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Development and Applicability of Artificial Reference Sediments for Interpretation of Results and Evaluation of Toxicity with the Microtox® Solid-Phase Bioassay

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Kristi Jill McAfee

May, 1994

Dedication

This thesis is dedicated to my parents, Dr. Wallace McAfee and Nelda McAfee, who always knew that I could succeed and who taught me the importance of continuing to learn throughout life; to my friend, James E. Egner, for support, encouragement, and advice; to Bingo Picard McAfee and Allister Bailey McAfee, for patience and unconditional love; and to the memory of Peggy Doherty.

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Abstract

The Microtox® Solid-Phase Test estimates the toxicity of sediments by measuring light reduction in the photoluminescent bacterium, species *Photobacterium phosphoreum*, upon exposure to the sediment. However, particles less than 45 microns in diameter often remain suspended in the diluent during testing and pass through the filter with the bacteria, scattering the luminescence of the bacteria and giving the illusion of toxicity. In order to determine the extent of this scattering effect, site-specific artificial reference sediments can be constructed from non-toxic components to provide a baseline of light inhibition by clay. As the concentration of clay in the sediment increases, the measurable light output decreases. From this relationship and the baseline light inhibition as determined from artificial sediments, the actual toxicity of the test sediments due to contaminants can be measured.

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Chapter 1

Introduction

The Microtox® Solid-Phase Test protocol evaluates toxicity of test sediments by measuring the metabolic inhibition due to sediment contaminants on the test organism, *Photobacterium phosphoreum*. This bacterium naturally luminesces as a function of its metabolic rate. The metabolic rate drops when exposed to contaminated sediments, and this drop is reflected in the amount of luminescence generated by the bacteria. The Microtox® measures the light output of exposed bacteria and assesses its difference from controls of unexposed bacteria, thus allowing the determination of EC50, the concentration of sediment at which there is a 50% reduction in light output from the controls, for the test sediment. Unfortunately, turbidity in the sample often gives the appearance of toxicity, due to the inability of light to penetrate suspended particulate matter. Therefore, it is necessary to have a reference sediment, with the same physical characteristics as the test sediment, tested in conjunction with the test sediment, to determine the effect of turbidity on the apparent toxicity of the sample. This reference sediment will reveal the amount of apparent toxicity due to turbidity, so that the actual toxicity of the test sediment can be determined. Usually "clean" sediments, those without the presence of contaminants, from a site near the test site are used as references. However, due to the extreme variation in sediments from site to site, and the uncertainty of possible

contamination, field sediments are, in reality, unsuitable for use as controls. Furthermore, obtaining two sediments from the field which have similar characteristics, yet different levels of toxicity is highly unlikely. Therefore, other means must be found to provide baseline light reduction information for test sediments.

Artificial sediments, created and carefully controlled in the laboratory, can be engineered to mimic specific test sediment characteristics. These artificial reference sediments are assured to have no toxicity due to contaminants, because only non-toxic components are selected in their creation. Therefore, the only light output reduction will be due to the presence of small clay particles which remain suspended during the test procedure. This amount of light reduction can be used as a baseline for the respective test sediment in order to determine actual toxicity. Any additional light reduction in the test sediment beyond the baseline value of the reference sediment can be contributed to the presence of contaminants in the sediment.

By establishing the relationship of light output to the concentration of a non-toxic clay component, a curve can be constructed which can then be used to predict the inhibition of light output due only to clay particles in the test sediments. This curve, together with artificially created references, can be used to determine actual levels of toxicity in sediments, thereby giving further significance to the results produced by the Microtox® Solid-Phase Test.

Chapter II

Literature Review and Background Information

1. About Sediments

The ability of a sediment to capture and hold a contaminant is a function of its physical characteristics, such as pH, particle size distribution, and organic content, and must be considered when assessing the toxicity of a sediment, as there will be variation among sediments (Soil Survey Staff, 1975; Håkanson, 1992). Movement of contaminants in and out of sediments is controlled by physical, chemical, and biological processes. Since sediment is composed of detritus and organic particles, as well as inorganic particles, it tends to be heterogeneous, varying spatially and seasonally, due to differences in salinity, species, water movement, sediment movement, and pore water residence time, etc. (Power and Chapman, 1992; Watson, 1985).

In recent years, there has been an awakened awareness of the need for toxicity testing of sediments, due to anthropogenic introduction of contaminants by industry, release of contaminants from sediments into aquatic systems due to dredging, and the subsequent likelihood of transport of these contaminants through the food chain (Burton, 1991; Cahill, et al., 1987; Couture, et al., 1989). Sediments are recognized as both repositories and sources for anthropogenic contaminants; therefore, certain

species, such as the burrowing *Chironomous tentans*, which in its adult stage leaves the aquatic system, pose as much more far-reaching problem in the food chain (Nebeker, 1984; Nelson and Coyle, 1990; Power and Chapman, 1992).

Due to their physical and chemical properties, sediments will attract certain contaminants, therefore the potential toxicity of a sediment will be decided by its characteristics. For example, clay particles usually carry a positive charge, and while repelling each other, are able to bind negative counterions (Singh, 1986). Therefore, different sediments will exhibit different toxicity levels for the same amount of contaminant (Di Toro, et al., 1990). These contaminants can reach benthic organisms by three primary routes, ingestion of the sediment itself, contact with overlying water, or contact with porewater, across respiratory surfaces and body walls (Power and Chapman, 1992). Relevant properties therefore include those which influence the distribution of chemicals between the solid and aqueous phases, such as organic carbon, which determines the extent of sorption of nonionic organic chemicals to sediments (Di Toro, et al., 1990). The amount of acid volatile sulfide (AVS) increases the metal-binding capacity of sediments, rendering metals unavailable to certain aquatic biota, but potentially very toxic to species which ingest sediment, such *Hyalella azteca* and *Chironomous tentans* (Howard and Evans, 1993). Similarly, contaminants such as PCBs and polyaromatic hydrocarbons also bind to the sediment, endangering benthic organisms which feed on the sediment (Brouwer, Murphy, and McArdle, 1990). Therefore, anthropogenic contaminants have the potential to severely impact sedimentary and benthic environments, which in turn will create a

cascade of effects throughout the food web.

2. Methods of Sediment Testing

With the increase in awareness of sediment contamination, the need to initiate sediment testing has arisen. The three primary methods for determining sediment toxicity testing are whole-sediment tests, elutriate tests, and interstitial or porewater tests. Porewater and elutriate tests, however, only identify non-sediment-bound contaminants, while whole-sediment tests can identify sediment-bound contaminants, such as the aforementioned polyaromatic hydrocarbons, as well as water-soluble contaminants (Brouwer, Murphy, and McArdle, 1990). Therefore, whole-sediment tests are generally more sensitive than extract tests. However, for many years porewater tests have been used to identify contaminants in sediments, due to the simple nature of the test, and the fact that whole-sediment tests have only recently become a more reliable form of toxicity assessment (Knezovich and Harrison, 1987). Several different test organisms have been included in sediment porewater and elutriate tests, including the Microtox® *Photobacterium phosphoreum* (Giesy, et al., 1988; Ankley, Schubauer-Berigan, and Dierkes, 1991)), the oligochaete *Lumbricullus variegatus* (Ankley, Schubauer-Berigan, and Dierkes, 1991), *Ceriodaphnia dubia,* and *Daphnia magna*. However, it is necessary to remove porewater from the sediment via centrifugation within 24-hours of collection, due to chemical changes in the

sediment that occur if this holding time is exceeded (Watson, Frickers, and Goodchild, 1984; Ankley, Schubauer-Berigan, and Dierkes, 1991).

Whole-sediment tests produce a more accurate assessment of sediment toxicity, utilizing a wide variety of test organisms. Aquatic organisms, such as *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas* (fathead minnow) larvae, are used to assess the effect of contaminants which leach out of the sediment into the overlying water (Suedel, 1993; Sasson-Brickson and Burton, 1991; Nebeker, et al., 1984). However, benthic organisms have proven to be more sensitive to sediment contaminants, both water-soluble and particle-bound, than aquatic organisms, and are therefore preferred in sediment testing. Test organisms include, but are not limited to, *Chironomous tentans, Chironomous riparius, Hyalella azteca, Hexagenia limbata, Rhepoxynius abronius,* and *Gammarus lacustric*. These organisms offer a wide variety of testing applications. Sensitivity to contaminants, as well as the degree of sensitivity, varies among organisms, thereby providing a method to screen sediments for particular contaminants (Suedel, 1993; Ciborowski, et al., 1991; Ciborowski, et al., 1992; Hanes, et al., 1990; Ditsworth, Schults, and Jones, 1990; Nebeker, et al., 1984; Giesy, et al., 1988).

The Microtox® Solid-Phase Test has also been used to assess the toxicity of whole-sediments. There have been only a few studies, however, thus far, concerning the solid-phase portion of this test, as it is a relatively new procedure. Some studies do show that the Microtox® Solid-Phase Test is more sensitive to contaminants than other forms of testing, but generally the results are difficult to interpret due to

improper, unsuitable, or absent reference sediments (Brouwer, Murphy, and McArdle, 1990). Testing of porewater and elutriates from sediments has been shown to be more sensitive than many whole-sediment assays, in spite of its susceptibility to low pH levels, but is generally only recommended as a screening method, to identify sediment toxicity "hot spots" (Giesy and Graney, et al., 1988; Giesy and Rosiu, et al., 1988; Bennett and Cubbage, 1992; Couture, et al., 1989).

3. The Microtox® Test

A. The Test Organism

The Microtox® test measures the metabolic inhibition of liquids and solids on *Photobacterium phosphoreum*. By exposing the organism to test samples, the toxic effect of the sample on the organism can be measured. The Microtox® Reagent used in these tests is living bioluminescent bacteria grown under optimal conditions, harvested, and lyophilized, or freeze-dried, at the Microbics Corporation in Carlsbad, California. The bacteria are rehydrated using Microtox® Reconstitution Solution, also prepared by the Microbics Corporation. The freeze-dried bacteria are stored at minus 20° C until ready for use. Each vial of bacteria is reconstituted only minutes before exposure to the test sample, and remains viable for approximately two hours after reconstitution.

Some toxic materials reduce the light output of the organisms instantaneously, while others may take five, fifteen, or even thirty minutes to solicit an effect (Microbics, Vol. 1, 1992). The toxic effect of the material is derived by measuring the reduction of light output by the organisms upon exposure to the test sample. Each cuvette of sample will contain approximately one million organisms, thereby rendering statistical variations among organisms insignificant (Microbics, Vol. 1, 1992). The Microbics Corporation assures homogeneity and uniformity among batches of test organisms. The system measures the combined, simultaneous light output of all organisms in the test sample, and records any reduction of light output due to exposure.

Photobacterium phosphoreum luminesces as a function of its metabolic activity. The Microtox® apparatus measures this light output and the effect, if any, that the test sample has upon it. A toxic test sample will reduce the metabolic activity of the bacteria, thereby reducing the light output. The effective concentration of a sample causing a 50% decrease in the light output of the bacteria from the control mean under a defined exposure time (EC50) is used to evaluate the toxicity of the sample.

B. Testing Apparatus

The Microtox® testing system used in this study consisted of the Microtox® Model 500 Analyzer, a Compaq® LTE Lap-top Computer, a Kodak® Diconix Printer,

and a Neslab® Endocal RTE-100LP incubator. The computer housed the Microtox® data analysis software, and was dedicated solely to this purpose in order to limit access to the test files.

The Microtox® Analyzer unit consists of thirty test wells, where tests are performed and test cuvettes are stored at a constant temperature of 15° C while being tested. There is also a REAGENT well, where reconstituted reagent is stored at the optimum temperature of 5.5° C until ready for use, and a READ well, where cuvettes are lowered into the analyzer unit for light output measurement. The latter is also maintained at 15° C. The analyzer is equipped with warning indicator lights, alerting the user to problems in the system, such as unstable temperature or a cuvette only partially in the READ well. The green READY light indicates that the unit is ready to read a cuvette, and the digital display registers the light reading for each cuvette (see Figure 1).

The Microtox® Analyzer is self-calibrating. Before beginning reading, the user places the first control cuvette in the READ well. When the SET button is pressed, the analyzer measures the energy-output from the photomultiplier circuitry when the light-measuring chamber is dark, and adjusts this amount to zero. The control cuvette is then lowered into the READ well, and its light output level is read. The analyzer assigns this control a value of 95 ± 5, no matter what the level is. Therefore, all later measurements are expressed as values on a comparative scale, based on the actual properties of the Reagent currently in use. This procedure calibrates the analyzer for each test, and must be repeated each time a new test is initiated. It is

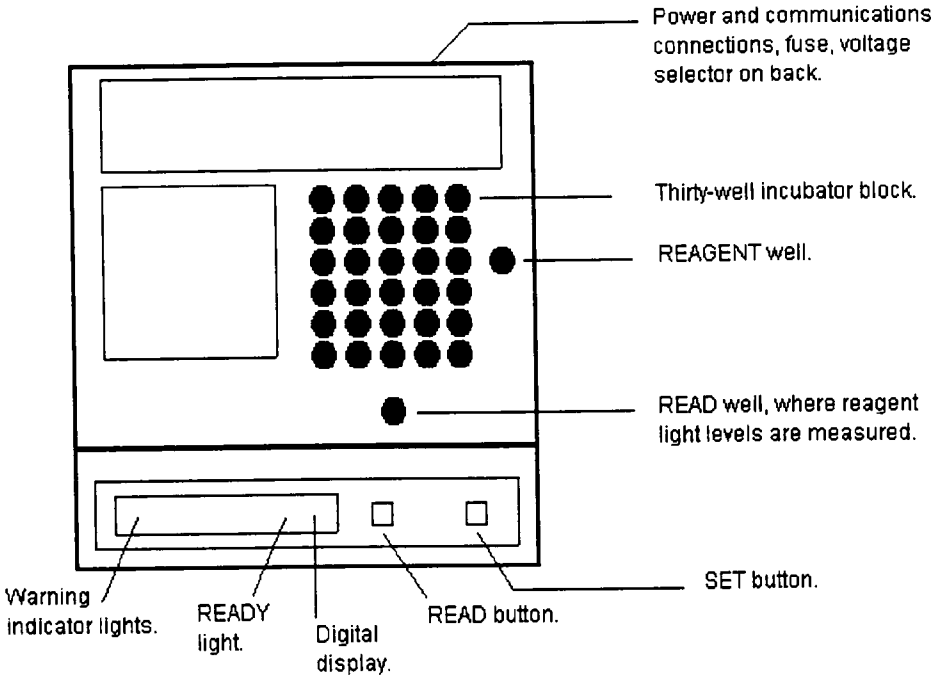


Figure 1: Diagram of the Microtox® Model 500 Analyzer (top view).

not necessary to calibrate before each timed set of readings within a test.

