MICROTOX AS AN INDICATOR OF CHRONIC TOXICITY

BY

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Bachelor of Arts

Washburn University

Topeka, Kansas

1989

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 1994

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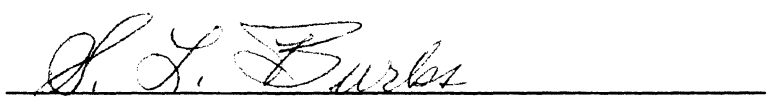
CHRONIC TOXICITY

Thesis Approved:



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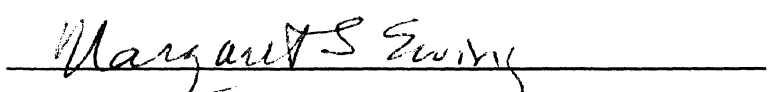

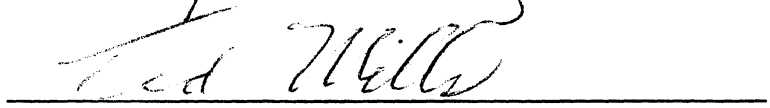

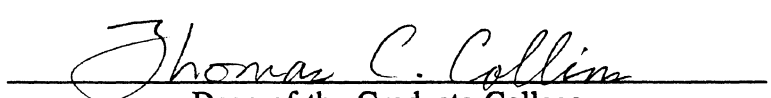

Dean of the Graduate College

ACKNOWLEDGMENTS

My committee members have shared invaluable advise and enthusiasm in their fields of expertise. Dr. Margaret Ewing went above and beyond her call of duty by offering editorial comments and encouragement when this document was rough. Dr. Ted Mills was always thrilled to explore new ideas and concepts in environmental education. And Dr. S.L. "Bud" Burks, my thesis/research advisor, always had time for that "one more question". In the past four years Dr. Burks has offered me the opportunities and respect of a fellow professional. Thank you to each of you, I am deeply indebted.

A special thank you goes to Elaine Stebler, Research Specialist of Oklahoma State University's Water Quality Research Laboratory. She hired me over four years ago, provided me with a wealth of learning opportunities, and introduced me to the Microtox study. If it wasn't for Elaine I would not have been able to accomplish the work presented in this thesis. Other staff members at WQRL who provided their expertise are Hildreth Overgard and Jane Wells. Thank you for assisting my endeavors and helping to make WQRL an enjoyable place to research.

God has taught me great patience during the process of my graduate work. I officially began study in Fall 1990, married in Fall 1991 and produced two boys in Fall of 1992. There are several support people that have enabled me to stay focused and complete my thesis during the intricacies of day-to-day life. Gayle and Jim

Sampley were always happy to help out with the kids and send food. Chris Dent provided financial wisdom and shared his wife Shelly Dent who herself provided childcare, house cleaning, transportation, artistic expertise, you name it Shelly did it. Glen Curtis provided the means for financial support since I was a wee thing on his knee. My father Gary Curtis has offered his love and support unconditionally. My mother Diane Curtis was there on the phone late at night to tell me to go on to sleep and everything would look better in the morning, and it always did. There are many other family members that have offered encouragement: Cindy and Lou Constantino; Clarence and Dorothy Renner; Lynnette, Jennifer, Renee, Melanie and Brandon Curtis; and others. To all of you, thank you for your support.

The two key people that enabled me to maintain my sanity during this long, drawn out process were my husband, Kurt Sampley, and daughter, Abrael. Kurt is everything one could ask for in a spouse: Supportive, kind, compassionate, devoted, chef, self-appointed head garbage-taker-outer, and gentle father. Abrael deserves a medal for patience. She has spent many hours in the laboratory with me entertaining herself with *Daphnids*, computers, overgrown cockroaches, minnows, and Frank, the lab's salamander mascot. She rarely complained, especially when promised a walk down to Theta Pond or the water fountain. Thank you Kurt and Abie-Baby-Cakes!

Two other special people are my sons Taush and Saxon. While they did not help directly, their love and joyful attitudes were contagious after a long day.

This work was made possible by an educational grant from Microbics Corporation, Carlsbad, California and a matching grant from the Oklahoma Oil Refiners Waste Control Council.

