data Reduction		30 min	
TOTAL		2 hr 15 min[1]	

1. Excluding sampling.

Table 15

Estimated Direct Costs to Conduct a Single Microtox Test[1]

Item		Quantity
A.	Capital cost to establish laboratory with one year's supplies[2]	$21,037
B.	Technician, annual salary	$15,000
C.	Number of assays conducted by one technician per year[3]	500
D.	Yearly supply costs	$11,181
	Cost per test, assuming capital is repaid during first year [(A + B)/C][4]	$72
E.	Cost per test after capital expense is repaid [(B + D)/C]	$52

1. Cost per test would be less if proportion of capital expenses assigned to each bioassay was distributed over more years. These costs exclude sampling.
2. Cost includes $10,750 equipment and supplies (40 tests) plus additional supplies to complete 500 tests = $10,287.
3. Assumes ten tests per week for one year.
4. Excluding interest.

XIII. References

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Appendix A: Case Study Water Quality Data

Sample	Date	pH	Dissolved Oxygen (mg/L)	Conductivity (μmhos)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)
Arnold Print Effluent	9/82					
Adams WWTP Effluent	9/82	-	-	-	-	-
Berkshire Tannery Effluent	9/82	-	-	-	-	-
James River Paper Effluent	9/82	-	-	-	-	-
Adams WWTP Influent	9/82	-	-	-	-	-
Hoosic WQ District WWTP Influent	9/82	-	-	-	-	-
Hoosic WQ District WWTP Effluent	9/82	-	-	-	-	-
Foxborough Plating Effluent	11/10/82	7.45	8.2	790	72.5	57.4
Fitchburg Leachate	7/7/82	5.8	4.4	900	902.4	687.4
Brockton WWTP Effluent	6/21-6/22/83	7.85	4.3	575	122	71
Palmer WWTP Effluent	2/14-2/15/83	6.6	10	255	37.8	75.2

Appendix A, continued

Sample	Date	pH	Dissolved *Oxygen* (mg/L)	Conductivity (µmhos)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)
Palmer WWTP Effluent	2/15 2/16/83	6.8	8.4	350	46.5	87.1
Omega Plating Effluent	2/15/83	6.1	10.4	155	16.5	33.7
Omega Plating Effluent	2/15- 2/16/83	6.6	9.3	170	24.3	33.7
Holyoke WWTP Effluent	2/14- 2/15/83	6.8	10.2	365	123.1	116.8
Holyoke WWTP Effluent	2/15- 2/16/83	6.85	9.0	550	115.4	134.7
Zero Manufacturing Effluent	2/15- 2/16/83	8.3	1.55	900	119.3	140.6
Unnamed Tributary to Bickford Pond	3/16/83	4.4	12	5	0	9.9
Raytheon Missile Systems Effluent	3/23/83	7.1	12.3	580	35	113
Hollingsworth and Vose Effluent	3/23/83	7.2	12.35	210	41	85
Oxford Pickle Effluent	4/28/83	4.0	4.4	17,000	0	440
Oxford Pickle Effluent	4/28/83	7.0	4.4	17,000	0	440

APPENDIX B

Studies on the Enhancement of the Microtox Bioluminescent Toxicity Test Using Two, Three and Four Component Chemical Systems

by

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Introduction

The Microtox toxicity assay is a method to assess the toxicity of an aqueous sample using lyopholized and reconstituted luminous marine bacteria. Upon exposure to a toxicant the amount of light emitted by these luminous bacteria is diminished in direct proportion to the toxicant concentration. The test is simple, accurate and reproducible and has therefore been suggested as a screening procedure to evaluate toxicity prior to the utilization of conventional fish or daphnid bioassays. One weakness of the Microtox assay, however, is that it is not as sensitive to some toxicants as are fish or daphnid bioassays. Many compounds tested to date exhibit an EC50 value (toxicant concentration resulting in a 50 percent light diminution in the specified time interval) equal to or less than corresponding fish or daphnid LC50 values (see Literature Review), the utility of the Microtox determination would be enhanced if the test could be modified to be more sensitive to such chemical toxicants. Possible modifications include the use of a more sensitive mutant bacterial strain, change in the test conditions, or co-exposure of the toxicant to a synergistic chemical. The objective of this study was to investigate several chemicals for their potential to exhibit a synergistic response with a few selected toxicants. The study used the Microtox bioluminescent test to assess the relationship between the toxicity exerted by chemical components singly, and in combinations of two, three and four chemicals. Co-exposure of a synergistic chemical and a toxicant under examination would result in greater light diminution during the Microtox assay and resulting enhanced sensitivity.

Methods

All tests were performed using a Beckman Instruments Microtox toxicity analyzer. Experiments were carried out at 15^{o}C according to the procedures described by the manufacturer (Beckman, 1982b). Several organic compounds, shown in Table 1, were selected for the study. These substances are known chemical toxicants which have been found in point source discharges. Additionally, single solute EC50 values have been previously determined for these chemicals. Three additional compounds (two antibiotic drugs, and one macromolecular dye) were selected to determine if they exhibit a synergistic response with the six other organic compound shown in Table 1. All toxicants were diluted in one percent phosphate buffer (pH = 6.9) and adjusted to two percent salinity by weight with NaCl. EC50 values were determined for single solute systems as well as for two, three and four component systems using the Microtox toxicity analyzer within three hours of dilution in the phosphate buffer. All reagents were of reagent grade, or were commercial pharmaceutical preparations. Both single and combined solute systems were analyzed in parallel to decrease variations