The test procedure requires that a non-toxic, positive Reagent Control, or blank, be tested concurrently with the test samples. In the case of the Solid-Phase Testing Procedure, three controls are recommended to better document variation among cuvettes. Light levels will become reduced over time due to a small amount of bacteria death, and the control allows the user to differentiate between light reduction due to normal drift with light reduction due to toxicity.

The Data Reduction program converts the raw light levels to gamma values. The gamma value is the ratio of the light inhibition to the light level after exposure of the bacteria to the test sample. The program captures the raw data from the analyzer and uses it to assess toxicity by calculating the EC50 of the test sample and the percent effect at each concentration (Microbics, Vol. 1, 1992). However, this system can only detect toxicity. When samples are not toxic, it considers the data points as invalid. The system will then run a message "Insufficient Data to Do Calculations". This message does not necessarily indicate a problem with the test. Review of the actual raw data (light readings) and the gamma values will provide information needed to determine if the sample was simply not toxic or if there is actually a systematic problem. This ability to interpret data reports produced by the Microtox® Data Reduction program is a skill acquired by the user through time.

C. Microtox® Solid-Phase Test

The Microtox® Solid-Phase Test is designed to measure the effects of toxicants, tightly bound to the soil or sediment particle, on the test organism. The procedure allows test organisms to come into direct contact with the particle-bound toxicants in an aqueous suspension of the test sample. This suspension, incubated for 20 minutes at 15° C in a incubator unit (see Figure 2), allows the bacteria to fully contact the sediment itself, not only the porewater or elutriate as found in other testing procedures (Bennett and Cubbage,1992). The Solid-Phase test utilizes one to three controls as prepared above, with no sediment added (Microbics, Vol. 2, 1992).

In spite of its usefulness, the Microtox® Solid-Phase Test is extremely problematic. Up until this time, it has been used as primarily a screening test, to identify "hot spots" of sediment contamination (Giesy and Rosiu, 1988). This is due to the presence of apparent toxicity in samples with a large percentage of silt particles, as will be discussed later.

Sediments can contain a high level of natural toxicity in areas that support abundant flora and fauna. Furthermore, these areas often support many microorganisms which can break down toxicants into non-toxic forms, thus giving the area a higher capacity to handle toxicity (Microbics, Vol. 2, 1992). The Microbics Corporation recommends obtaining control or reference sediments from the same area to help distinguish between natural and anthropogenic toxicity. According to

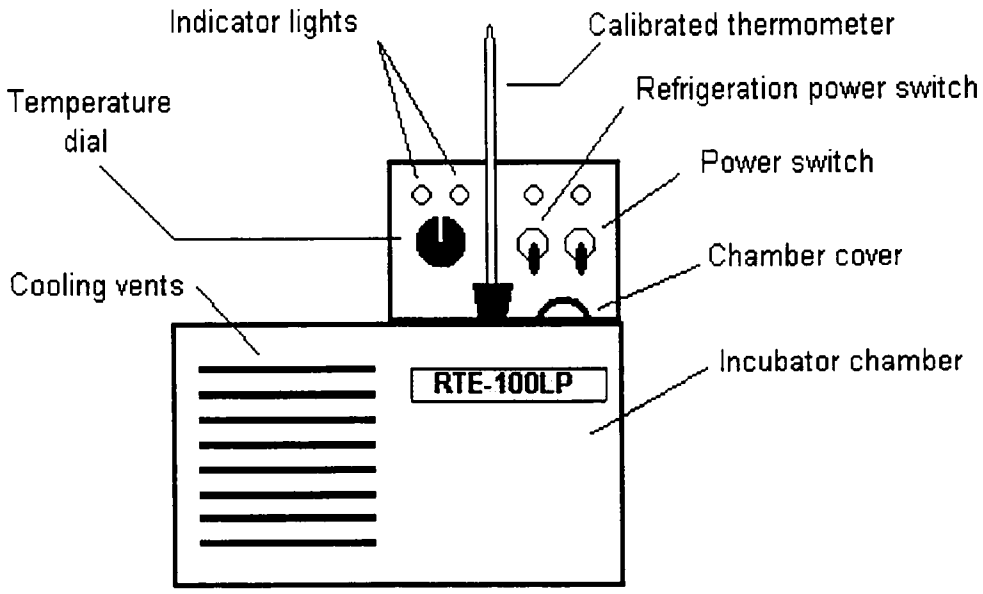


Figure 2: Neslab® Endocal RTE-100LP Incubator (front view).

various sources, a good reference sediment should contain the same particle-size distribution, organic composition, and moisture content as the test sample (Microbics, Vol. 2, 1992; Burton, 1992; Power and Chapman, 1992). Due to the complexity of the sampling regime described in the next section, and the actual difficulty of locating "clean" sediments similar to the test sediments in the sampling area, the idea of procuring reference sediments from field sites was quickly squelched. However, in order to determine the actual anthropogenic toxicity of the test sediments some form of reference sediment had to be devised.

As the size of the particle decreases, the ratio of surface area per unit volume becomes larger. Thus, per unit volume, a smaller particle has more binding sites to which toxicants could readily affix themselves than a larger particle, and are therefore able to hold more toxic material. However, due to the nature of the Microtox® Analyzer, these same smaller particles could also indicate apparent toxicity in a non-toxic same. These particles remain suspended in the test solution, thereby creating turbidity, which blocks the ability of the analyzer to read the light output of the bacteria as they luminesce. Thus, an apparently high level of toxicity could be due to an actual reduction in light output of the bacteria due to a toxicant, or simply the inability of the analyzer to detect light output due to suspended particles in the sample. It is therefore necessary to have a reference sediments with the same physical characteristics as the test sediment. By comparing results of testing from the reference and the sample, the user could determine if the apparent toxicity of the test sample was due to the physical characteristics of the sediment or actual

anthropogenic toxicity. Thus, without a reference by which to "gauge" the results of the test sediments, the tests produce virtually no applicable conclusions for the user.

Chapter III

Materials and Methods

1. Sampling of Sediments from Clinch River and Poplar Creek

Sediments for testing purposes were sampled from Poplar Creek and Clinch River by a sampling team from the Clinch River Environmental Restoration Program (CRERP) of the Oak Ridge National Laboratory (ORNL) Environmental Sciences Division (ESD). The sites for sampling were chosen by CRERP. Sediment samples were taken using a sampling dredge, then placed in 4-L Nalgene® containers with a layer of overlying water from the sampling site to prevent drying of the sediment, and transported to ORNL at 4° C. Upon arrival at ORNL-ESD Toxicology Laboratory, samples were refrigerated at 4° C until ready for use.

Before these sediments could be used in any kind of testing, homogenization was required. This simply consisted of removing the overlying water from each sample, transferring the sample to a large, plastic bucket, and mixing the sample thoroughly using a spatula. Once the sediment was homogenized, portions were transferred to testing dishes for chronic tests with *Daphnia magna*, *Hyalella azteca*, *Adontis imbecellis*, and *Chironomous tentans*. The remaining sediment was returned to the Nalgene® container and the overlying water was replaced carefully, avoiding any mixing with the sediment. These sediments were returned to storage at 4° C.

It was from these larger samples that subsamples were taken for Microtox® testing.

Eight sites were included in the sampling regime, three sites from the Clinch River and five sites from Poplar Creek. Site designations were measured in miles, with the source abbreviated **PC** (Poplar Creek) or **CR** (Clinch River). Thus, site **CRM 9.0** designated Clinch River mile 9.0. Sampling sites were as follows: from Poplar Creek, PCM 1.0, PCM 2.9, PCM 4.3, PCM 5.1, and PCM 6.0; from Clinch River, CRM 9.0, CRM 19.0, and CRM 22.0.

2. Microtox® Testing of Clinch River and Poplar Creek Sediments

As part of the sediment testing regime required by CRERP, sediments were analyzed using the Microtox® Solid-Phase Test. Though different variations of the procedure outlined in the Microtox® Detailed Protocols Manual (Brouwer, Murphy, and McArdle, 1990; Bennett and Cubbage, 1992) were attempted, the most consistent results were obtained by the method outlined in the manual.

The Solid-Phase Testing was conducted using the incubator and Microtox® testing unit described in Chapter 2. Both the incubator and testing unit were maintained at a constant temperature of 15° C, the recommended optimum temperature for *Photobacterium phosphoreum* testing with this apparatus (Microbics, Vol. 1, 1992). The Microtox® testing unit is self-calibrating. The temperature of the Neslab® Endocal RT100-LP incubator was monitored constantly using a mercury

thermometer inserted into the refrigerated incubation chamber. The calibration of this thermometer was checked against a NIST Standard thermometer.

In preparation for testing, approximately 10-13 mL of sediment was placed in a 15-mL disposable plastic centrifuge tube. The sediment was centrifuged for 20 minutes at 3500 rpm using an Octagon small-volume centrifuge with a three-inch diameter. This removed porewater from the sample, allowing for a drier sediment to be used in the testing. Excess moisture in the sediment reduces the actual concentration of the test sample, yielding inaccurate results (Microbics, Vol. 3, 1992). Two samples of each sediment were tested and analyzed separately.

Following centrifugation, the pore water was removed from the tube using a polished glass pipette affixed with a pipette bulb. The sediment in the tube was carefully mixed using a scoopula to create a homogenous sample. Using a Mettler® AE 163 balance, .3 grams of centrifuged sediment was isolated and placed in a Microtox® Solid-Phase Filter Tube. The exact weight of the sediment used was recorded. The filter tube was transferred to the incubator to allow temperature equilibration while the remaining equipment was prepared.

Nine empty Microtox® Solid-Phase filter tubes were placed in a holding rack inside the incubator. Using an Oxford 1-5 mL adjustable pipette affixed with a disposable pipette tip, 1.5 mL of Microtox® Solid-Phase Diluent was pipetted into each of the nine tubes. Finally, 3.0 mL of diluent was pipetted into the filter tube containing the sediment. Using Parafilm® to seal the tube, the sediment-diluent mixture was thoroughly shaken to obtain a homogenous 10% dilution of sediment.

The tube was returned to the holding rack, and using the same pipette as above, the sediment was mixed yet again by filling and dispensing the pipette several times. Then, while the sediment was still suspended throughout the diluent, 1.5 mL of sediment-diluent mixture was transferred to the next filter tube. The contents of this tube were mixed using the pipette, and then 1.5 mL was transferred to the next filter tube. This procedure was repeated until seven decreasing serial dilutions, including the initial 10% dilution, were prepared. 1.5 mL of the sediment-diluent mixture was discarded from the last tube, so that each of the seven tubes contained 1.5 mL of sediment-diluent mixture. The remaining three tubes containing only diluent were used as controls, as seen in Figure 3. All dilutions were allowed to equilibrate to 15° C for 10 minutes.

While the samples were equilibrating, ten Microtox® cuvettes were placed in ten wells of the Microtox® testing unit. One cuvette was placed in the well marked "Reagent". Into this cuvette, 1000 μL of Microtox® Reconstitution Solution were added using a 1000 μL Oxford® pipette affixed with a disposable tip. Nothing was added to the other ten cuvettes.

The Microtox® software was prepared next. The Solid-Phase Test program was loaded, and file names were assigned to each test. The initial dilution, calculated from the actual recorded weight above, was entered, as well as the number of controls, in this case three, and the number of dilutions and dilution factor, in this case seven dilutions with a dilution factor of two. The program automatically calculated the subsequent dilutions and entered the file information into memory.

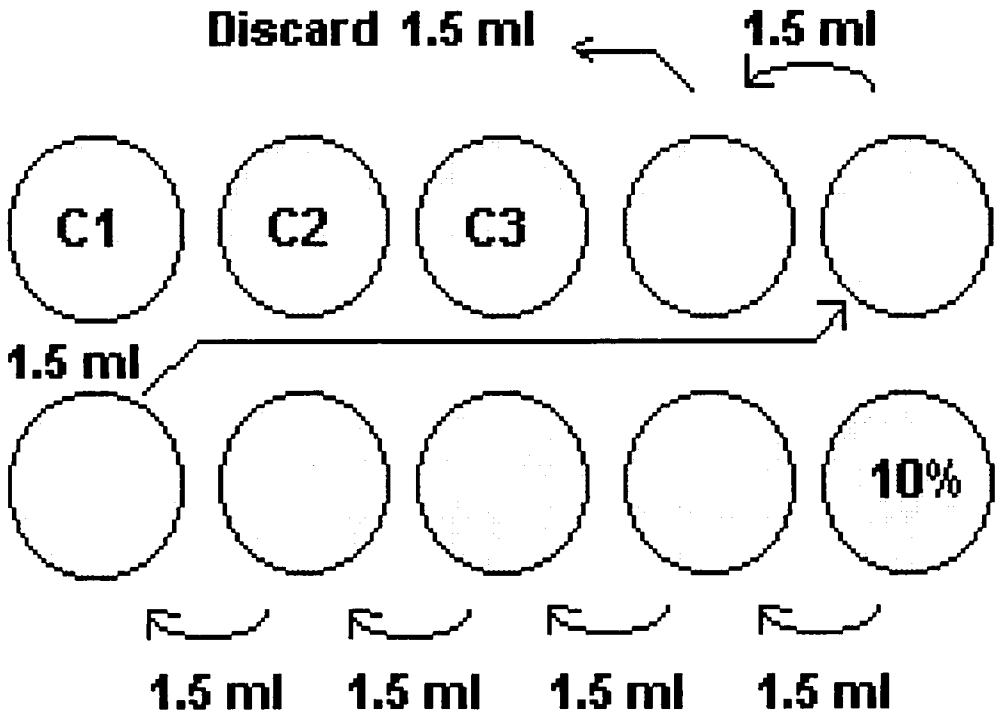


Figure 3: Diagram of serial dilution protocol for Microtox® Solid-Phase Test

A vial of freeze-dried *Photobacterium phosphoreum* was removed from the freezer, where it had been kept at minus 20° C. The cap was removed and the contents of the cuvette in the "Reagent" well of the testing unit were quickly added to the vial. The mixture was swirled gently, then returned to the cuvette. Using a 500 μL Oxford® pipette affixed with a disposable pipette tip, the reconstituted bacteria were gently mixed by filling and dispensing the pipette about 20 times. Next, using a repeat pipettor affixed with a .6 mL pipette syringe, 20 μL of reconstituted bacteria was pipetted quickly into each filter tube, including the controls. The bacteria were mixed with the sediment using the 500 μL pipette to allow maximum contact of bacteria with sediment. The tubes were allowed to incubate for 20 minutes. During the incubation period, Microtox® Solid-Phase Filter Columns were inserted into the filter tubes until the bottom of the column lay above the sediment-diluent mixture, but <u>not</u> in contact with it.