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CHAPTER I

INTRODUCTION

The Federal Water Pollution Control Act of 1972 states as its objective to "restore and maintain the chemical, physical and biological integrity of the nation's water." In order to meet this objective it states as a national policy "that the discharge of toxic pollutants in toxic amounts be prohibited." Unfortunately, implementation provisions of the act were not designed to specifically meet this goal (Freeman 1990). The control of toxics in wastewater relied upon the setting and maintaining of effluent limitations, guidelines and standards for selected specific toxicants in the primary industrial point sources (Bishop 1987). These limitations and guidelines were set under the auspices of the National Pollution Discharge Elimination System (NPDES). All dischargers into United States waterways were required to hold a permit through the NPDES. The late 1970's brought an increase in the realization that there were far too many unknown chemicals and chemical interactions within a wastestream to properly base water quality judgements on physical and chemical criteria alone. In 1984 the Environmental Protection Agency (EPA) began requiring the use of living organisms in addition to chemical assays to assess the quality of the nation's wastewater discharges via the NPDES program (EPA 1987). These organismal tests, or bioassays, employ the use of specific native species, ubiquitous within a region in order to provide consistency of testing procedures. These bioassays are time and

labor intensive lasting anywhere from 48 hours to 7 days depending upon the EPA region and require a complex system of sample collection over a representative time frame.

Toxicity of a chemical may manifest itself in several ways. It can produce either lethal or sublethal effects upon an organism. Lethal effects are those that cause death to the organism. Sublethal effects are deleterious to the organism, affecting it behaviorally, physiologically, or morphologically, but will not cause death directly. The mechanism of action of lethal and sublethal effects may occur within a short (acute) or long (chronic) period of exposure. Acute effects have been defined as occurring in less than 96 hours of exposure. Chronic toxicity will affect the organism over a period of time, i.e., exposures lasting anywhere from weeks to years depending on the life cycle of the organism (Rand 1985).

Methods for evaluating toxicity are as diverse as the different forms of toxicity. Two of the most common measures of response are the median effect concentration (EC50) and the no observed effect concentration (NOEC). The term "effect" used in both methods may be anything in which the particular researcher is interested, i.e., death, immobility, decreased reproduction, stunted growth, etc. When death is the effect studied the term LC50 (median lethal concentration) is used interchangeably with EC50. The important difference between the EC50 and NOEC methods is the point at which they indicate toxicity. The EC50 is defined as the point at which 50% of the population is adversely affected by treatment. The NOEC is defined as the level of toxicant or wastewater whose effect is not statistically significantly different from that of the control at the 95% level of confidence.

As industrial and municipal dischargers became aware of impending NPDES

permit requirements for biomonitoring, they quickly recognized the need for alternative toxicity tests. The existing standard toxicity tests were time and labor intensive and required a cumbersome volume of wastewater sample when shipping to remote testing labs. In addition to the necessity of a rapid and inexpensive test for obtaining biomonitoring results for their NPDES permits, there was also the need for a screening test to locate and reduce sources of toxicity within their facilities.

Anthony A. Bulich introduced Microtox (MTX) in 1979. The Microtox system consists of a self-contained photometer that quantifies the light output of the luminescent marine bacterium *Photobacterium phosphoreum* upon 5-, 15- and/or 30-minute exposures to an aqueous sample. Traditionally MTX has used the EC50 to report phosphorescence inhibition. The MTX EC50 has been reported to be a reliable indicator of acute toxicity for specific pure chemicals and complex chemical mixtures that are commonly found in wastestreams (Munkittrick 1991).

National Pollution Discharge Elimination System (NPDES) permitting in EPA Region 6, including Oklahoma, requires 7-day biomonitoring of whole effluents using *Ceriodaphnia dubia* and *Pimephales promelas* as the test organisms. The Water Quality Research Laboratory (WQRL) of Oklahoma State University has been involved in biomonitoring for over six years. The availability of effluents with proven histories of chronic toxicity to traditional biomonitoring organisms, and the facilities to perform 7-day biomonitoring provided an excellent setting to study the potential for Microtox as an indicator of chronic toxicity. In order to carry out this study the following null hypotheses were formulated and tested:

Ho: There is no significant correlation between MTX EC50 and *P. promelas* NOEC survival and growth.

Ho: There is no significant correlation between MTX EC50 values and *C. dubia* NOEC survival and reproduction.

Ho: There is no significant correlation between MTX NOEC and *P. promelas* survival and growth.

Ho: There is no significant correlation between MTX NOEC and *C. dubia* NOEC survival and reproduction.

CHAPTER 2

LITERATURE REVIEW

The use of *P. promelas* in bioassays was originally recommended for standard toxicity tests by a joint task force established between the American Public Health Association and the American Waterworks Association (Burks et al. 1981). The two major advantages to this organism are that it is considered ubiquitous throughout the United States and is readily obtainable through commercial minnow dealers. In 1969, spurred by the questionable health of minnows obtained from bait shops, the WQRL established a successful breeding population of *P. promelas* that supplied year-round organisms (Burks et al. 1981). This organism is currently used in both acute and chronic toxicity bioassays.