Table 1. Organic Chemicals Tested

Compound	Grade	Supplier
Group 1: Known Chemical Toxicant		
1. Phenol	reagent	Fisher Scientific Co., Inc.
2. Acetone	reagent	Fisher Scientific Co., Inc.
3. Chloroform	reagent	Fisher Scientific Co., Inc.
4. Endrin	analytical reference standard	U. S. Environmental Protection Agency
5. Toluene	reagent	Fisher Scientific Co., Inc.
6. Dimethyl formamide (DMF)	reagent	Fisher Scientific Co., Inc.
Group 2: Compounds Tested For Synergistic Response		
1. Methylene blue	88 percent dye	Fisher Scientific Co., Inc.
2. Achromycin	---	Pharmaceutical Supply
3. Choramphenicol	reagent	Aldrich Chemical Co., Inc.

attributable to solute volatility or reagent variability. Toxicity was evaluated by observing light diminution at a constant toxicant concentration over time or by measuring light diminution after 5, 15, and 30 minutes of exposure over a toxicant concentration range. In all tests, light diminution was measured relative to the standard Microtox control.

Chemical Interactions

Several possible types of chemical interactions can occur in mixed solute systems:

1. Simple additive interaction in which the toxicity of the combined toxicants is merely equal to the sum of their individual toxic effects;
2. Synergistic interaction in which the toxicity of the combined toxicant solution is greater than the sum of their individual toxic effects; and
3. Antagonistic interaction in which the toxicity of the combined toxicant solution is less than the sum of their individual toxic effects.

A mathematical model was therefore developed (Cristensen, 1983) to predict light diminution of solutions containing toxicant mixtures assuming simple additive (no interaction) toxicity. The type of chemical interaction was then determined by comparing the observed response with that predicted by the model as shown in Table 2. To construct the additive model it is assumed that light remaining after exposing the Microtox reagent to a combination of toxicants $\prod_{n=1}^{i}$ Y is the same as light remaining after a sequential exposure to each single solute (Y_s). If we let Y designate the fraction of light remaining at a stated time, t, then the above assumption can be stated mathematically as:

$$\prod_{n=1}^{i} Y = (Y_{S1})\,(Y_{S2}) \ldots (Y_{Si}) \qquad (1)$$

where $\prod_{n=1}^{i} Y$ = Fraction of light remaining after exposure of Microtox reagent to i chemicals in solution,

Y_s = Fraction of light remaining after exposure of Microtox reagent to single toxicant at concentration, X_i.

Table 2. Determination of Chemical Interactions

Observed Response	Interpretation
Toxicity < Value Predicted By Additive Model	Antagonistic
Toxicity = Value Predicted By Additive Model	Additive
Toxicity > Value Predicted By Additive Model	Synergistic

Light diminution of the Microtox reagent often follows a first order rate of decay with time as well as with increasing toxicant concentration. We can therefore represent the relationship between the fraction of light remaining (Y) and time (t) at a given toxicant concentration (X_i) as:

$$Y_1 = Ae^{-zt} \tag{2}$$

$$Y_2 = Ae^{-wt} \tag{3}$$

where Y_1 and Y_2 are the values for the fraction of light remaining at time, t, for toxicant X_1 and X_2, respectively. A is a constant which is equal to 1 (100 percent light output at t = 0). Combining equations 1-3 to obtain the light remaining for a mixture of toxicants X_1 and X_2, assuming an additive (no interaction) effect one obtains equation 4:

$$Y_3 = \prod_{n=1}^{2} Y_i = Ae^{-(w + z)t} \tag{4}$$

where w and z are the rate decay constants for the single solute species.

Similarly, one can derive the relationship between the fraction of light remaining (Y) and toxicant concentration (X_i) at some constant time by the following equations:

$$Y_1 = Ae^{-ZX_1} \tag{5}$$

$$Y_2 = Ae^{-WX_2} \tag{6}$$

$$Y_3 = \prod_{n=1}^{2} Y_i = Ae^{-(ZX_1 + WX_2)} \tag{7}$$

Equation 7 represents the light remaining at some exposure time to a mixture of two toxicants, having concentration of X_1 and X_2,

respectively and Z and W are the concentration decay constants. It assumes a simple additive (no interaction) relationship. Equation 7 can be expanded into:

$$Y_3 = \{Ae^{-ZX_1}\} \{Ae^{-WX_2}\} \quad (8)$$

This represents the product of the light remaining at some time for toxicant X_1 and toxicant X_2. Thus the light remaining for a mixture of "n" toxicants can simply be obtained by multiplying by the percent light remaining for each single solute toxicant for n solutes in solution. This approach gives the 'predicted' light remaining for a solution containing n toxicants, assuming simple additive interaction.

Equations 2-8 are only valid if light diminution follows a first order rate of decay with time or toxicant concentration. The model can be expanded to predict light diminution of toxicant mixtures which do not obey this first order decay, using the graphical approach shown in Figure 1. The decay pattern for compound 'B' is graphically subtracted from the decay pattern for compound 'A' to obtain the predicted light decay pattern for the mixture of compound A + B, assuming an additive interaction. This is performed by superimposing the light decay pattern for compound B from data points located on the light decay pattern for compound A and then transposing the light decay pattern for compound A onto that for compound B. An example of this is shown in Figure 1. The percent differences in predicted vs. observed values for the mixtures were calculated using equation 9.

$$\text{Percent Difference} = \left[\frac{\text{Predicted \% light remaining} - \text{Observed \% light remaining}}{\text{Predicted \% light remaining}}\right] \times 100 \quad (9)$$

A negative value indicated that the observed toxicity was less than that predicted by simple additive interaction (antagonism) while a positive value indicated a synergistic interaction.