At the end of the incubation period, the computer timer was started, and one at a time each filter column was gently pressed into the filter tube, allowing filtered diluent and bacteria to rise into the filter column, as demonstrated in Figure 4. Using a 500 μL Oxford® pipette affixed with a disposable tip, 500 μL of the filtered mixture was transferred from each filter column into the corresponding cuvette in the testing unit. As soon as the transfer was complete, the computer timer was halted. After 5 minutes of incubation in the testing unit, each sample was read by placing it in the "Read" well of the unit and pressing the "Read" button. The testing unit self-calibrates using the first cuvette, which is always a control.

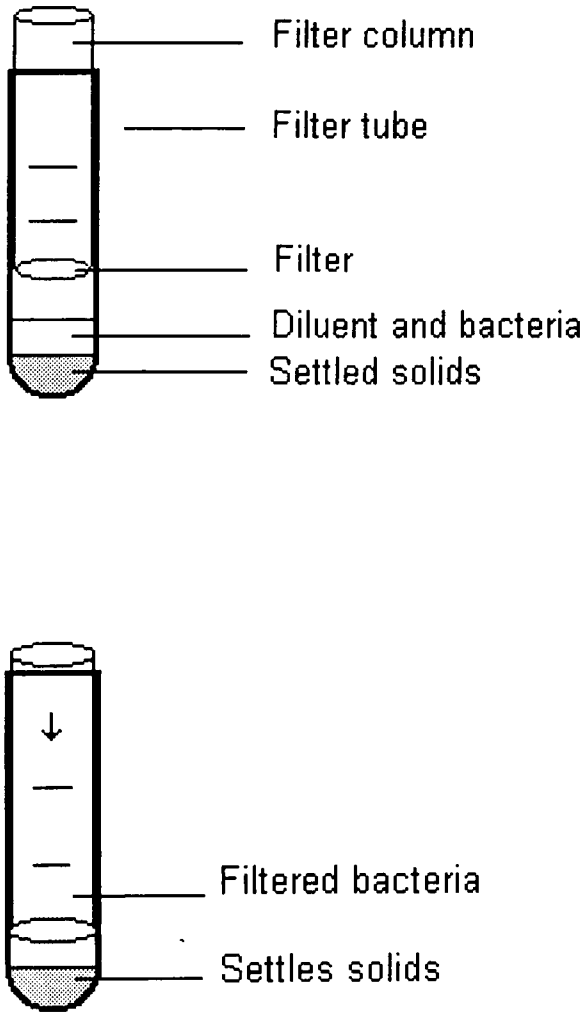


Figure 4: Diagram of filtering procedure in Microtox® Solid-Phase Test, before and after

When all the samples had been read, statistics for the sample were obtained using the Microtox® program. The output of this program was printed for each file. These output files showed actual light readings for each dilution and the EC50 as calculated by the program.

3. Characterization of Clinch River and Poplar Creek Sediments

Sediments were isolated and centrifuged as described above. Following removal of the portion for Microtox® testing, the remainder of the centrifuged sediment underwent characterization. The sediment was placed in a labelled, dried, and pre-weighed aluminum weigh pan. The exact weight of the sediment and pan, as determined using the Mettler® AE balance, were recorded. The pan was then wrapped in aluminum foil, ensuring that the foil did not contact the sediment, and placed in a drying oven at 100° C for 24 hours. At the end of the 24-hour drying period, the pan was removed and placed in a desiccator for 15 minutes to allow it to cool to room temperature. The pan was removed from the aluminum foil and weighed again using the Mettler® AE balance. This weight was recorded as the dry weight of the sediment. From this value, percent by weight of moisture in the sediment could be calculated (Ciborowski, et al., 1992; Microbics, Vol. 3, 1992).

The pan was again wrapped in aluminum foil and this time placed in an ashing furnace at 500° C for 2 hours. Though alternate ashing periods were tried, it was

determined that 2 hours was sufficient to remove organics from the sediment. This method is widely used for determining organic content of soils by loss-on-ignition (Davies, 1974; Bremner and Tabatabai, 1971; Ciborowski, et al, 1992; Ditsworth, Schults, and Jones, 1990). After two hours, the pan was removed from the oven and placed again in a desiccator for 15 minutes to allow cooling. The pan was then removed and reweighed. This weight was recorded as the ashed weight.

The weights of the pan, pan and wet sediment, pan and dry sediment, and pan and ashed sediment were entered into a LOTUS 123® worksheet along with the site and designation and replicate number. Two replicate characterizations were performed for each sediment, using the two independently centrifuged sediment subsamples described in Section 2. The LOTUS 123® worksheet was then imported into a SAS® program designed to calculate the percent moisture and percent organics of the sediment. These values for percent moisture and percent organics were used in creation of the artificial sediments.

Subsamples of the sediment were again removed from the original sample. This subsample was rinsed using distilled water through a series of sieves. Each portion was then rinsed into a clean, dried Pyrex® beaker, the weight of which had been previously recorded. The beakers were labelled with the particle size range and placed in a drying oven at 100° C until thoroughly dried, usually about 36-48 hours. Once dried, the sieved samples were removed from the oven, allowed to cool, and weighed again to determine the dry weight of each portion of the sediment.

The weights of the empty beaker and the beaker with dried sediment were

entered into a LOTUS 123® worksheet, along with the site designation and particle size range of each sample. This worksheet was then imported into a SAS® program designed to determine the percentage of each particle size range in the sediment. These values of particle size distribution were used in creation of the artificial sediment. The following sieve sizes were used to break down the sediments: 2000 μm, 850 μm, 425 μm, 250 μm, 150 μm, and 45 μm.

4. Creation of Site-Specific Artificial Sediments

Artificial sediments were created to imitate the physical characteristics as determined by the characterization methods above. Particle size distribution is the most important of these parameters, due to erroneous readings by the Microtox® unit in the presence of small suspended particulates. Therefore, the assessment of particle size distribution was the first consideration in creating the artificial sediments.

A. Selection of Clay Component

Three basic components were chosen as non-organic particulates. All three clays were tested independently using the Microtox® Solid-Phase Test. The results of these tests, as well as the evaluation of the clay's ability to readily combine with

other components, were considered in the final choice of the clay component. Originally, for particle sizes below 45 μm, Kaolinite, a ceramic clay, was chosen. However, due to difficulties with hydration of its Kaolinite particles, it did not readily mix with other components. Therefore, Amaco® sculptor's clay, a "wet" clay, was chosen to replace Kaolinite. Two colors were used, red and grey. Each was tested by itself, as well as mixed in equal proportions to obtain a color similar to that of the test sediments. Since the particles were already hydrated, the sculptor's clay mixed easily with the other components. When generating artificial sediments, the moisture content of the clay was taken into consideration, as were the minute amounts of organic material (@ 3%).

In addition to testing, the grey sculptor's clay was treated with sodium hexametaphosphate in order to remove any additives which might prove to be toxic. This procedure consisted of mixing 250 mL of a 40 g/L dilution of sodium hexametaphosphate with 100 g of sculptor's clay. The chemical reduces the clay to separate particles, suspended in a solution of dilute sodium hexametaphosphate. The mixture was centrifuged at 3500 rpm for 12 minutes, and the supernatant discarded. The remaining clay was rinsed with distilled water to remove any remaining chemicals, and labelled as single-rinsed clay. Next, a portion of the clay was again centrifuged as above, and the supernatant discarded. This clay was rinsed again with distilled water to become double-rinsed clay. This process was repeated yet again on a portion of the clay to yield triple-rinsed clay. All three rinses of clay were dried thoroughly in a drying oven at 100° C, ashed to remove any organics, allowed to cool,

and then carefully homogenized with a mortar and pestle. The three rinse regimes were tested to determine which best removed remaining sodium hexametaphosphate and other residual chemicals from the clay.

Generic gardening sand represented particle sizes ranging from 45 μm up to 850 μm. Talus®, a texturizing rock used in models and found in most hobby shops, was chosen for the 850 μm to 2000 μm range of particles. All these components were selected due to homogeneity, low organic content, and of course, availability. Prior to use, all components except the sculptor's clay were sieved using the same sieve sizes as above, to remove unwanted particle sizes, and were dried and ashed in an ashing furnace at 500° C for two hours, to remove excess moisture and organics. Upon thorough cooling, each "range" was stored separately in a covered container. From the Talus® rock, all particles below 850 μm and above 2000 μm were discarded. From the gardening sand, all particles above 850 μm and below 45 μm were discarded. The sculptor's clay consisted only of particles 45 μm or less, therefore no sieving was necessary.

B. Selection of Organic Component

Alfalfa, Cerophil®, yeast, algae, and peat moss, as well as a mixture of algae, species *Selenastrum capricornutum*, Cerophil®, and yeast, were considered for the organic portion of the sediment. Alfalfa was difficult to work with and did not blend

readily with other components. Cerophil®, yeast, and algae, and the mixture of the three, were unable to provide consistent amounts of organics and were therefore considered unreliable sources. However, peat moss, due to its homogeneity and ability to become hydrated quickly with the application of heat, proved to be an ideal source of organic content for the artificial sediments. A sample was ashed to assure that it was 100% organic. The peat moss was milled with a 2 mm mesh to break up any portions above 2000 μm. The remainder was sieved as needed when creating artificial sediments, assuring that the percent by weight of each particle size range was equal to the percent by weight determined in the archetype sediment.

C. Creation of Artificial Sediments

In order to have a sufficient volume of artificial sediment to test, but eliminate excess, all sediments were created to have an approximate final dry weight of around 10 grams. Thus, all percent-by-weight measurements were reduced to this scale. The results from characterization analysis were analyzed using a SAS® program. This program generated the amount of each component range needed, as well as the amount of organics needed, to create an artificial sediment with the characteristics of a specific test sediment. Using the output generated by this program, artificial sediments were created.

Sized dry, non-organic components were obtained for each size range needed.

All particles larger than 2000 μm were not included in the artificial sediment since this size is too large to affect the bacteria in the test procedure. Each percent by weight, as determined by characterization, was divided by 10. This allowed for weights of each component, when combined, to yield approximately 10 grams of sample, though somewhat less for sediments with large percentages of $>2000\ \mu$m particles.

It was assumed that each size range would have an equal percentage of organic components. Thus, the total weight of the component needed in each size range was multiplied by the percentage of organics to obtain the weight of organics needed in each range. This weight was subtracted from the total weight for that range. This new figure was the weight needed of non-organic components in that size range.

All components were combined in a 50 mL Pyrex® beaker with enough deionized distilled water to suspend the particles and facilitate moisture absorption. A stir bar was placed in the beaker, and the beaker, covered loosely to block settling dust, was placed in a water bath at approximately 90° C, and allowed to gently stir for 2-4 hours. The heat combined with the stirring allowed the organics to become thoroughly dispersed throughout the mixture. After the organics became hydrated, the warm water bath was removed, but stirring continued for another 24 hours.

At the end of 24 hours, the mixture was centrifuged at 3500 rpm for 15 minutes to remove excess overlying water. The sediment was then homogenized by mixing by hand, as described in Section 1 of this chapter, and immediately tested

using the Microtox®. Two small portions were dried and ashed to determine the precision of the measurement of organics. Excess sediment was refrigerated until needed.

Chapter IV

Results and Discussion

1. Testing of Clinch River and Poplar Creek Sediments

A. Results

July testing of sediments from Clinch River and Poplar Creek initially yielded ambiguous results. EC50 values were much lower than expected. Sediments collected during the July sampling period yielded EC50 values ranging from .3910% for Poplar Creek site 4.3 to 9.6161% for Clinch River site 9.0. Site 6.0 from Poplar Creek and site 22.0 from Clinch River were designated reference sites by the Clinch River Environmental Restoration Program, and therefore were expected to have very low or no toxicity. However, both of these sites demonstrated EC50 values around 1%.