Cladocerans such as the daphnids have been widely accepted for bioassays due to their minimal space demands. Numerous organisms may be maintained in a small container and it is easy to obtain organisms of a known age. *Daphnia magna* was originally employed in acute toxicity work by Bertle Anderson in 1944 (Burks et al. 1981). *D. magna* bioassays were further developed by the EPA for chronic toxicity work (EPA 1982). Mount and Norberg (1984) developed a bioassay using *C. reticulata* for chronic toxicity estimates noting increased reliability and decreased test time compared to *D. magna*. Mount and Norberg (EPA 1989) later used *C. dubia* in a 7-day subchronic assay citing the same benefits as *C. reticulata*, plus good

reproducibility between laboratories and relatively easy food preparation.

The observation of bioluminescence can be dated as far back as Aristotle who referred to "cold light" or phosphorescence of flesh (Harvey 1952). Boyle (1672) has been credited with performing the first toxicity tests using luminescent bacteria. Boyle noted that light was produced on rotten wool (luminous fungi) and shining flesh (luminous bacteria) without perceptible heat and that the light was adversely affected by certain chemical agents. Ironically, Boyle's work never suggested that the light might be coming from living organisms. Baker, in 1742, was the first to suggest that phosphorescence on dead fish and flesh was due to living organisms (McElroy 1961). He identified "animicules" as the source of light. Later researchers showed that the luminescent organisms could be filtered and cultured on different media (Harvey 1952).

Despite the relatively early discovery of luminescent organisms and the many hypotheses suggested for their source of light, it was not until about 1920 that the process was characterized for bacteria (Bulich 1986). Bacterial luminescence is a product of the electron transport system. Light along with FMN (flavin mononucleotide) and acid are produced when the enzyme luciferase catalyzes the oxidation of $FMNH_2$ and a long chain aldehyde (Bitton 1986). The reaction can be summarized as follows (Hastings 1977):

$$FMNH_2 + O_2 + RCHO => 0.1\ hu + FMN + H_2O + RCOOH$$

This bacterial luciferase system is coupled to respiration via NADH and the flavin nucleotide (Hastings 1977). Thus the relative amount of light emitted is directly linked to the metabolic state of the cell.

One of the first practical applications of luminescent bacteria was in the study

of air quality. A study by Sie et. al. (1966) involved exposure of *P. fischeri*, grown on a solid medium, to toxic fumes. A photomultiplier tube within a light-tight container was used for monitoring light levels. Toxic vapors were introduced to the system then purged with clean air. In this manner the same culture could be reused numerous times. Bacterial test response time was 1-3 seconds with recovery time being dependent on the age of the culture. Serat (1965) also studied the effects of air pollution upon luminescent bacteria. Serat was able to determine the presence and relative concentration of a toxicant by monitoring the changes in light output.

Bulich (1979) was the first to report the use of luminescent bacteria in the evaluation of water quality. The testing equipment was a photometer consisting of a rotary shutter built around a photomultiplier tube. Bulich's total study involved 17 different species of luminescent bacteria but the responses of only five species were reported. Test refinement was performed with *P. phosphoreum* due to its stable light production and sensitivity to a broad range of toxicants. Initially fresh cultures from agar-grown cells had to be created daily. The test was made more reliable and repeatable when lyophilized cells were reconstituted. Testing temperatures were set at $15 \pm 0.5^{0}C$ when it was discovered that all toxicants tested had a different temperature-response curve. Good sensitivity was found with most of the 16 chemicals tested. Comparisons made between *P. phosphoreum* and 4-day *P. promelas* acute toxicity tests revealed that the bacterium was more sensitive to malathion and phenol than the fish. In 1980 Bulich introduced the lyophilized *P. phosphoreum* along with the materials and equipment necessary for testing of aqueous samples marketed as Microtox (MTX) originally through Beckman Instruments, Inc., and currently through Microbics Corp.

MTX system's software calculation of the MTX EC50 follows the procedure described by Johnson et. al. (1974). In this procedure the percent light decrease is replaced by a gamma function. A gamma (G) value of one is assigned when the amount of light lost is equal to the amount of light remaining. The values for G were plotted against sample concentration on log-log graph. A best fit line is created and the EC50 determined by interpolation at G = 1.