Results and Discussion

Table 3 presents EC50 values for single components at 15°C, for various exposure times, reported as mg/L and moles/L. Some of the observed EC50 values were in close agreement with previously reported values (phenol, acetone), while others were significantly higher (chloroform) or lower (toluene, DMF). Dutka and Kwan (1981) reported intra and inter-laboratory analysis variability of up to 65

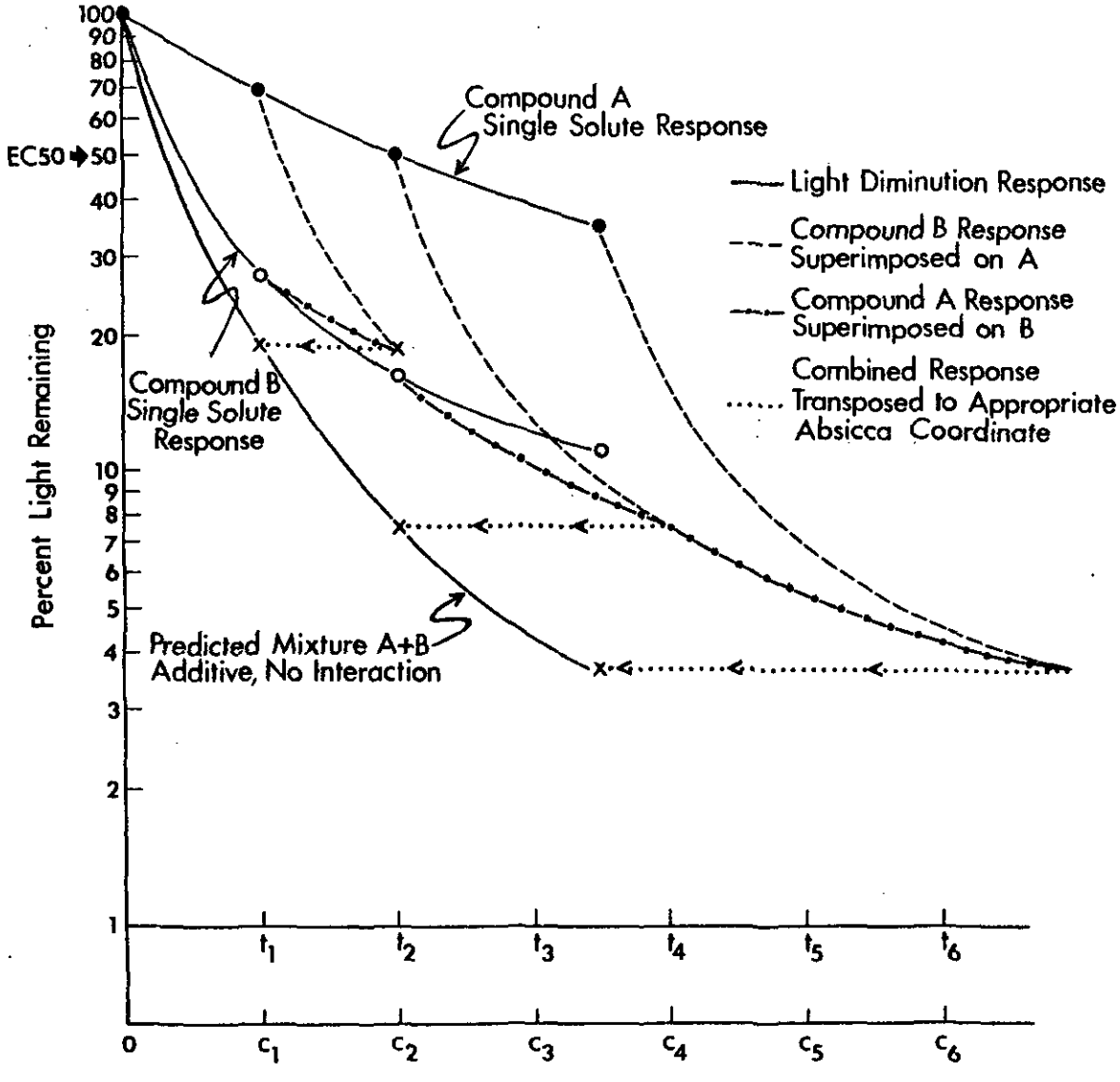

Figure 1. Graphical Determination of Light Diminution for Two Component Solute Mixture from Single Solute Data.

Table 3. EC50 Values for Single Components at 15°C

Compound	Exposure Time,(min)	Literature EC50[1] Values, (mg/L)	Experimental EC50 Values Replicate 1 mg/L	(moles/L)	Replicate 2 mg/L	(Moles/L)
Phenol	5	25(B),40.2(C)	25	(2.7×10^{-4})	26	(1.8×10^{-4})
	15	28(B)	27	(2.9×10^{-4})	27	(2.9×10^{-4})
	30	--	27	(2.9×10^{-4})	28	(3.0×10^{-4})
Chloroform	5	435(B)	730	(1.5×10^{-2})	583	(1.2×10^{-2})
	15	914(B)	660	(1.3×10^{-2})	876	(1.8×10^{-2})
Toluene	5	43.5(B)	11.1	(1.2×10^{-4})	14.0	(1.5×10^{-4})
	15	--	15.0	(1.6×10^{-4})	18.0	(2.0×10^{-4})
	30	--	18.5	(2.0×10^{-4})	--	--
Dimethyl	5	18,685(B)	11,800	(1.6×10^{-1})	13,300	(1.7×10^{-1})
Formamide	15	--	13,000	(1.8×10^{-1})	13,600	(1.9×10^{-1})
(DMF)	30	--			14,000	(1.9×10^{-1})
Acetone	5	22,000(B),21500(C)	22,000	(3.8×10^{-1})	--	--
	15	--	22,000	(3.8×10^{-1})	--	--
Endrin[2]	5	7.3(B)	0.69		0.51	
	15	--	0.37		0.31	
	30	--	0.31		0.30	
Achromycin	5	--	74.6	(1.7×10^{-4})	68.6	(1.6×10^{-4})
	15	--	45.1	(1.0×10^{-4})	46.6	(1.0×10^{-4})
	30	--	33.8	(7.6×10^{-5})	--	