Testing of sediments collected in August yielded results similar to those from July testing, with EC50 values ranging from .5325% at CRM 22.0 to 5.7938% at CRM 9.0. Unlike samples from July, August sediments were analyzed for particle size distribution to determine the percentage of particles less than 45 microns. Figure 5 demonstrates the particle size breakdown for all sediments from Poplar Creek and for Clinch River sites 9.0 and 22.0. Samples from Clinch River site 19.0 were of

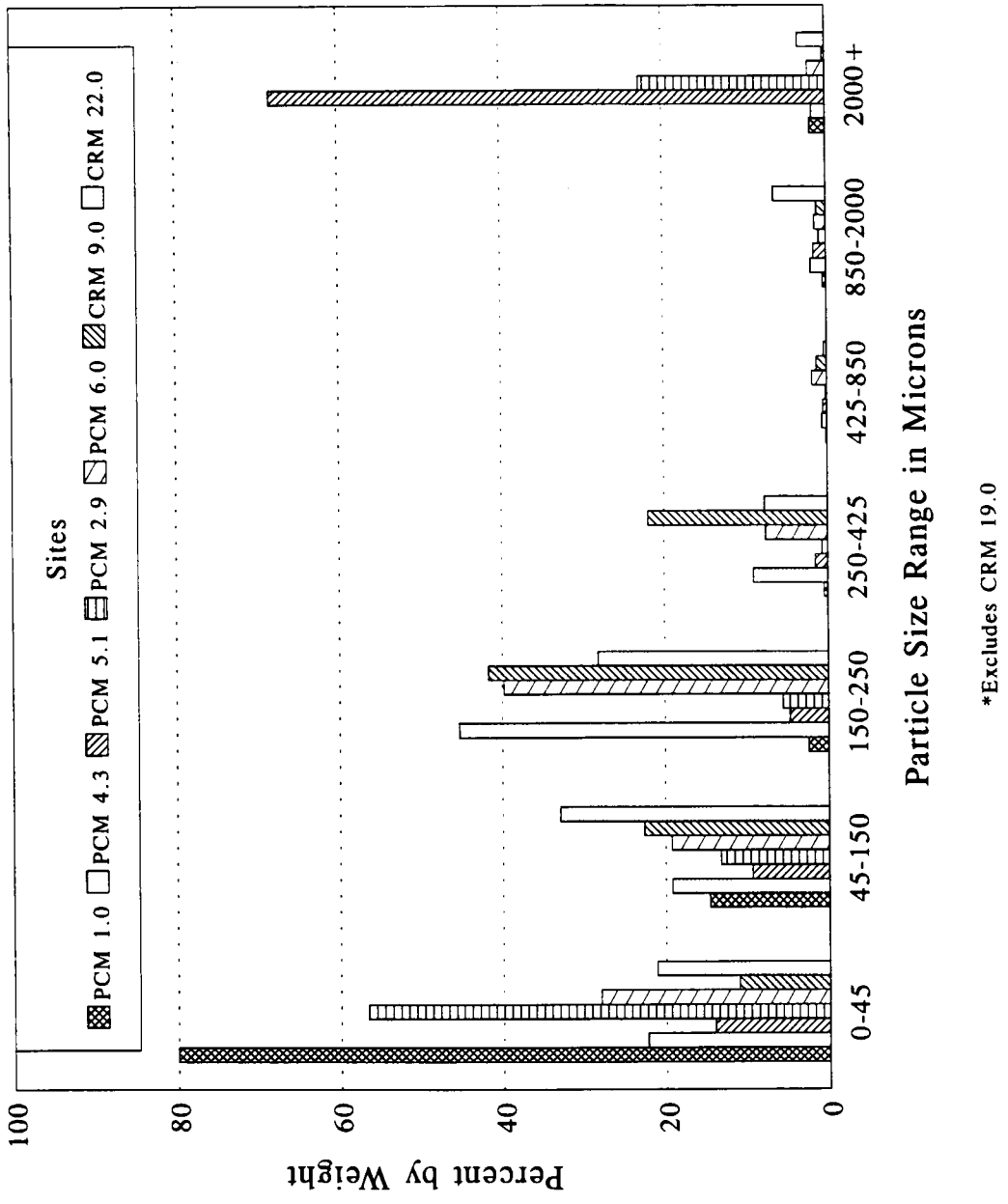


Figure 5: Particle size distribution analysis for Clinch River and Poplar Creek sediments*

insufficient volume to undergo particle size distribution analysis. Of the samples analyzed, the percent by weight of particles less than 45 microns ranged from 80% to 11%. Total organic matter percentages ranged from 14.2% at PCM 5.1 to 2.4% at CRM 9.0.

The final samples, collected in February, yielded results consistent with those from July and August. EC50 values ranged from .58% at Clinch River site 9.0 to 7.8% at Clinch River site 21.2. The percentages of total organic matter ranged from 1.2% at CRM 21.2 to 5.6% at PCM 5.1. Percent by weight of particles less than 45 microns ranged from 68% at PCM 1.0 to 19.6% at PCM 6.0. Table 1 presents the results of Microtox testing for all sediments, with and without correction for moisture content. Similarly, Tables 2 and 3 summarize the results of sediment characterization, moisture content and total organic matter respectively, from all three sampling periods.

B. Discussion

As demonstrated in Table 1, EC50 values generally dropped substantially as a result of moisture correction. In the initial test weight of the sediment, moisture accounted for a percentage of the total weight. Therefore, when the test concentration was recalculated to disregard moisture weight, the actual weight of sediment was lower than initially indicated in the computer input. Subsequently,

Table 1: EC50 results of Microtox® testing for Clinch River and Poplar Creek sediment samples collected July 1993, August 1993, and February 1994, with and without moisture correction

July 1994	Replicate 1		Replicate	
	No Moisture Correction	Moisture Correction	No Moisture Correction	Moisture Correction
PCM 1.0	1.5072	0.9376	1.8774	1.1679
PCM 2.9	2.7299	1.3537	2.4729	1.2262
PCM 4.3	0.6123	0.3910	0.7719	0.4930
PCM 5.1	6.9023	4.7301	7.5875	5.2000
PCM 6.0	1.8632	1.3225	1.7814	1.2644
CRM 9.0	12.5464	9.6161	10.1391	7.7771
CRM 19.0	3.8831	2.9135	5.2828	3.9636
CRM 22.0	1.1972	0.8908	1.4575	1.0753
August 1993				
PCM 1.0	0.7746	0.5380	2.477	1.6709
PCM 2.9	0.9911	0.6392	1.6735	1.0147
PCM 4.3	6.1468	4.1165	4.7587	3.2443
PCM 5.1	3.5345	2.5152	3.4190	2.4896
PCM 6.0	3.1728	2.5224	2.0814	1.2598
CRM 9.0	4.7881	3.7184	6.0930	5.7938
CRM 19.0	1.5564	1.1541	2.3294	1.7568
CRM 22.0	0.7016	0.5325	1.0922	0.8315

Table 1: (con't)

February 1994	Replicate 1		Replicate 2	
	No Moisture Correction	Moisture Correction	No Moisture Correction	Moisture Correction
PCM 1.0	1.5319	0.9097	1.8822	1.1178
PCM 2.9	2.3390	1.5337	1.6168	1.0602
PCM 4.3	1.5308	0.9432	1.6089	0.9913
PCM 5.1	2.1019	1.5674	0.8410	0.6271
PCM 6.0	4.3798	2.9653	7.4922	5.0725
CRM 9.0	1.0040	0.7269	0.8034	0.5817
CRM 19.0	1.6270	1.1756	1.5119	1.0925
CRM 21.2	10.9133	7.7684	10.5951	7.5419

Table 2: Results of moisture content characterization for Clinch River and Poplar Creek sediments from three sampling periods

Sites	Moisture Content in Percent-by-Weight		
	July	August	February
PCM:			
1.0	37.788	31.545	40.614
2.9	50.413	37.436	34.430
4.3	36.135	32.426	38.386
5.1	31.467	28.011	25.431
6.0	29.023	33.762	32.297
CRM:			
9.0	23.357	13.625	27.598
19.0	24.972	25.215	27.742
21.2	***‡	***‡	28.817
22.0	25.593	23.985	***‡

‡ indicates that no testing of this site was performed for the sampling period

Table 3: Results of total organic matter characterization for Clinch River and Poplar Creek sediments from three sampling periods

Sites	Total Organic Matter in Percent-by-Weight		
	July	August	February
PCM:			
1.0	4.185	3.997	3.973
2.9	11.045	6.266	4.616
4.3	5.020	5.734	4.824
5.1	2.606	14.196	5.645
6.0	3.985	2.735	4.623
CRM:			
9.0	0.7878	2.433	3.420
19.0	1.600	2.958	2.760
21.2	***‡	***‡	1.248
22.0	3.909	3.811	***‡

‡ indicates that no testing of this site was performed for the sampling period

adjusted EC50's were lower than initial EC50 values. In sediments with high moisture content, the difference between initial and adjuted EC50's proved to be quite substantial.

When comparing sediment characterization results from August and February, there was a large amount of variation in the levels of total organic matter and of particles less than 45 microns. Variation in sediment characteristics are expected to occur as a result of continually shifting sediments, fluctuating seasonal rainfall patterns and flow volume, and seasonal variation in organic input (Watson, et al., 1985). Figure 6 illustrates the variation of total organic matter between August and February. Similarly, Figure 7 shows the temporal variation in percentage of particles less than 45 microns. While variation at some sites was minimal, other sites showed a more substantial increase or decrease in a certain characteristic. For example, Poplar Creek site 5.1 showed a reduction in total organic matter from 14% to 6%, and an increase in particles <45 microns from 14% to 67% by weight. Though the sampling site did not vary from August to February, natural influences caused a significant amount of temporal variation.

Due to the extreme variations in sediment characteristics, such as illustrated in Figures 6 and 7, it becomes necessary to analyze each test sample, regardless of origin or testing frequency. However, a full particle size analysis need not be performed on every sample. The only particles of interest are those less than 45 microns in diameter, as these are the particles which can pass through the Microtox Solid-Phase filter and inhibit light readings. Therefore, February sediments were

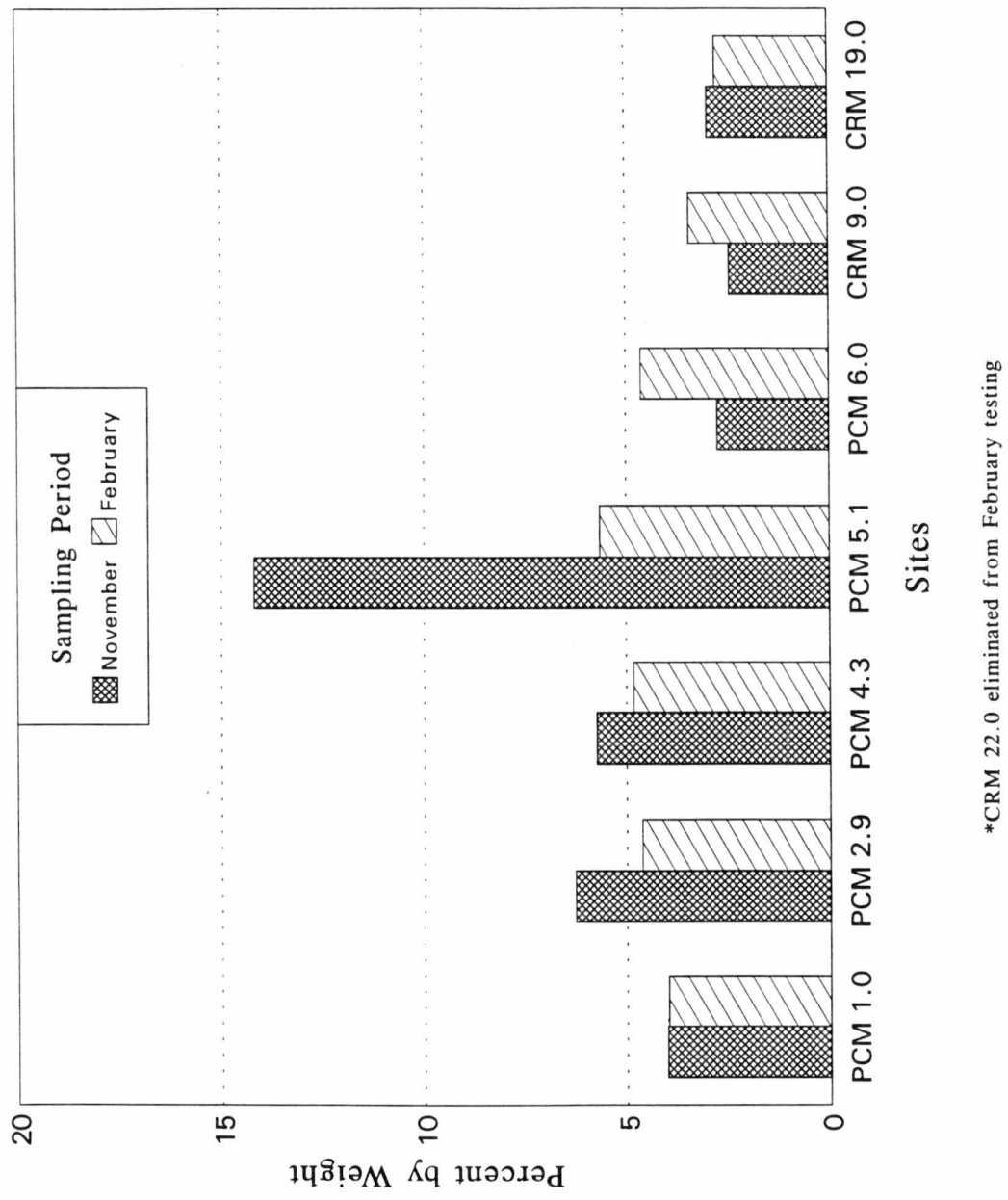


Figure 6: Temporal variation in total organic matter of Clinch River and Poplar Creek sediments