Cronin (1991) studied the toxicity of several common organic pollutants upon *P. promelas* , the cladoceran *D. magna* and *P. phosphoreum*. Data was compiled from the literature except for 40 experimentally determined data for MTX. The study found encouraging correlations between the toxicities to fish and the lower organisms. Bulich et. al. (1981) compared MTX assay values for pure compounds to fish LC50 values. They also compared MTX assay values for complex effluents with simultaneously run fish assays. They reported good correlation between the MTX and fish values although no correlational values were provided. Neiheisel et al. (1983) used MTX, *P. promelas* and *D. magna* to quantitate the toxicity of influent and effluent samples from two conventional activated sludge pilot wastewater treatment systems. The influent and primary effluent samples were slightly more toxic to *P. phosphoreum* than the other two species. However, the data from all three species for the secondary effluents were similar indicating little or no toxicity.

Some authors have suggested the importance of MTX as a prescreening tool in the hazard assessment of chemicals. DeZwart and Slooff (1983) compared MTX to 20 other standard aquatic toxicity test species. MTX was found to yield replicable results which were comparable to those obtained from the standard tests. The authors recommended that MTX be used as a primary test to quickly determine which

compounds yield certain risks to the aquatic environment. The standard bioassays could then be used on the limited samples which warranted further analysis. Qureshi et. al. (1982) also found that the MTX test sensitivity was comparable to that of *P. promelas* and *D. magna* tests particularly for pure compounds and complex effluents. The study found that MTX is a poor indicator for substances such as ammonia and cyanide. Therefore, they recommended that MTX only be used in a battery of screening tests or to supplement other well-established toxicity bioassays.

There is no one organism that can indicate all possible toxicants. What is lethally toxic to one species may have no detectable deleterious effects upon another species. This is why a battery of organisms is often used for the detection of toxicity. Typically, a battery will be composed of at least three species comprising various trophic levels. Since its introduction MTX has been included in numerous batteries with a wide variety of species.

Hill (1987) used MTX, *C. dubia*, and *P. promelas* to evaluate the toxicity of a simulated in situ retorting of a western oil shale. Toxicity was also evaluated after three different treatments. It was found that these treatments reduced toxicity to MTX but not *C. dubia* nor *P. promelas*.

Giesy et. al. (1991) used MTX, *D. magna* and two other species to delimit the extent of further sediment investigations. Since perfect predictability cannot be expected even with a battery, prioritization of further investigations was based on the screening assays and chemical analyses.

PEEP (Potential Ecotoxic Effects Probe) introduced by Costan et. al. (1993) integrates the results of MTX, *C. dubia* and two other species. The resulting index

number (ranging from 0 to infinity but generally no more than 10) indicates the persistence of chemical constituents, their ability to affect multiple trophic levels and the level of toxic expression.

Volterra (1992) found that MTX could be used to screen within water treatment facilities for cyanophyte blooms harmful to human health. Although MTX results did not always agree with results from high-performance liquid chromatography, the authors were satisfied with the high sensitivity of MTX to algal toxins.

Casarini et. al (1991) used MTX to determine initial loading rates in a land treatment unit. Detoxification, degradation and immobilization of hazardous waste constituents to protect surface water, groundwater and soil rely upon the presence of healthy, active soil microorganisms. Test loading rates that did not impact the biological activities of these soil microorganisms were determined by comparing MTX results to the EC50 or toxic unit (TU). It was found that the loading rates in practice were three times above the advisable level, possibly compromising the biodegradation processes and causing accumulation of organic compounds.

Researchers such as Eisman et. al. (1991) have found MTX to be a very effective bioassay tool for specific chemical groups. They used MTX for successfully assessing the toxicity of hydrocarbon fuels, fuel components and water soluble fractions and soil column effluents of these components.

Research with MTX has been so extensive and correlations with traditional organisms so good that MTX is being used by researchers as a calibrating tool for relatively new screening systems. The MetPAD bioassay kit (Bitton 1992) is one such system. MetPAD and MTX were compared in toxicity screens of sediments

contaminated with heavy metals. The authors were pleased that the relative levels of toxicity evidenced by MetPAD were confirmed with MTX.

Microtox is relatively simple to perform and requires much less time and sample volume than the traditional assays. Due to these advantages numerous chemicals have been documented with MTX EC50 data in the relatively short time MTX has been on the market (Kaiser 1991). The MTX bacteria represent the lowest trophic level. Understanding the impact at this level may help in understanding the potential a certain chemical or group of chemicals has in total impact on the environment.

The MTX assay is used with two major variations (Microbics 1990). The "standard method" uses a maximum dilution of 45% and the "100% method" uses a maximum dilution of 91% or 98%. Tarkpea and Hansson (1988) found that the confidence intervals (CI) generated by the 100% method could be as much as 10.4 times larger than the CI for the standard method. However, the EC50 values were not drastically different between the two methods.