Table 3, Continued

Compound	Exposure Time,(min)	Literature EC50[1] Values, (mg/L)	Experimental EC50 Values			
			Replicate 1		Replicate 2	
			mg/L	(moles/L)	mg/L	(Moles/L)
Methylene Blue	5	--	2.6	(8.2×10^{-6})	--	
	15	--	2.1	(6.6×10^{-6})	--	
Chloram-phenicol	5	--	375	(1.2×10^{-3})	--	
	15	--	298	(9.2×10^{-4})	--	
	30	--	240	(7.4×10^{-4})	--	

1. B = Beckman Instruments, Inc., (1983).
   C = Curtis, Lima, et al., (1982).

2. Based on aqueous solubility = 0.26 mg/L at 25°C, U.S. Environmental Protection Agency, 1980.

percent for the Microtox test. Beckman Incorporated (1983) additionally reported an 11 percent coefficient of variation for representative analyses. The observed discrepancy with some previously reported EC50 values, therefore, seems reasonable in light of the observed variability of the test data. Such variability may be attributable to slight time variations, pipetting precision, variations in Microtox reagent, and in some cases, solute volatility. In addition, variances in data reported for this research can be attributed to different periods of data generation.

Tables 4, 5, and 6 present observed and predicted EC50 values for systems of test compounds combined with 12 mg/L achromycin, 0.723 mg/L methylene blue, and 55 mg/L chloramphenicol, respectively. Predicted EC50 values were calculated using the additive graphical approach described earlier. The data is further illustrated in Figures 2-4. Percent differences values indicate either synergistic (+ value) or antagonistic (- value) interactions. These values however, were almost all less than five percent, with the exception of chloroform which displayed a ten percent value (antagonistic) after 30 minutes of exposure with chloramphenicol. Considering the precision and accuracy reported by Beckman (1983), and Dutka and Kwan (1981), the percent differences are most likely insignificant, indicating that the toxicity of the two component systems are additive for the concentration tested. Figures 2, 3 and 4 clearly illustrate the finding that none of the three 'Group 2' compounds tested (Table 1) displayed a synergistic response in combination with the tested chemical toxicants. Instead, a simple additive response was observed. While the addition of chloramphenicol, achromycin or methylene blue failed to enhance the sensitivity of the Microtox test for the chemicals tested, the data did verify the additive graphical mathematical model developed to predict light diminution of solutions containing toxicant mixtures assuming simple additive (no interaction) toxicity for toxicant mixtures which do not obey first order decay.

In addition to examining the relationship between light diminution and toxicant concentration, the study investigated the time dependency of light diminution for single toxicant solutions. Figure 5 shows the effect of increasing exposure time on microbial bioluminescence for 9.69 mg/L chloroform, 23 mg/L achromycin, 72 mg/L phenol, 54.6 mg/L chloramphenicol and 0.727 mg/l methylene blue. Chloroform resulted in a rapid decline in bioluminescence in the first five minutes followed by a slight recovery. Achromycin showed a steady decline in bioluminescence over the entire 30 minute exposure period while methylene blue, chloramphenicol, and phenol resulted in the greatest percent light diminution during the first five minutes of exposure and was fairly constant, thereafter. These data emphasize the importance of measuring light diminution at several periods of exposure.

Several three and four component mixtures were tested to determine the types of interaction within such systems. Table 7

Table 4. EC50 Values for Test Components plus 12 mg/L Achromycin[a] at 15°C

Compound	Exposure Time (min)	EC50-Observed for Mixture (mg/L)	EC50-Predicted[b] for Mixture (mg/L)	Percent Difference[c]
Phenol	5	28	27	-4
	15	32	31	-3
Chloroform	5	540	560	+4
	15	750	792	+5

a. 12 mg/L Achromycin resulted in 7, 10, and 20 percent light diminution at 5, 15, and 30 minutes, respectively.

b. Predicted graphically

c. $\frac{\text{EC50 Predicted-EC50 Observed}}{\text{EC50 Predicted}}$ x 100 percent

Table 5. EC50 Values for Test Components plus 0.727 mg/L Methylene Blue[a] at 15°C

Compound	Exposure Time (min)	EC50-Observed for Mixture (mg/L)	EC50-Predicted[b] for Mixture (mg/L)	Percent Difference[c]
Phenol	5	18	19	+5
	15	20	19	-5
	30	20	20	0
Chloroform	5	530	540	+2
	15	850	820	-4
Acetone	5	19,000	18,500	-3
	15	19,000	18,500	-3

a. Methylene blue at 0.727 mg/L resulted in 8, 9, and 10 percent light diminution at 5, 15, and 30 minutes respectively.
b. Predicted graphically.
c. $\frac{\text{EC50 Predicted-EC50 Observed}}{\text{EC50 Predicted}}$ x 100 percent