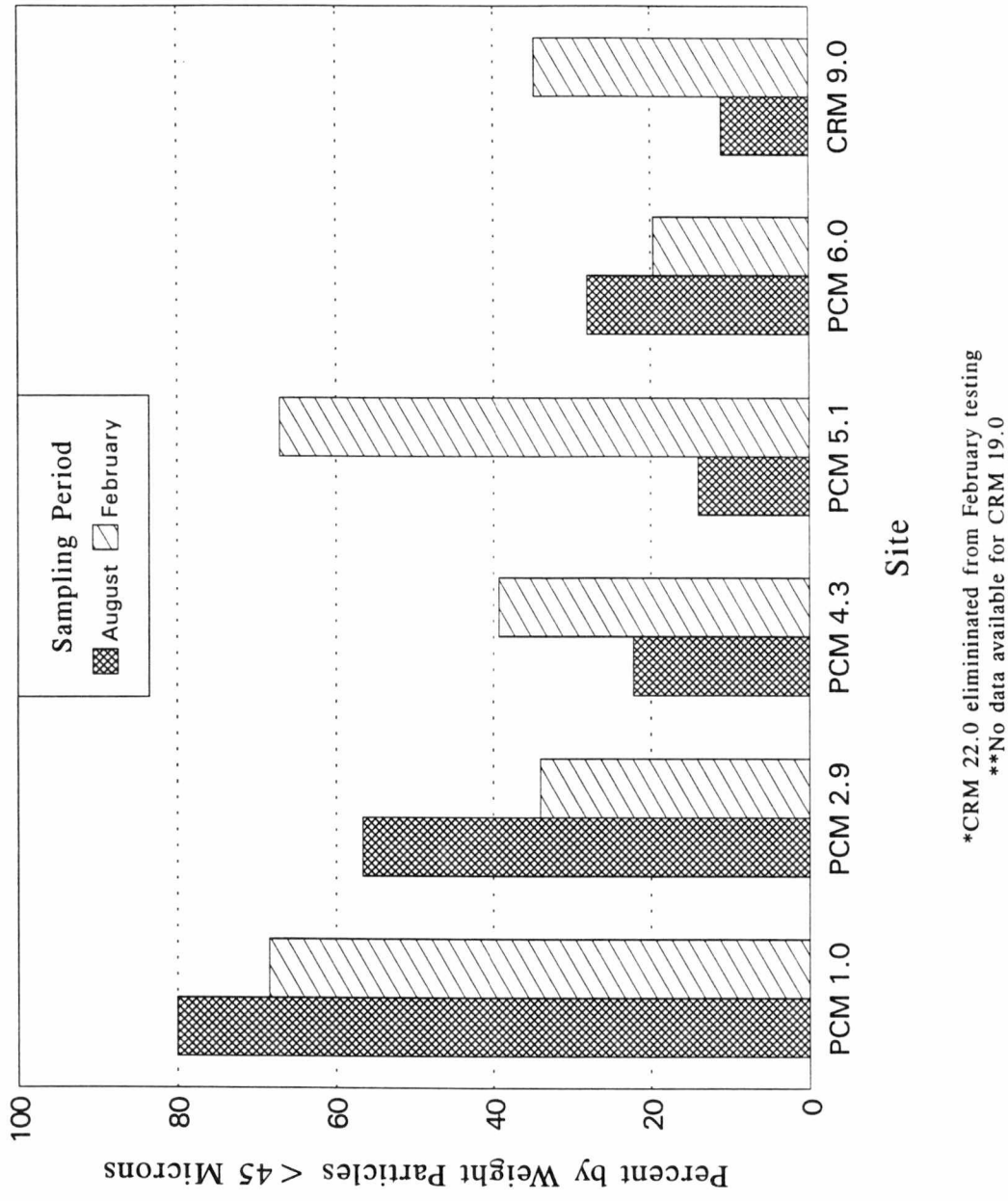


Figure 7: **Temporal variation in fraction of particles <45 microns in Clinch River and Poplar Creek sediments**

analyzed only by the percentage <45 microns, the percentage from 45 to 150 microns, and the the percentage >150 microns. Total organic matter levels are necessary to accurately reproduce sediment characteristics in artificial sediments.

The most important result of sediment testing and characterization is the relationship which can be seen between the EC50 value and the percent of particles less than 45 microns. As the concentration of these particles increases, the test EC50 decreases. As previously mentioned, this effect is caused by the scattering of light being emitted from the photoluminescent bacteria. Figure 8 illustrates this relationship between particle size composition and test EC50. It is important to note, however, that while this relationship does indeed exist, there may be toxicity in these sediments due to the presence of contaminants as well. Therein lies the necessity for non-toxic reference sediments, with the same charateristics as the test sediments, to determine the extent of light reduction due to <45 micron particles. Any reduction of light output in the test sediment beyond that of the reference sediment will be due to contaminants in the sediment. An assessment of the toxicity of sediments due to contaminants will be discussed further in Section 4 of this chapter.

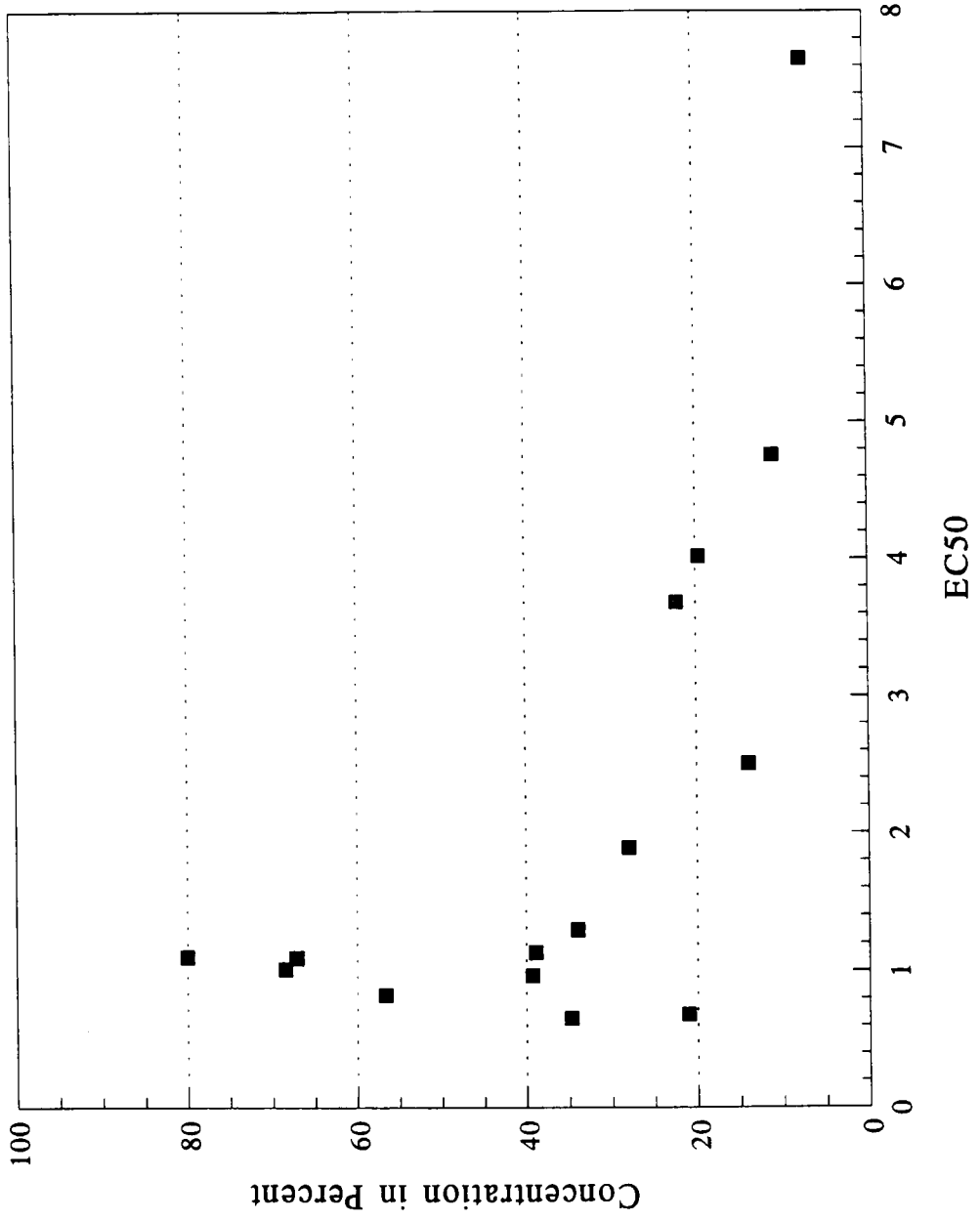


Figure 8: Relationship of test EC50 to percent by weight of particles <45 microns in sediment

2. Testing and Selection of Clay Components for Creation of Artificial Sediments

A. Results

Many different clay components were tested before a suitable non-toxic clay was selected. Kaolinite, chosen for initial testing due to its homogeneity, resulted in EC50 values much lower than expected, around .75%. "Mexican" sculptor's clay, or red clay, yielded EC50 values ranging from .10% to .12%. Grey sculptor's clay demonstrated the highest EC50 values of the three clays, ranging from .22% to .25%. However, these values were still unacceptably low for a non-toxic component.

The grey sculptor's clay, with its higher EC50, was treated with sodium hexametaphosphate in order to remove any additives from the clay which might induce inhibition of light output or otherwise interfere with test results. Three different rinse regimes were tested. Single-rinsed clay yielded EC50 values from .99% to 1.2%. Double-rinsed clay demonstrated EC50s ranging from 1.9% to 2.1%. This rinse regime proved to be the most effective of the three. Triple-rinsed clay produced lower EC50s than double-rinsed clay, with EC50 values ranging from .78% to 1.0%.

2. Discussion

Though kaolinite was chosen initially to act as the clay component in the creation of artificial sediments, its dry, powdered form prevented hydration of the particles. Subsequently, the particles would not mix with other components, but generally remained suspended on the surface of the mixture. The same problem arose during Microtox testing of kaolinite. The Microtox diluent, even with its trace sodium content, could not force the hydration of the particles. Particles remained on the surface of the diluent, did not mix with the bacteria properly, and inhibited filtration of the bacteria by clogging the filter column. Though the kaolinite was homogenous and convenient to weigh and measure, its inability to absorb water disqualified it from candidacy as an artificial sediment component.

Red sculptor's clay, or "Mexican" clay, was initially selected because of its proximity in color to the test sediments from Clinch River and Poplar Creek. However, the low EC50 values seen in testing indicated early in this investigation that red clay was inappropriate for use in artificial sediments. It was suspected that the red color was due to high iron content, not artificial coloration. The Amaco Corporation laboratories confirmed this hypothesis, and the red clay was eliminated from further testing.

The Amaco Corporation laboratories also confirmed that grey sculptor's clay, while from the same company, did not have the high iron content of the red clay. Testing of this clay yielded the best results of the three clays tested. However, when

used in the creation of site-specific artificial sediments, EC50 values were low. For example, a site-specific reference created for PCM 1.0, collected in August, yielded an EC50 of only .3%. The EC50 of the test sediment was around .9-1.2%, indicating that some ingredient of the artificial sediment was inhibiting light output of the bacteria. Since all organic components and particles greater than 45 microns tested as non-toxic using the Microtox, the grey clay was assumed to be the source of the toxicity.

However, the grey clay did have the lowest toxicity of those tested, therefore, it was selected for the sodium hexametaphosphate treatment. Of the three different rinse regimes used, the double-rinse regime appeared to be the most effective. Triple-rinsed clay produced highly variable results, while the single-rinsed clay yielded lower EC50s. Double-rinsed clay, with its higher EC50 values and consistent and repeatable results, also mixed effortlessly with other artificial sediment components to produce non-toxic and homogenous reference sediments. The results of testing of these reference sediments will be discussed further in Section 4.

3. Statistical Analysis of Double-Rinsed Clay Data

A. Results

Microtox testing of double-rinsed grey sculptor's clay produced light output readings for 78 concentrations of clay. Light output was plotted as a function of concentration, and the curve produced was analyzed by second-degree regression. The model sum of squares was 68238 and the error sum of squares was 3190. The R-square value for the model was .95.

In order to simplify the model and eliminate the quadratic term, the log of the light output was plotted against the concentration and analyzed by linear regression. The model sum of squares from this model was 21.55 and the error sum of squares was 1.12. The R-squared value was again .95, indicating a fit equally as good as the quadratic model. Figure 9 summarizes the statistical analysis of the data for the final linear model. Figure 10 is a graphic representation of the linear regression model.

B. Discussion

The r-squared value of the linear model indicates that 95% of the variation in the data is accounted for by the model. In other words, the light output of a particular concentration of clay can be predicted from the model with 95% accuracy.