P. phosphoreum is a marine organism which can be adapted to test freshwater sources by osmotic adjustment with sodium chloride to maintain the organism. Hinwood (1987) has questioned the validity of such adjustment as it may compromise the composition of the sample tested. It would be very difficult to determine what interactions may take place between other chemicals present and the added sodium chloride.

The Southern California Coastal Wastewater Research Project (SCCWRP 1987) used MTX in a battery of tests in order to document changes in wastewater toxicity. In their annual report they noted that expressing MTX toxicity in terms of the NOEC

made the test much more sensitive than evaluation with the EC50. This finding suggests that comparisons of NOEC and EC50 values for MTX, *P. promelas*, and *C. dubia* bioassays should be studied.

CHAPTER 3

MATERIALS AND METHODS

Wastewater samples were collected by municipal and industrial dischargers and shipped via special overnight services to our lab in accordance with protocols specified in their NPDES permits (EPA 1989). The samples were mechanically composited over a 24 hour period at volumes proportional to the flow of the effluent. Samples were collected into polyethylene cubitainers, placed on ice and transported to the laboratory. According to EPA protocol each 7-day bioassay required three subsamples (EPA 1989). Figure 1. indicates a typical scenario for introduction of individual subsamples to the 7-day tests. The first of the three subsamples was used to initiate the static removal bioassay tests. Subsamples were used for daily exchanges in the 7-day bioassays for up to 2 or 3 days depending upon sampling and shipping schedules. The MTX assay was used to analyze all subsamples.

The wastewater samples used for exposing *P. promelas* and *C. dubia* studies were exchanged daily. Aliquots of subsample were slowly brought to $23^0C \pm 1.5$ in a water bath. Dissolved oxygen (D.O.) content of the aliquot was measured and when necessary purified air was bubbled through to maintain proper D.O. values (6.0-8.0 mg/l). pH and chlorine levels were also measured. Dilutions were made using either receiving stream water or synthetic mineral water prepared in lab. Effluent/dilution water concentrations were determined by individual NPDES permits. Each dilution was analyzed for D.O., pH, conductivity, hardness and alkalinity. Exposure rooms

were kept at constant temperature , $25^0C \pm 1.5$, and constant photoperiod, 16 hours light and 8 hours dark.

P. promelas larval survival and growth tests were conducted according to EPA specifications (EPA 1989). All available larvae were collected at less than 24 hours old into a common container. Random samplings of ten larvae were introduced into bowls containing 250 ml of diluted effluent, with 4 replicates of each dilution. Daily dilution exchanges with identical concentrations were conducted after organisms were counted and the dead removed. *P. promelas* was fed twice daily with live brine shrimp cultured in laboratory. On the seventh day all living organisms were killed by thermal shock and dried. *P. promelas* weights were measured to determine significant differences in growth between controls and effluent exposed fish.

C. dubia survival and reproduction tests were also conducted according to the EPA specifications (EPA 1989). *C. dubia* neonates less than 24 hours old and shed within 8 hours of each other were collected. One neonate was placed in 15ml of effluent concentration with 10 replicates of each dilution. Daily dilution exchanges were conducted after the general health of the original neonate was recorded and any new generation neonates were counted. Once the control organisms had three broods and approximately fifteen neonates, the test was terminated; this generally occurred on day 6 or 7. *C. dubia* was fed daily with *Selenastrum capricornutum* and TCY (Trout chow-Cerophyl-Yeast) digest, both prepared in accordance with EPA protocols.

Four sets of data were collected from the 7-day tests: *P. promelas* survival and growth, and *C. dubia* survival and reproduction. Each set of data was statistically analyzed using TOXSTAT version 3.2 (Gulley 1990). An NOEC value for each

biomonitoring parameter was determined following the decision flowchart established by the EPA for statistical analysis of biomonitoring data (EPA 1989).

The Microtox (MTX) assay system utilizes lyophilized marine bacteria (*P. phosporeum*) which emit light upon rehydration. All samples were osmotically adjusted to 2% sodium chloride with either Microtox Osmotic Adjustment Solution (MOAS) or solid sodium chloride to accomodate the osmotic requirements of the marine *P. phosphoreum*. Each subsample was initially screened at 91% effluent. Effluent dilutions were created using aliquots from each of the three subsamples. All samples were adjusted using MOAS for an excess of dilution at 91% or 98% effluent. Subsequent dilutions were made using the excess. Twenty microliters of reagent, which contained millions of bacterial cells, were added to 1ml of test dilution, with four replicates per dilution. Each cuvette was incubated at 15° C for five and 15 minutes after exposure prior to measurement of phosphorescent light output. Raw light values and all pertinent information were recorded on the Microtox data sheet designed in the lab (Appendix). Raw light values were entered into TOXSTAT version 3.2 (Gulley 1990) for calculation of mean light values per dilution. TOXSTAT was further used for analysis of MTX NOEC following the EPA decision flowchart (EPA 1989). Mean light values generated by TOXSTAT were also entered into the MTX software to establish EC50 values.