Table 6. EC50 Values for Test Components plus 55 mg/L Chloramphenicol[a] at 15°C

Compound	Exposure Time (min)	EC50-Observed for Mixture (mg/L)	EC50-Predicted[b] for Mixture (mg/L)	Percent Difference[c]
Phenol	5	19.5	20.5	+5
	15	18.5	20.0	+8
Chloroform	5	650	650	0
	15	900	845	-7
	30	1100	1000	-10
Acetone	5	18,700	18,700	0
	15	19,000	17,500	-9

a. Chloramphenicol at 55 mg/L resulted in 6, 9, and 16 percent light diminution at 5, 15, and 30 minutes, respectively.

b. Predicted graphically.

c. $\frac{\text{EC50 Predicted-EC50 Observed}}{\text{EC50 Predicted}}$ x 100 percent

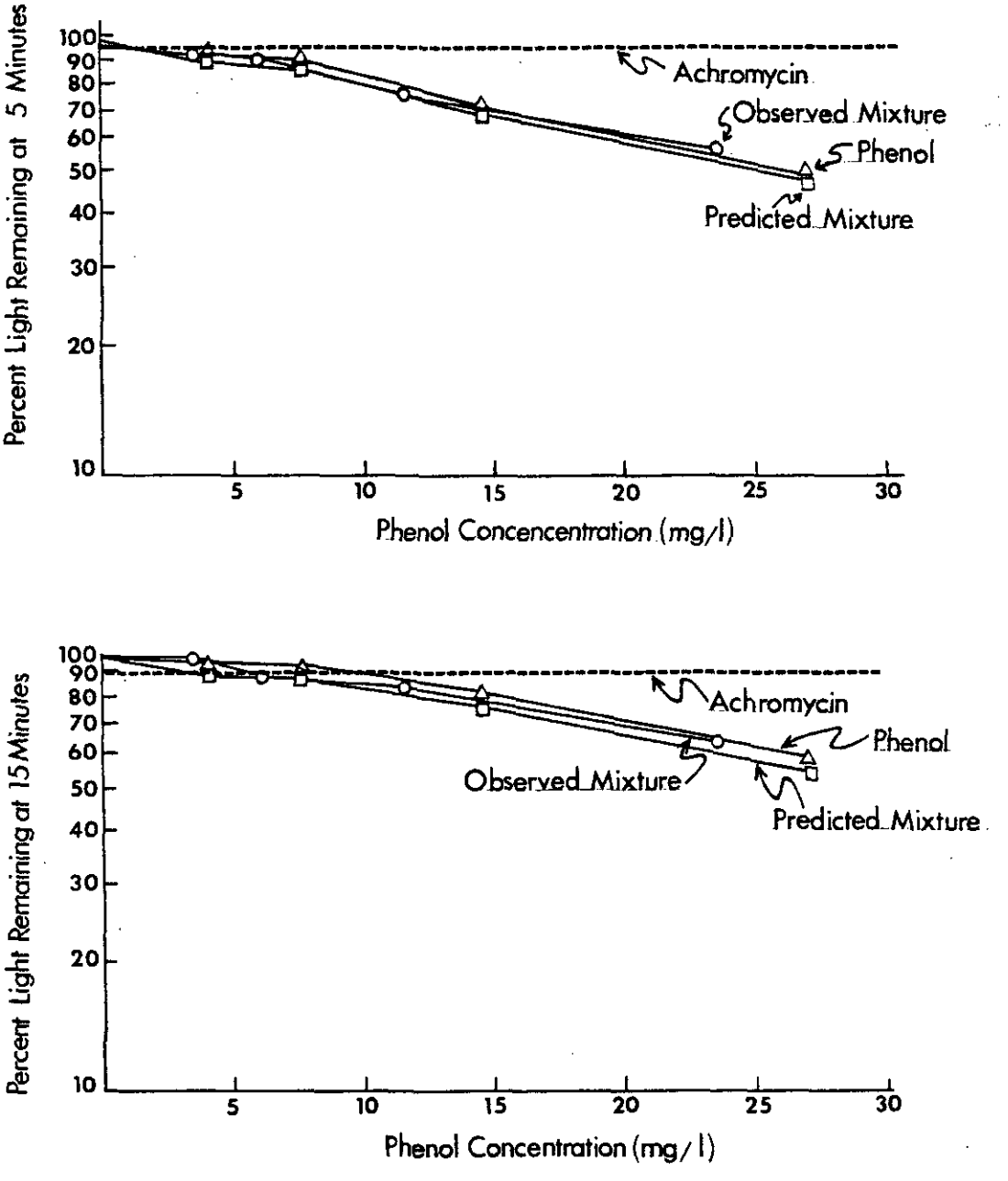

Figure 2. Log Percent Light Remaining after Exposure of 5 and 15 Minutes to 11 mg/L Achromycin, Phenol, and Phenol plus 11 mg/L Achromycin