Expected EC50s for Test Sediments

Model: MODEL1
Dependent Variable: ITLOG

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	1	21.55013	21.55013	1451.163	0.0001
Error	75	1.11377	0.01485		
C Total	76	22.66390			

Root MSE	0.12186	R-square	0.9509
Dep Mean	1.62098	Adj R-sq	0.9502
C.V.	7.51776		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEP	1	1.973590	0.01668945	118.254	0.0001
CONC	1	-0.184663	0.00484755	-38.094	0.0001

Figure 9: Statistical analysis of testing results of treated grey sculptor's clay using linear regression and semilog scale

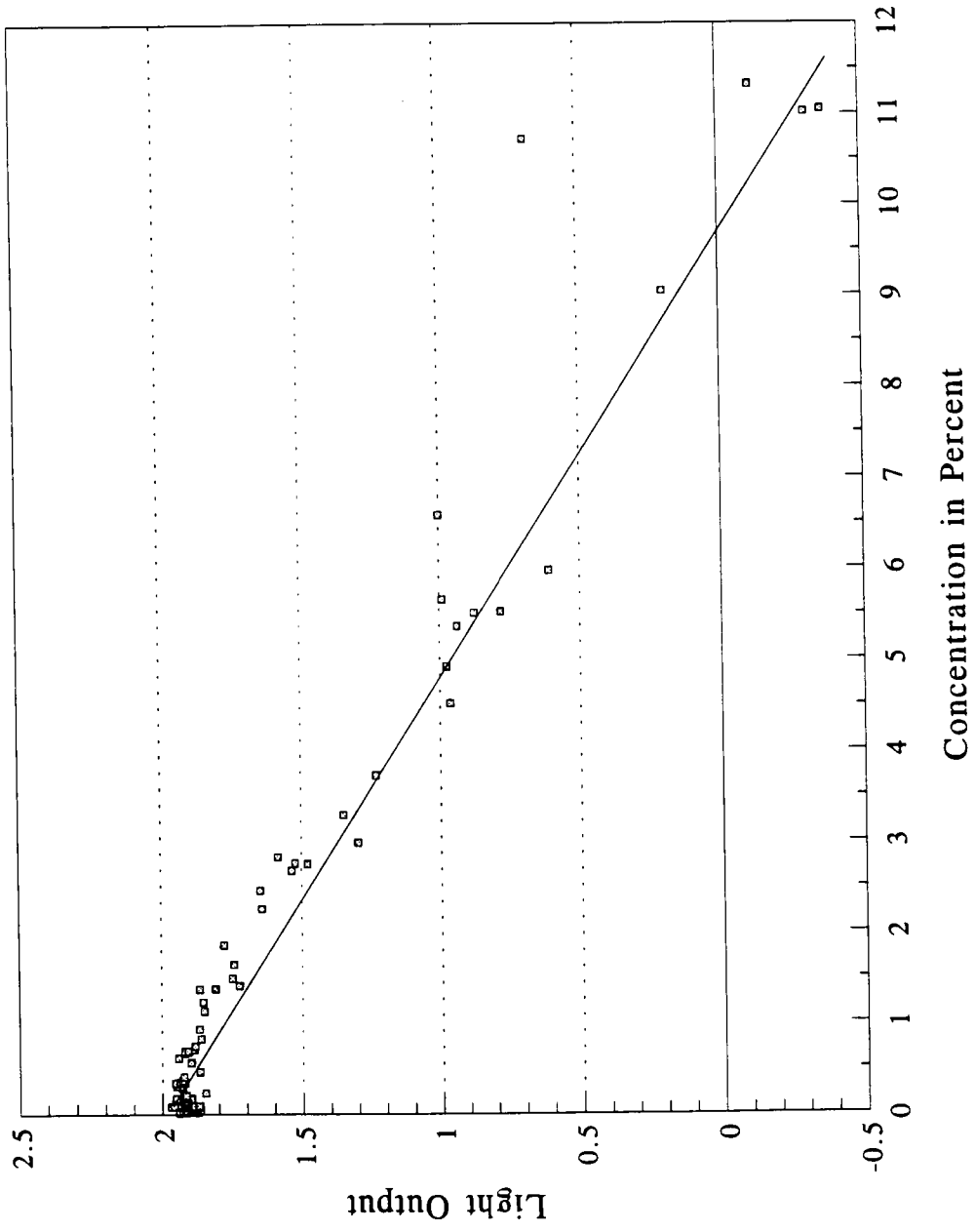


Figure 10: Variation in log of light output with concentration of double-rinsed clay

If the concentration of clay in a test sediment is known, the light output of that concentration can be predicted from the model, given that the sediment has no toxicity due to contaminants. This value accounts for any reduction in the light output of the test sediment due to the presence of particles less than 45 microns scattering the luminescence from the bacteria. If the measured light output in the sediment is below the predicted value, then any additional reduction in light output can be attributed to contaminants in the sediment. Thus, the greater the difference between the predicted and actual light output values, the higher the toxicity of the sediment due to contaminants.

This model can be used to evaluate the light output reduction due to the presence of <45micron particles, much in the same way the reference sediments can be used to assess the same phenomenon. The results of artificially created reference sediments are discussed in Section 4.

4. Testing of Artificial Sediments and Evaluation of Toxicity for Sediments from Clinch River and Poplar Creek

A. Results

Before any artificial sediments were created, the amount of variation among the controls for all Microtox® tests, including clay tests, was evaluated. The mean light output value for all controls was 87.93 with a standard deviation of ± 7.82. Figure 11 illustrates the variation seen in the controls for each test. Since 95% of the controls fall between the mean ± two standard deviations, in this case 72.30 and 103.56, it was concluded that concentrations of sediments which produced light output values above 72.30 did not demonstrate significant light reduction. Therefore, any sediment which in a sample prepared for Microtox® testing contained less than 2.2% of particles <45 microns, did not express significant light reduction due to the presence of those particles. In other words, any light reduction seen in these sediments could not be differentiated from sampling noise.

Based on these conclusions, artificial sediments were created for those sediments whose concentration of particles <45 microns was above the crucial concentration of 2.2%. Table 4 lists the percent by weight of particles <45 microns in whole sediments and the subsequent percentage of same particles in an actual test sample. Figures 12 and 13 illustrate which sediments from each sampling period fell

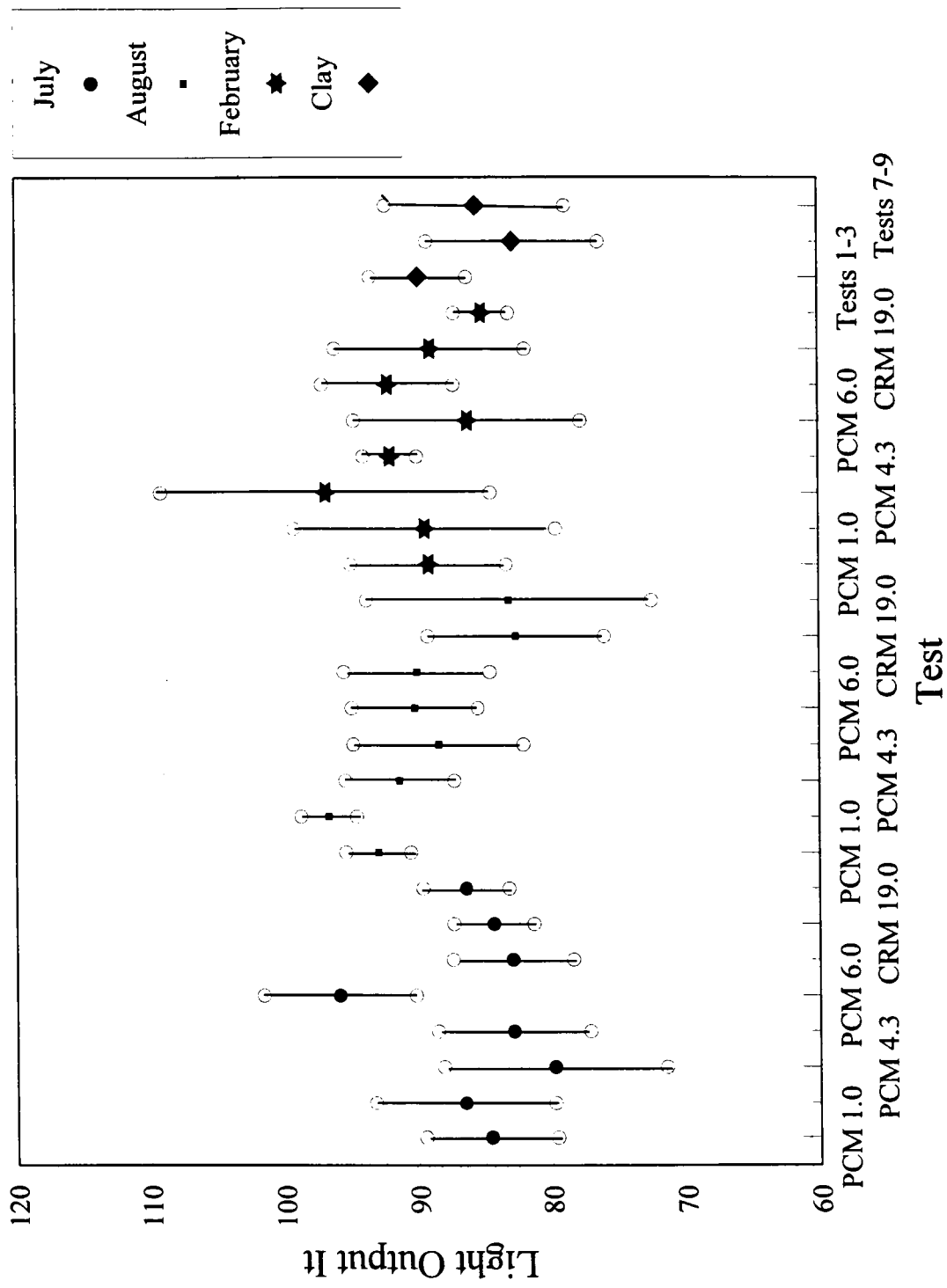


Figure 11: Control light output means and standard deviations

Table 4: Concentration of particles less than 45 microns in a sample of sediment from each site† as prepared for Microtox® Solid-Phase testing. 300 mg (or .3 g) per 3.0 mL of diluent represents a 9.868% sample.

Site	Percent by Weight of Particles <45 microns in Whole Sediment	Concentration as Percent in a Test Sample of Particles <45 microns
August		
PCM 1.0	80.014	7.896
PCM 2.9	56.638	5.589
PCM 4.3	22.249	2.196
PCM 5.1	14.004	1.382
PCM 6.0	28.025	2.766
CRM 9.0	11.016	1.090
CRM 22.0	21.100	2.082
February		
PCM 1.0	68.473	6.757
PCM 2.9	34.011	3.356
PCM 4.3	39.289	3.877
PCM 5.1	67.144	6.626
PCM 6.0	19.649	1.939
CRM 9.0	34.745	3.429
CRM 19.0	38.918	3.841
CRM 21.2	7.335	0.724

† sediments from August sampling period exclude CRM 19.0

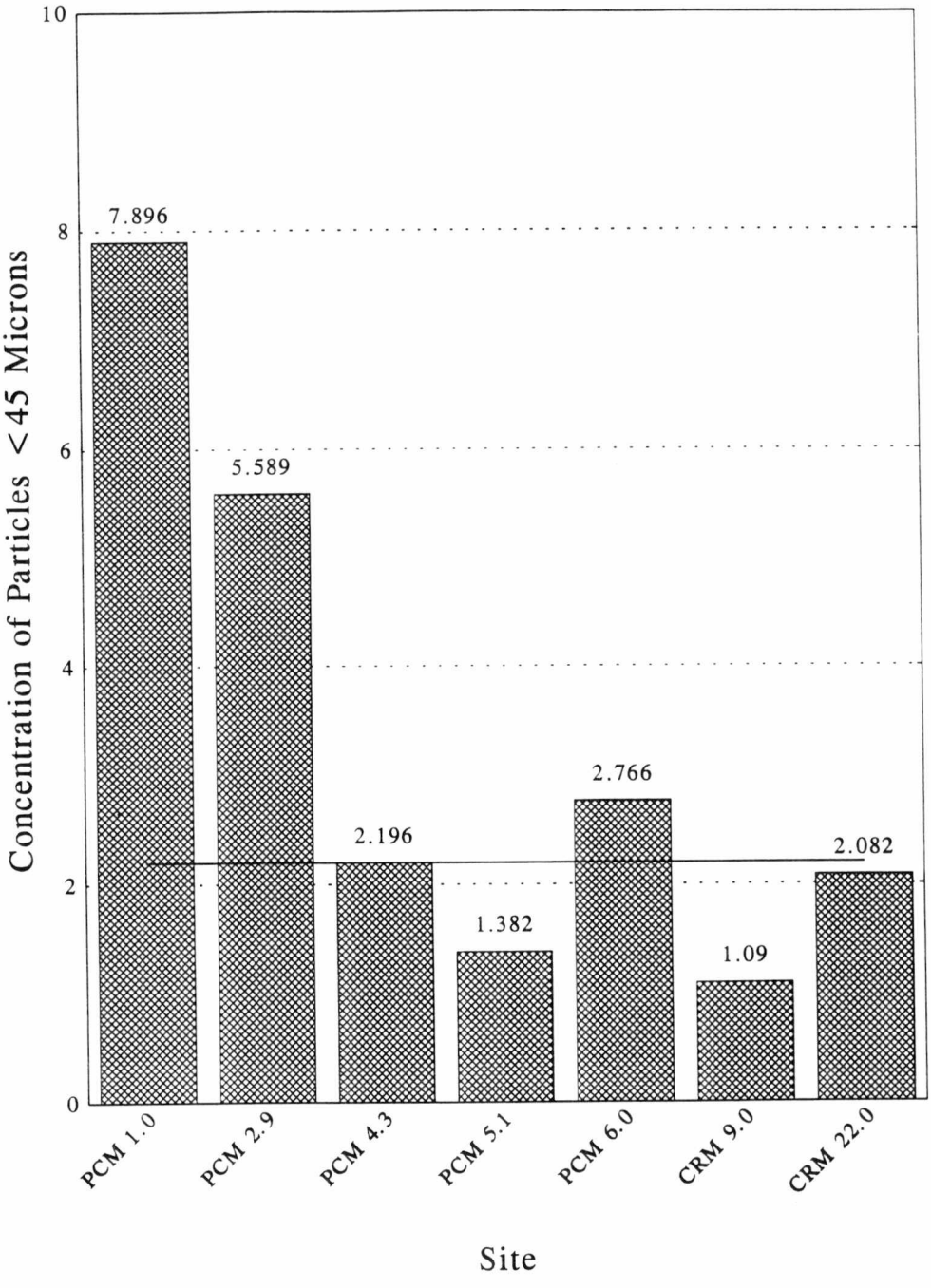


Figure 12: Deviation from crucial concentration of the percentage of particles <45 microns in test samples from August sampling period