Two different methods were explored in analyzing the MTX EC50/ MTX NOEC data versus the 7-day NOEC data. First we applied a binary approach of toxicity identification and compared the percent agreement and disagreements between different tests. Second we ranked and correlated the data using Pearsons's correlation

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biomonitoring parameter was determined following the decision flowchart established by the EPA for statistical analysis of biomonitoring data (EPA 1989).

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The first approach was to designate whether the MTX endpoint indicating the

presence or absence of toxicity was in agreement with the presence or absence of toxicity as indicated by the traditional organisms. The data was further categorized as to the presence or absence of toxicity as indicated by the MTX endpoint. This provided four categories of data: Agree - Toxic, Agree - Nontoxic, Disagree - Toxic to MTX, Disagree - Nontoxic to MTX. This approach identified samples as toxic whenever the endpoint was less than 100% effluent (or the highest concentration tested).

Ranking the data prior to statistical work was essential due to the different ranges of sensitivity expressed by the different organisms. Systat version 5.02 (Systat 1993) was used to rank and correlate the data. The Pearson's test was chosen to provide a correlational value based on the organization of the ranks in respect to the various categories. The data were ranked in the following categories: MTX EC50 subsample 1; 2; and 3; low MTX EC50; high MTX EC50; MTX NOEC subsample 1; 2; and 3; low MTX NOEC; high MTX NOEC; *P. promelas* survival; *P. promelas* growth; *C. dubia* survival; *C. dubia* reproduction; 7-day low; and 7-day high.