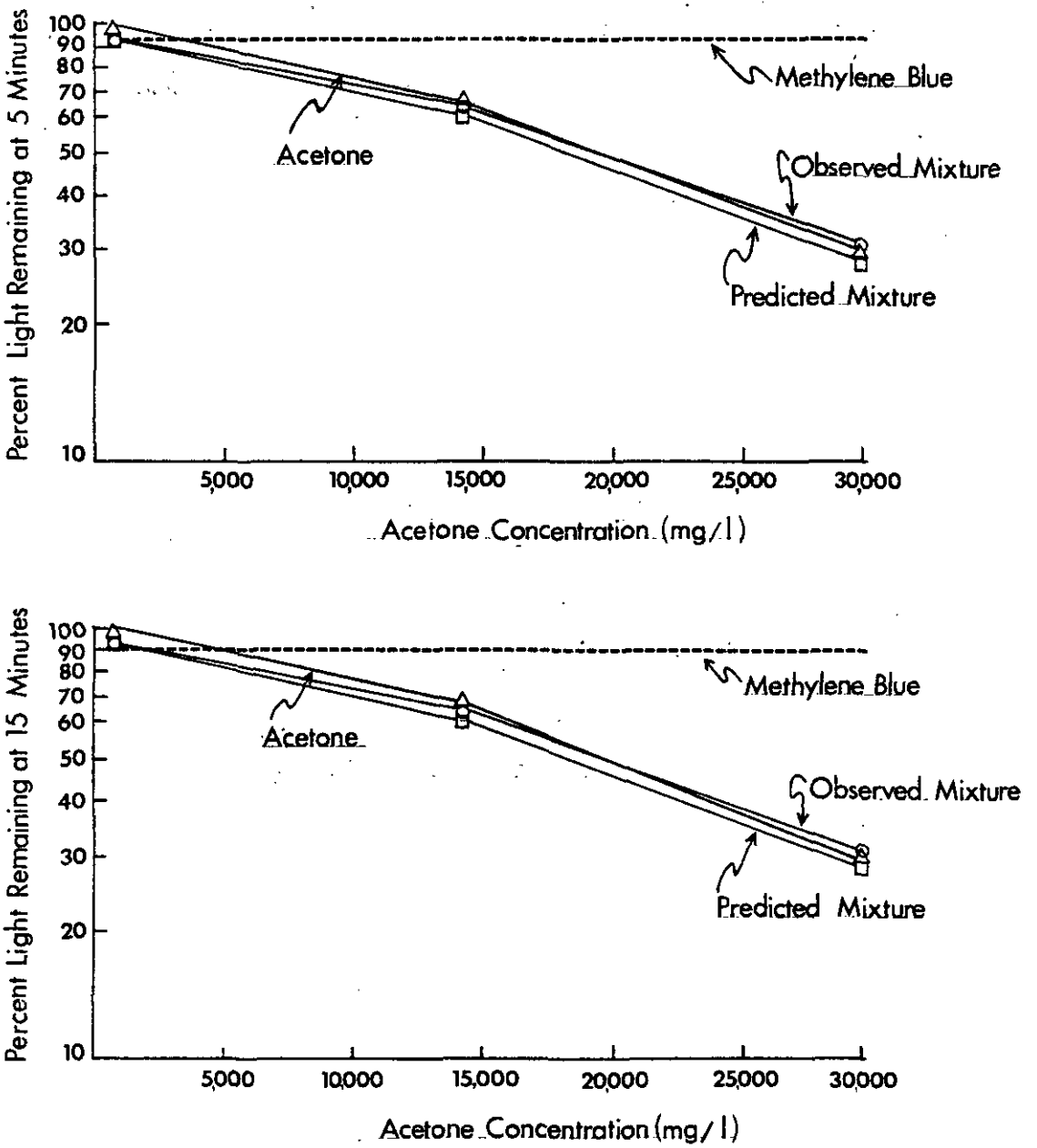

Figure 3. Log Percent Light Remaining after Exposure of 5 and 15 minutes to 0.723 mg/L Methylene Blue, Acetone, and Acetone plus 0.723 mg/L Methylene Blue