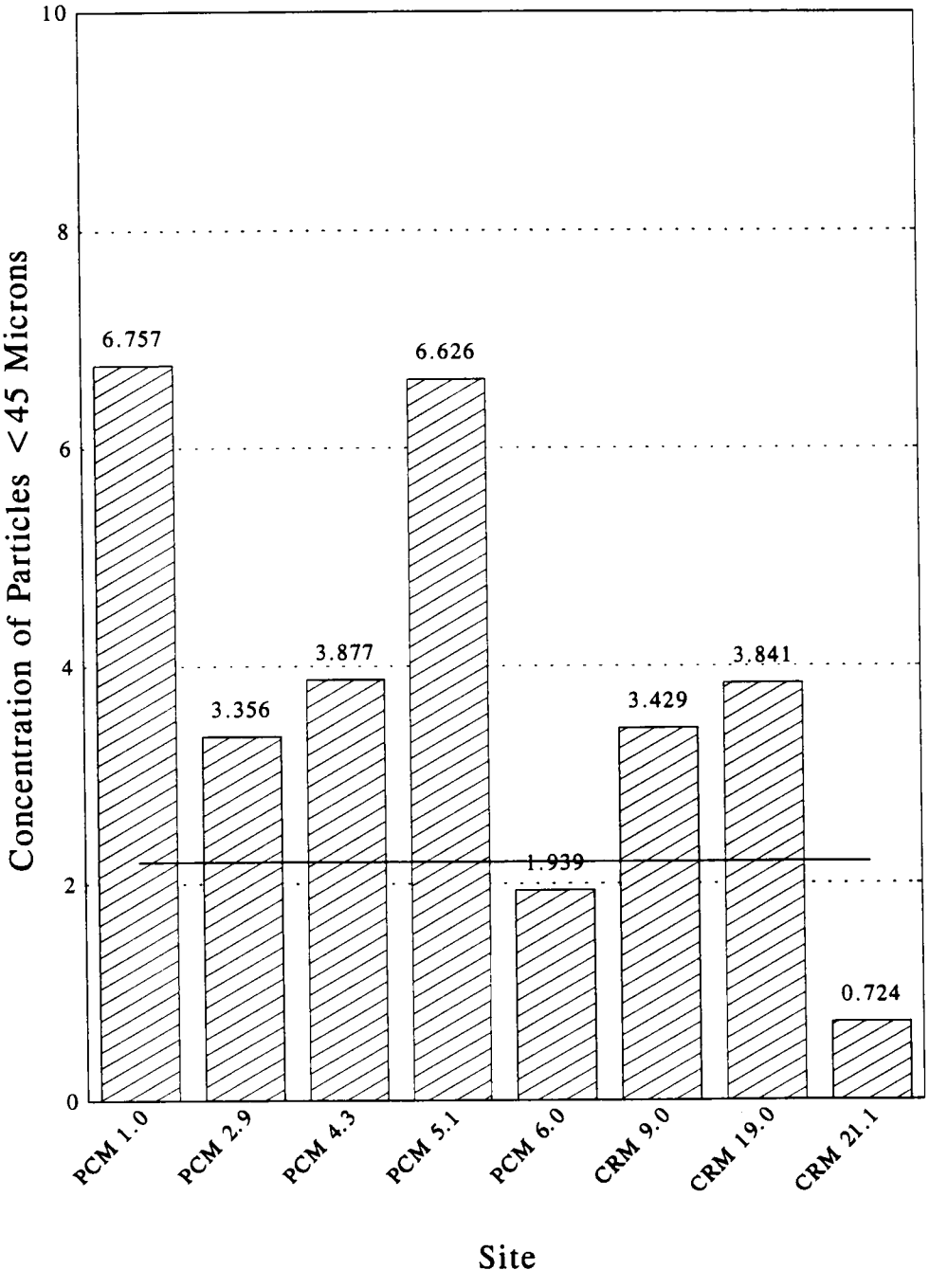


Figure 13: Deviation from crucial concentration of the percentage of particles <45microns in test samples from February sampling period

below the crucial concentration. From August sampling period, PCM 4.3, PCM 5.1, CRM 9.0, and CRM 22.0 yielded less than 2.2% particles <45 microns in a test sample. In February sampling period, only two sites fell below the crucial concentration, PCM 6.0 and CRM 21.2.

Artificial sediments were created for all test sediments above the crucial concentration. Each sediment was compared with predicted values from the model discussed in Section 3 of this chapter. EC50 values were not found to be significantly different from one another. Sediments below the crucial concentration were analyzed with the model as well. Table 5 shows predicted or reference EC50 values, actual test EC50 values, and the percent difference for sediments from August and February sampling period. Percent differences for August sediments ranged from approximately 46% at PCM 1.0 to 92% at CRM 22.0. February percent differences ranged from 51% at PCM 6.0 to 86% at CRM 9.0. Figures 14 and 15 graphically illustrate the differences between predicted or reference and actual EC50 values for test sites from both August and February sampling periods.

The Clinch River Environmental Restoration Program requests that results from all sediment testing be adjusted against background variation as indicated by the two reference sites, PCM 6.0 for Poplar Creek sites and CRM 22.0 or CRM 21.1 for Clinch River sites. This adjustment was made by allowing the percent difference of predicted and actual values of the two test reference sites to equal one unit, and then evaluating the number of units deviation in these percent differences for each test sediment. Table 6 demonstrates the percent difference and deviation expressed as

Table 5: Comparison of actual and predicted EC50 values for sediments collected from Clinch River and Poplar Creek in August and February sampling periods, including percent differences. * indicates that predicted EC50 value is calculated from the model.

Site	Actual EC50	Predicted EC50	Percent Difference
August			
PCM 1.0	1.1045	1.6598	46.76
PCM 2.9	0.8270	1.5683	70.13
PCM 4.3	3.6804	1.7432*	53.03
PCM 5.1	2.5024	1.7759*	80.27
PCM 6.0	1.8911	1.7293	69.35
CRM 9.0	4.7561	1.7336*	69.78
CRM 22.0	0.6820	1.9210*	92.51
February			
PCM 1.0	1.0138	1.8098	61.65
PCM 2.9	1.2970	2.0102	78.06
PCM 4.3	0.9673	1.8760	79.74
PCM 5.1	1.0973	1.6718	55.93
PCM 6.0	4.0189	1.6244*	51.39
CRM 9.0	0.6543	1.5900	85.70
CRM 19.0	1.1341	1.6455	73.18
CRM 21.2	7.6112	1.8315*	69.34

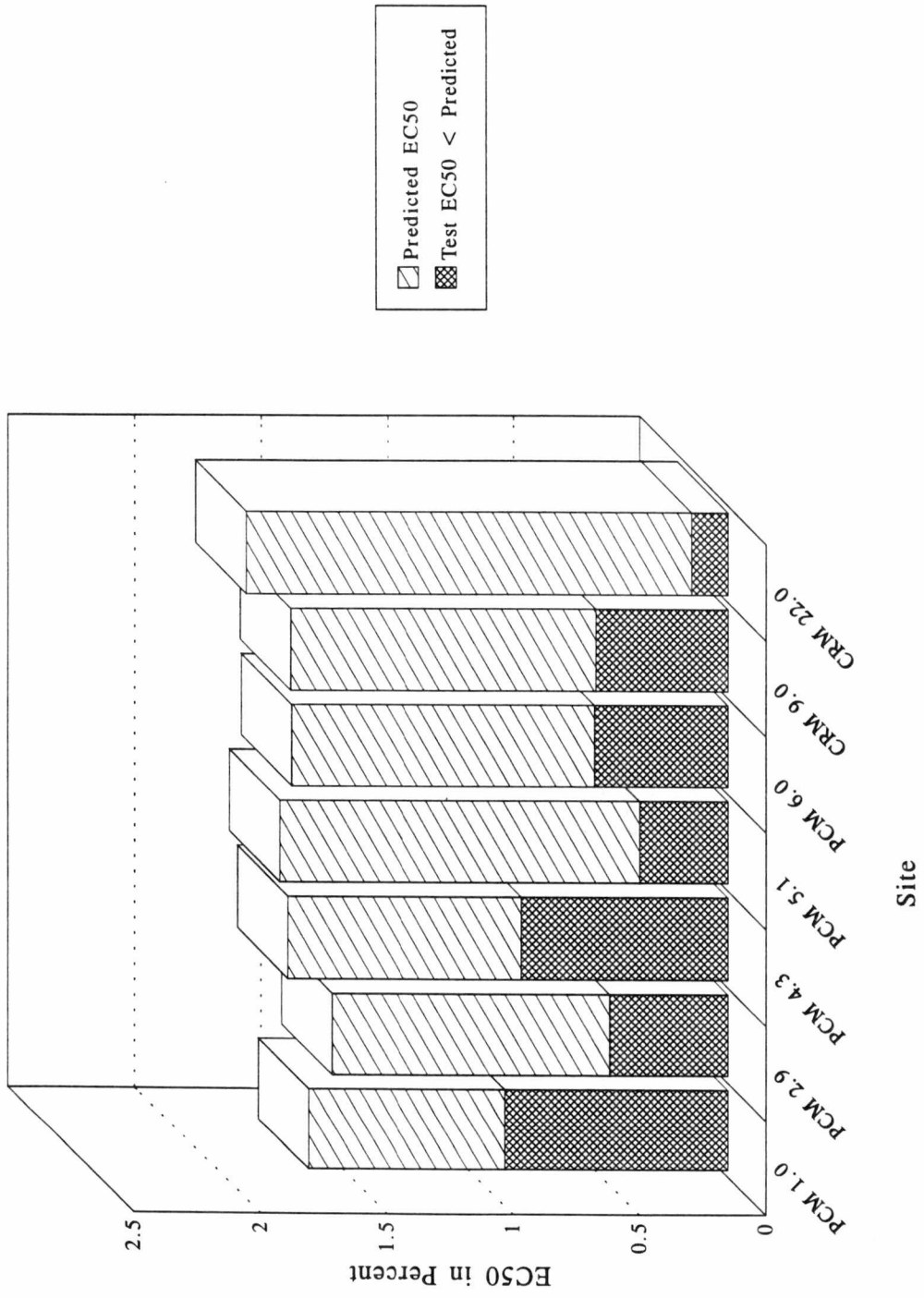


Figure 14: Comparison of test EC50 values and predicted EC50 values for Clinch River and Poplar Creek sediments from August sampling period

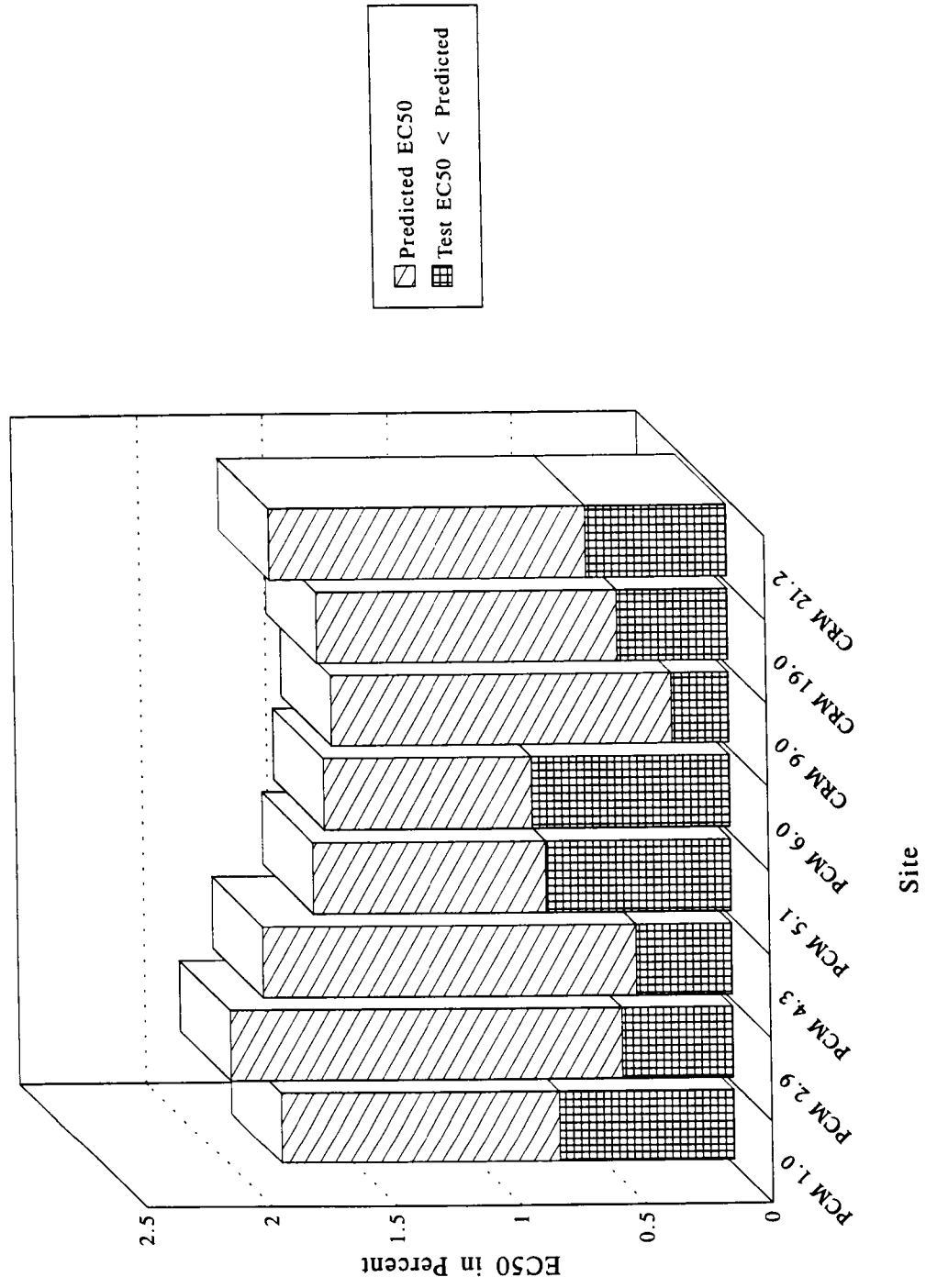


Figure 15: Comparison of test EC50 values and predicted EC50 values for Clinch River and Poplar Creek sediments from February sampling period

Table 6: Deviation ratios of the percent difference between actual and predicted EC50 values for test sites to the percent difference between actual and predicted EC50 values for reference sites PCM 6.0 and CRM 21.2 or CRM 22.0*.