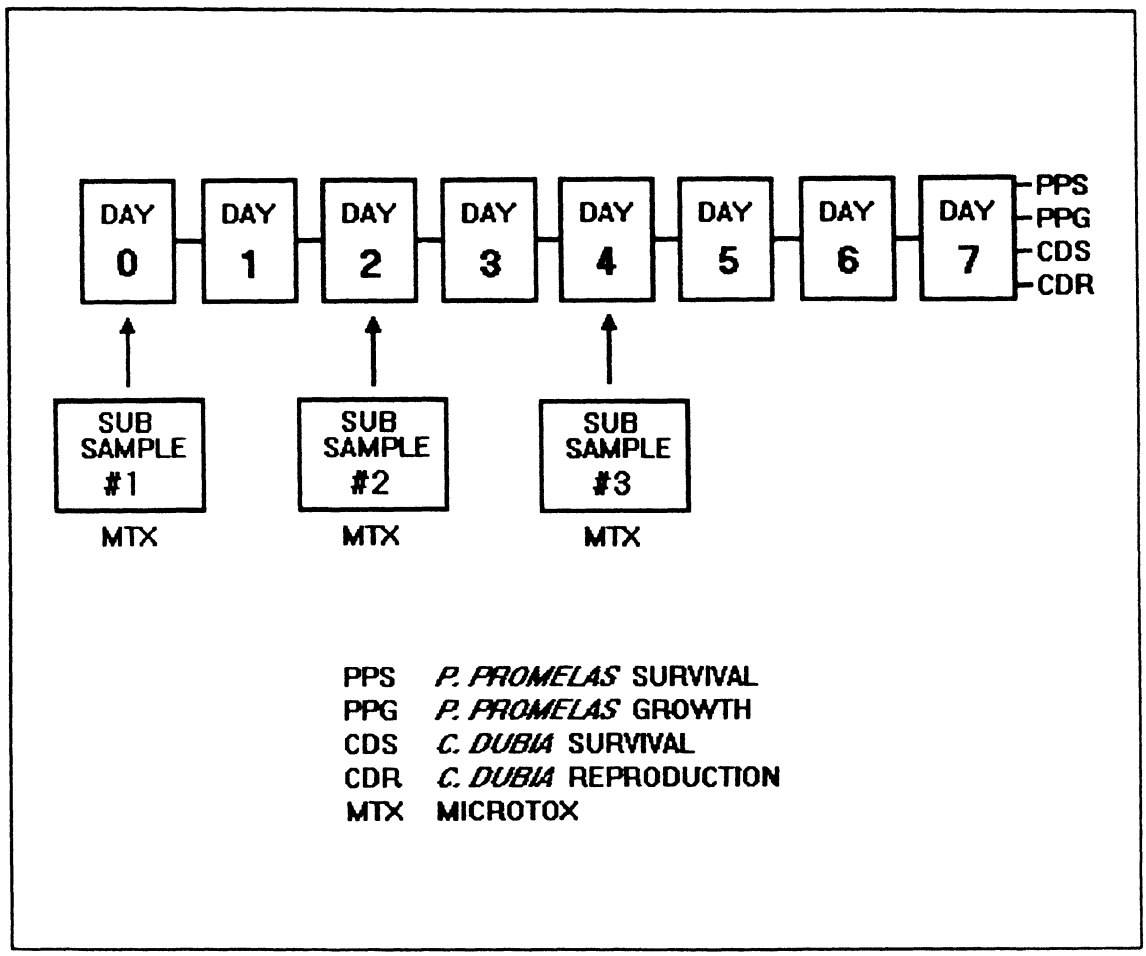

Figure 1. Introduction of subsamples to the static renewal seven-day bioassays.

CHAPTER 4

MANUSCRIPT

INTRODUCTION

The Microtox (MTX) assay system has been used in a wide variety of aquatic applications to determine the concentration of a toxicant which causes a 50 percent reduction in phosphorescence (EC50). Microtox EC50 data for organic compounds have been favorably compared to acute lethality toxicity tests using fish and *Daphnia* (Qureshi et al. 1982, Cronin et al. 1991). Even better comparisons have been made for complex effluents and process waters tested simultaneously with standard fish or *Daphnia* toxicity tests (Kovacs and Voss 1992, Vasseur et al. 1986, Vasseur et al. 1984, Bulich 1982, Qureshi et al. 1982, Dutka and Kwan 1981, Lebsack et al. 1981). Munkittrick et al. (1991) provide a good summary of over 70 comparative studies performed with MTX EC50 and *Daphnia*, *Oncorhynchus mykiss*, and/or *Pimephales promelas* acute lethality bioassays. The studies found MTX to be more or as sensitive to pure organic chemicals as the higher organisms. Microtox was found to be less sensitive than the higher organisms to most inorganics. Overall, MTX correlation with other organismal tests and its sensitivity appeared to improve as the complexity and toxicity of industrial effluents increased. These comparative studies suggested that the MTX assay might be useful as an exploratory screening tool in the hazard assessment of chemicals or effluents (De Zwart and Slooff 1983, Firth and Backman

1990). Researchers (Vasseur et al. 1986, Casseri et al. 1983) have expounded upon the potential for MTX in evaluating the toxicity of, and treatment techniques for, complex industrial wastewaters. This substantial work supported Bulich and Isenberg (1980) who stated that MTX was a useful bioassay when applied in the analysis of acute toxicity.

Some investigators have found that the MTX EC50 does not work for some applications (Mazidji 1990). However, little work has been done with MTX and chronic or sublethal toxicity. The Southern California Coastal Water Research Project (SCCWRP) in their 1987 annual report noted that expressing MTX toxicity in terms of the "no observed effect concentration" (NOEC) made the test much more sensitive to toxicants than evaluation with the EC50. This apparent increase in sensitivity was the result of using the endpoint of the first treatment that was not significantly greater in light inhibition than that of the control (NOEC) rather than the conventional 50% light inhibition (EC50). While no one organism can effectively indicate all possible toxicants, the benefits of a rapid screening system such as Microtox cannot be overlooked and, when possible, its potential should be explored.

The Water Quality Research Laboratory (WQRL) of Oklahoma State University has been conducting acute toxicity tests for over 20 years and the seven-day toxicity tests involving *Ceriodaphnia dubia* and *P. promelas* for the past six years. In these tests NOEC values were generated as an endpoint which could be statistically tested for significance. These seven-day tests were designed to provide chronic and sublethal toxicity estimates by evaluating *C. dubia* survival and reproduction and *P. promelas* survival and growth. Many of the effluents tested by the WQRL staff did not produce significant effects upon the higher organisms until day 6 or 7. It was our

desire to determine the potential of the MTX assay for rapidly predicting trends in toxicity of these effluents.