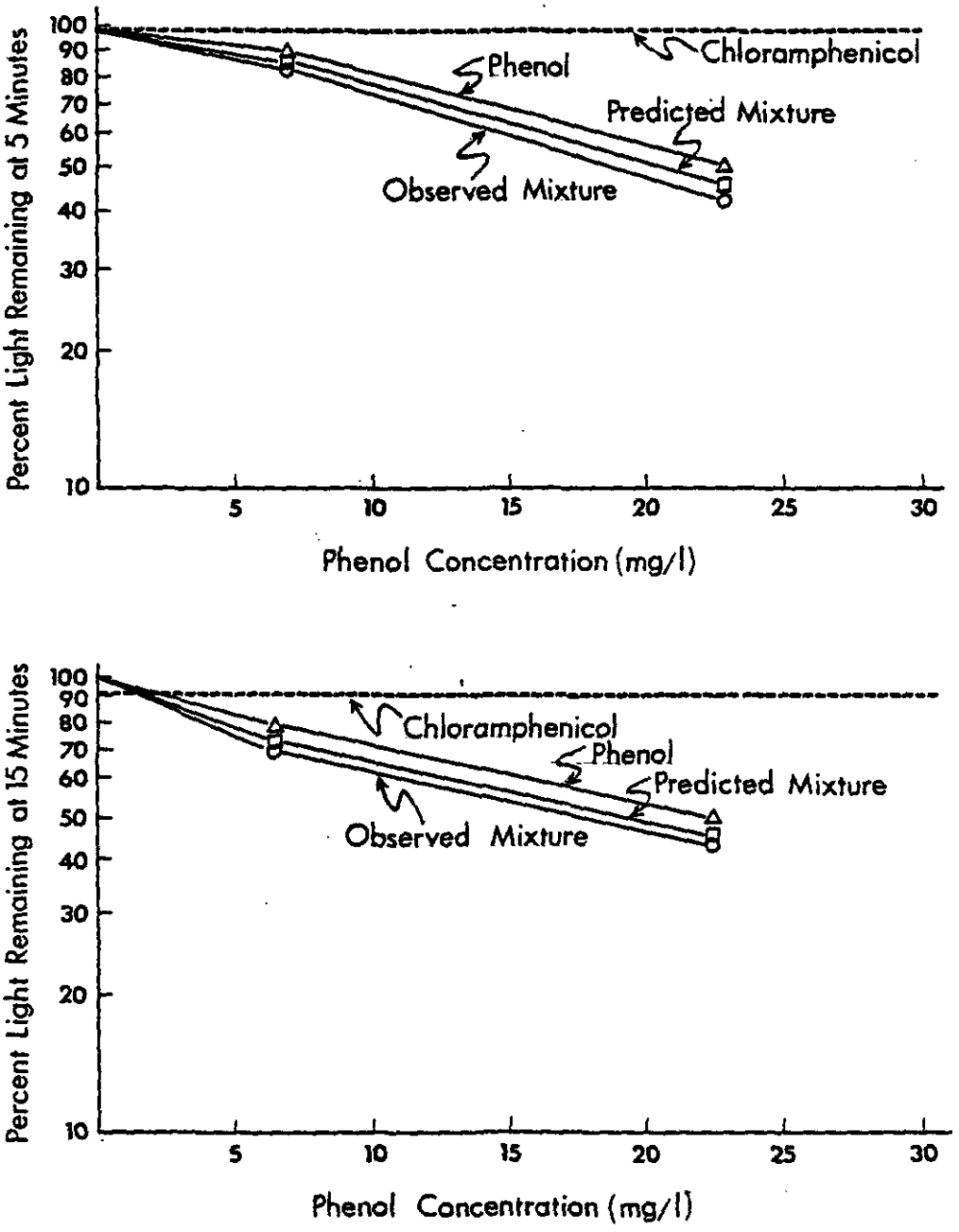

Figure 4. Log Percent Light Remaining after Exposure of 5 and 15 minutes to 55 mg/L Chloramphenicol, Phenol, and Phenol plus 55 mg/L Chloramphenicol

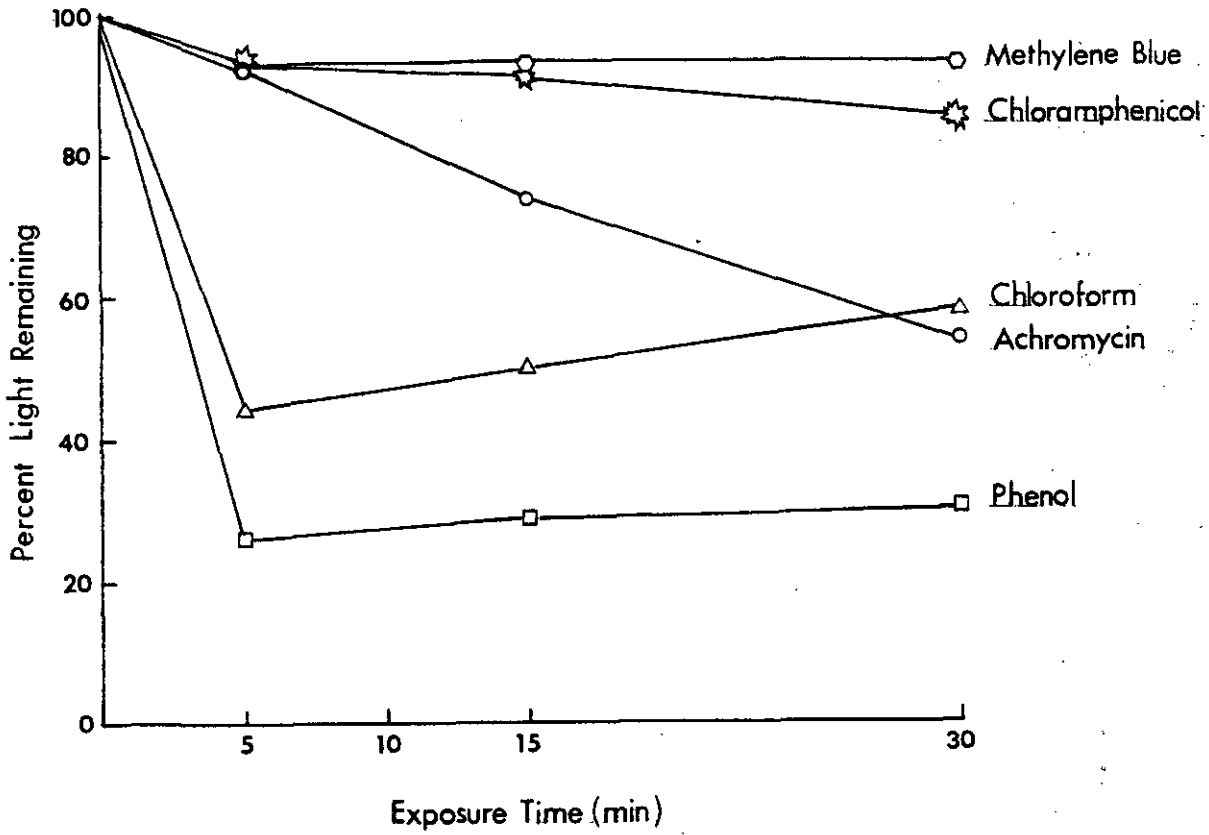

Figure 5. Percent Light Remaining after Exposure to 9.69 mg/L Chloroform, 23 mg/L Achromycin, 72 mg/L Phenol, 54.5 mg/L Chloramphenicol, and 0.727 mg/L Methylene Blue