Site	Deviation Ratio from PCM 6.0	Deviation Ratio from CRM 22.0 or CRM 21.2
August		
PCM 1.0	0.6742	
PCM 2.9	1.0113	
PCM 4.3	0.7646	
PCM 5.1	1.1574	
CRM 9.0		0.7543
February		
PCM 1.0	1.1996	
PCM 2.9	1.5190	
PCM 4.3	1.5518	
PCM 5.1	1.0884	
CRM 9.0		1.2360
CRM 19.0		1.0553

* CRM 22.0 is the reference for Clinch River sites collected in August sampling period; CRM 21.1 is the reference for Clinch River sites collected in February sampling period.

a ratio of the percent difference of the test site to the percent difference of the respective reference site. Figures 16 and 17 graphically depict this deviation ratio for each sampling period, with one unit equal to the percent difference of the reference site. From August sampling period, two sites have greater deviation than their respective reference, PCM 2.9 and PCM 5.1. From February sampling period, all sites have greater deviation than their respective references.

B. Discussion

The crucial concentration at 2.2% basically provides a reference or baseline for the natural variation of light output inherent to the Microtox® Solid-Phase Test. Sediments are diluted with Solid-Phase diluent for testing, so the highest sample concentration is 10%, the equivalent of 300 mg of whole sediment in 3.0 mL of diluent. If the total concentration of particles <45 microns in this sample is less than 2.2%, then any effect of the suspended clay particles on light reduction of the bacteria can not be isolated from the background variation of the testing procedure. Therefore, the creation of artificial sediments will yield no significant information about predicted light output reduction since they too are subject to this crucial concentration. In order to determine the predicted EC50 values for these sediments, the light output value equal to 50% of the control mean for that test was determined. Using SAS®, the concentration of clean sediment which should have produced this

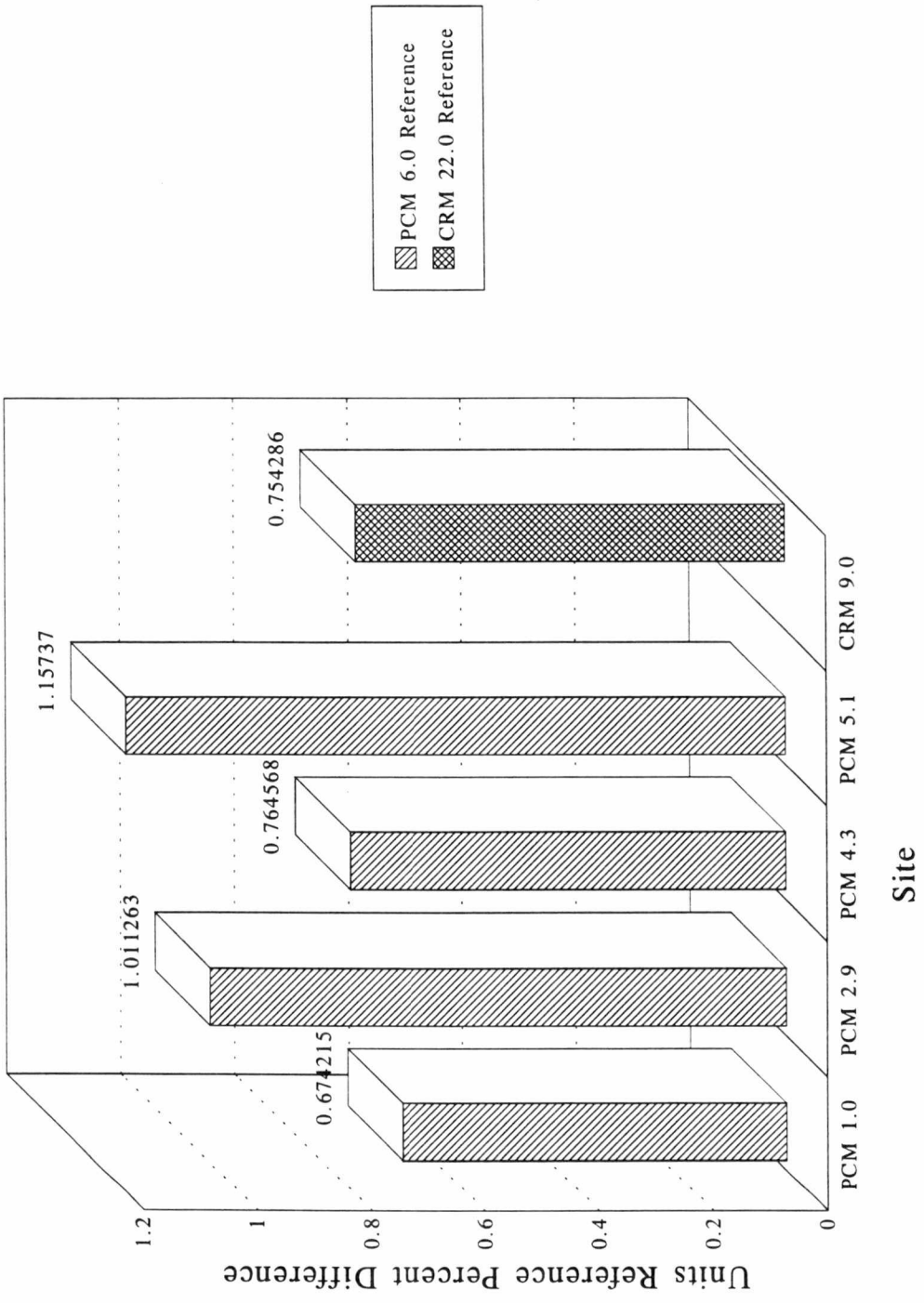


Figure 16: Deviation in reference percent difference of actual and predicted EC50 values for test sediments from August sampling period

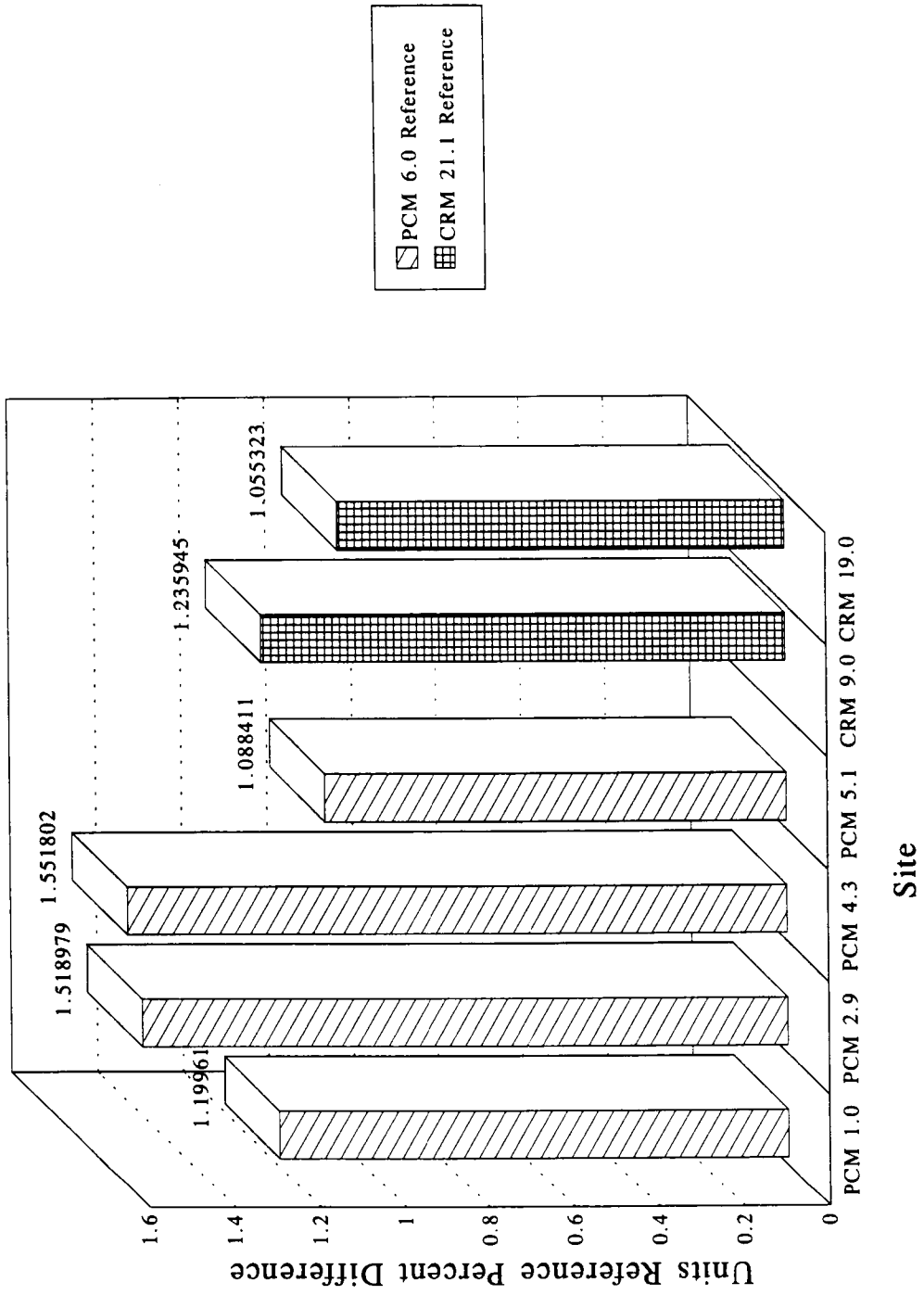


Figure 17: Deviation in reference percent difference of actual and predicted EC50 values for test sediments from February sampling period

light output value was predicted from the double-rinsed clay model. The actual EC50 value was adjusted for the percentage of particles <45microns for that sediment, and then compared to the predicted EC50, producing the percent differences seen in Table 5. Artificial sediments were used to predict EC50 values for those sites above the 2.2% crucial concentration of particles <45microns. It is important to note that these values did not differ from those predicted by the model, thereby confirming that particles greater than 45 microns to not reduce light output at any noticeable level.

Aside from the presence of suspended clay particles, background variation for a certain creek or river also allows a comparison of toxicity. The reference sites PCM 6.0 and CRM 21.2 or 22.0 are labelled "no toxicity" by the Clinch River Environmental Restoration Program. However, variation from the predicted EC50 values does exist in these sites. The Clinch River/Poplar Creek system is an area of high recreation, including boating, fishing, swimming, and camping along the shores. It is therefore reasonable to assume that, those these reference sites may lie upstream of the contaminant sources in question, they will very likely have high toxicity levels due to "recreation waste": motor oil, boat fuel, discarded refuse, and runoff from camp sites and parking lots. Therefore, even though the Clinch River Environmental Restoration Program considers this level of variation to be "background variation", these sediments can not be considered "clean" and should not be used as a standard by which to evaluate the toxicity of the other sites. These are the exact problems which have created the need for artificial sediments in toxicity analysis. *In situ* references simply do not provide an accurate standard by which to assess sediment

toxicity.

In spite of this discrepancy, the Clinch River Environmental Restoration Program insists on the use of *in situ* references for toxicity evaluation. Therefore, the ratio of the percent difference between actual and predicted EC50 values for test sites and that for reference sites was used to determine if the variation between actual test EC50 values and predicted values is greater than that produced by "natural background variation". If this ratio is greater than one, as it is for PCM 2.9 and PCM 5.1 from August sampling period and all sites for February sampling period, then the variation between actual and predicted EC50 values is considered above normal. Therefore, these sites are considered to have various levels of toxicity. In other words, the excess variation is due to influences other than suspended clay particles and natural background variation. The sites mentioned above with higher than normal variation therefore require more indepth study to determine the source and nature of their toxicity. This information could best be achieved by utilizing other sediment testing protocols which are designed to evaluate the presence of particular contaminants.

Chapter 5

Conclusions

Through testing and experimentation, the applicability of artificial sediments to interpret results from the Microtox® Solid-Phase Test has been determined. Though artificial sediments are useful for those test sediments which have high concentrations of particles <45 microns, the predicted values obtained from the model of light output to concentration of these particles are equally helpful in interpreting testing results. The most valuable aspect of this study is the characterization of test sediments to determine the concentration of particles <45 microns. A sufficient concentration of these particles in a sediment, that is, a concentration above the crucial concentration of 2.2%, does produce reduction in the measurable light output produced by the bacteria. Therefore, the percentage of this particle size in a test sediment in highly relevant to the results of initial testing.

The background variation, accepted by the Clinch River Environmental Restoration Program as a standard for toxicity assessment, and defined by the percent difference between actual and predicted EC50 values for the reference site sediments, is an excellent indicator of the need for artificial reference sediments. The high levels of toxicity in these sediments can be accounted for by anthropogenic contaminants, albeit of a different nature than seen in test sites. Therefore, the value of these reference sites in evaluating toxicity is highly doubtful, since there is considerable

evidence of contamination at these sites. Furthermore, when an "all pairwise comparison" analysis was performed on these sites, it was noted that all sites had similar levels of toxicity, and that any site, even one known to be contaminated, could be used as a reference and generate the same results. This is an area which requires further investigation utilizing other modes of sediment testing.

As for the Microtox® Solid-Phase Test, it is a useful tool to quickly determine approximate toxicity levels of sediments. Other sediment tests in current use require from seven to fourteen days to complete. Though the Microtox® test requires substantial time to sieve sediment and arrive at predicted EC50 values, it is more time efficient than other tests. Adversely, the small sample size, while convenient, limits the test capabilities by reducing variations within the sample. The test analysis software in its current form is very limited, requiring strict adherence to the written protocol and a large amount of independent interpretation of results by the technician. The use of the standard curve created in this study considerably reduces the amount of "guesswork" involved in the interpretation of Solid-Phase Test results. Without this means of adjustment, the resulting EC50 values were essentially meaningless. However, in spite of the upgrade produced by the standard curve, the Microtox® test is best suited to serve as merely one component of a battery of sediment testing.

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Vita

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