Table 7. *Percent Light Remaining Values for Phenol (7.27 mg/L) and Chloroform (27 mg/L) Combined With Either Methylene Blue, Chloramphenicol, $NaAsO_2$ or Na_2HAsO_4, to form a 3-Component System, 15°C*

Third Component	Concentration (mg/L)	Exposure Time(Min)	% Light Remaining for Single Solute	% Light Remaining for Mixture (Observed)	% Light Remaining for Mixture (Predicted)	Percent Difference[a]	Date of Data Generation
Methylene blue	1.09	5	88	55	44	-25	6/15/83
		15	86	59	48	-23	
		30	85	62	56	-11	
Chloram-phenicol	54.5	5	93	59	47	-26	6/15/83
		15	88	61	51	-20	
		30	82	64	56	-14	
$NaAS(III)O_2$	72.7	5	74	46	46	0	6/24/83
		15	56	39	36	-8	
		30	47	37	30	-23	
$Na_2HAS(v)O_4$	145	5	100	63	60	-5	6/24/83
		15	92	60	58	-4	
		30	88	57	55	-4	

a. $\frac{\text{EC50 Predicted-EC50 Observed}}{\text{EC50 Predicted}}$ x 100 percent

indicates the light remaining at varying exposure intervals for a three component system consisting of phenol (7.27 mg/L) and chloroform (27 mg/L) plus either methylene blue (1.09 mg/L), chloramphenicol (54.5 mg/L), arsenic + III (72.7 mg/L), or arsenic + V (145 mg/L). The three component mixtures displayed antagonistic interactions at almost all exposure periods, with methylene blue and chloramphenicol resulting in the greatest antagonistic response. The percent difference for the methylene blue-phenol-chloroform and chloramphenicol-phenol-chloroform mixtures, for example were -25 percent and -26 percent, respectively at five minutes, and -23 percent and -20 percent, respectively, after 15 minutes of exposure. The arsenic (+ V)-methylene blue-phenol mixture displayed only slight antagonism with percent differences ranging from -4 to -5 percent. The arsenic (+ III) - methylene blue-phenol mixture displayed variable interactions with a zero percent difference (additive) at five minutes and a -23 percent difference (antagonistic) at 30 minutes. Only percent differences greater than 15 percent were considered to represent antagonistic (-) or synergistic (+) interactions because of Beckman's (1983) previously reported 11 percent coefficient of variation for representative analyses.

Table 8 indicates the light remaining at varying exposure intervals for a four component system consisting of phenol (7.27 mg/L) plus combinations of three of the following compounds: chloroform (271 mg/L), acetone (5,710 mg/L), DMF (4,300 mg/L), achromycin (21.8 mg/L), methylene blue (1.09 mg/L) or Na_2HASO_4 (145 mg/L). Both antagonistic and synergistic interaction were observed. Synergistic responses were observed only in the four component system containing arsenic (+V). Noteworthy was the pronounced antagonistic interaction (-85 percent) for the methylene blue-chloramphenicol-achromycin-phenol mixture after 30 minutes of exposure.

Conclusions

The toxic effects exerted by single solute systems on Microtox bioluminescence were additive for all two component mixtures examined. The interaction of constituents within mixtures of three or four chemical components was variable. The three component mixtures tested displayed antagonistic interaction while four component mixtures displayed either antagonistic or synthergistic interaction. The interaction of mixtures is therefore dependent upon the chemical properties of the constituent components. EC50 values for the toxicants listed varied over five orders of magnitude with endrin being most toxic (5EC50 = 0.7 mg/L) and acetone being least toxic (5EC50 = 22,000 mg/L).

The compounds tested additionally demonstrated varying time dependency on bioluminescent diminution. None of the three

Table 8. Percent Light Remaining Values of Four Component Mixtures Containing 7.27 mg/L Phenol plus Three Additional Compounds at 15°C

Phenol plus	Concentration (mg/L)	% Light Remaining for Single Solute After 5/10/15 Minutes of Exposure[a]	Exposure Time of Four Component Mixture (Min.)	% Light Remaining for Mixture (Observed)	% Light Remaining for Mixture (Predicted)	Percent Difference[b]
Chloroform	271	74/81/85	5	44	39	-13
Acetone	5710	92/94/94	15	48	45	-7
DMF	4300	76/77/76	30	51	47	-9
Methylene Blue	1.09	89/85/84	5	54	44	-23
Chloramphenicol	54.5	91/88/85	15	44	36	-22
Achromycin	21.8	90/66/38	30	37	20	-85
AS(V)	145	100/91/88	5	52	55	+5
Chloroform	271	76/79/89	15	46	51	+10
Achromycin	21.8	98/84/60	30	46	39	-18
AS(V)	145	100/91/88	5	58	54	-7
Chloroform	271	76/79/89	15	51	56	+9
Methylene Blue	1.09	94/95/94	30	48	63	+24

a. The chloroform/acetone/DMF and methylene blue/chloramphenicol/achromycin single solute values were generated on 6/23/83 while the AS(v)/chloroform/achromycin and AS(v)/chloroform/methylene blue single solute values were generated on 6/4/83.

b. $\frac{\text{EC50 Predicted-EC50 Observed}}{\text{EC50 Predicted}}$ x 100 percent

compounds investigated (chloramphenicol, methylene blue, achromycin) enhanced the sensitivity of the Microtox test via synergistic interaction with the test compounds.