Preliminary work led us to believe that the MTX NOEC would be more useful than the MTX EC50 as a surrogate index of potential chronic toxicity and sublethal toxicity to the higher organisms. This work suggested that the MTX EC50 might be the best method for evaluating potential acute toxicity. Based upon these observations our aim was to evaluate whether the MTX NOEC endpoint would be an improvement over the MTX EC50 endpoint for predicting toxicity of wastewater samples. Chronic/acute toxicity was evaluated by comparing MTX results with *C. dubia* and *P. promelas* survival. Sublethal toxicity was evaluated by comparing MTX results with *C. dubia* reproduction and *P. promelas* growth. We analyzed the MTX data by running statistical tests for significant reductions in light output of treatments when compared to a control, analogous to the procedure used in calculating NOEC values for *C. dubia* and *P. promelas*. Organismal mortality may not exceed 50% in conventional toxicity tests. However, there may still be a statistically significant reduction in survival when compared to the control. We suspected MTX EC50 was not adequate since values greater than 100% effluent (considered non-toxic) had been measured on several wastewater samples, yet survival of *C. dubia* and *P. promelas* was affected in the seven-day tests. In many of these cases, a trend of increased light inhibition with increased effluent concentration was observed with the bacteria. We chose to modify Microbics' Microtox 100% assay slightly (increased the number of replicates to 4) and analyze the data for a NOEC endpoint according to the protocol outlined in EPA/600/4-81/001 for *P. promelas* survival. We made comparisons

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between MTX EC50, MTX NOEC and NOECs of the traditional organisms. A total of 53 wastewaters were tested representing 34 oil refinery effluents, 14 municipal effluents, two industrial effluents, and three cooling tower effluents.

METHODS AND MATERIALS

Microtox Assay

The MTX bacteria, *Photobacterium phosphoreum*, is a marine organism which would be osmotically stressed by exposure to freshwater. All of the wastewater samples were derived from freshwater sources and were osmotically adjusted to 2% sodium chloride. This was performed using either Microtox Osmotic Adjustment Solution (MOAS), allowing a maximum dilution of 91%, or solid sodium chloride, allowing a maximum dilution of 98%.

Generally, once effluent samples had been warmed and the dissolved oxygen (D.O.) adjusted for the 7-day tests, an aliquot was collected for MTX analysis. Before subjecting a sample to the complete MTX test, it was osmotically adjusted to 91% in duplicate then screened for toxicity. This procedure is described in the Microtox manual as the 90% screen protocol (Microbics 1992a). Light values were compared to the screening reference table (Table 1) constructed at WQRL for aid in choosing the appropriate dilution scheme. When the initial concentration was very low ($\leq$ 22%), a stock dilution of 91% was created and subsequent dilutions were made from the stock. Dilutions and controls were then prepared using Microtox diluent and following the Microbics 100% assay with the exceptions of dilution concentrations prepared and test dilutions were performed in quadruplicate. The raw

light values to be used for statistical analysis were collected at 5 minutes of exposure using a Microtox Model #500.

Microtox Data Analysis

The raw light values generated by the MTX 500 were entered into Toxstat software, Version 3.2 (Gulley 1990) to determine mean values and data distribution. Toxstat was further used to calculate a MTX NOEC by testing for significant differences between treatments and controls following the decision flowchart established by the EPA for statistical analysis of biomonitoring data (EPA, 1989). Mean light values generated by Toxstat were also entered into the MTX software to establish EC50 values.

Conventional *C. dubia* and *P. promelas* Assays

Seven-day static renewal biomonitoring was conducted on *C. dubia* and *P. promelas* according to procedures outlined by the EPA (EPA 1989). Test dilutions were created based upon requirements for individual National Pollutant Discharge Elimination Systems (NPDES) permits. Most dilution schemes for a particular effluent were identical for both *C. dubia* and *P. promelas*. Both organisms were cultured in laboratory, collected and exposed to dilutions at <24 hours old with an eight hour span for collecting *C. dubia*. Organisms were kept in constant temperature rooms of $25^0C \pm 1.5$. *P. promelas* was fed twice daily with live brine shrimp cultured in laboratory. *C. dubia* was fed once daily with *Selenastrum capricornutum* and TCY (Trout chow-Cerophyl-Yeast) digest, both prepared in laboratory. *C. dubia*

tests were terminated once controls had 3 broods and an average of 15 neonates, this usually occurred by day 6 or 7. *P. promelas* tests were terminated on day 7, when the surviving fish were killed by thermal shock in an ice bath, removed, dried and weighed. NOECs were calculated for *P. promelas* survival and growth and *C. dubia* survival and reproduction and tested for significance at $p=0.05$. Acute 48 hour toxicity was determined for each sample by graphing percent survival vs. log-percent effluent volume for interpolation of median lethal concentration (LC50) values.

Final Data Analysis

Each seven-day test required three sub-samples of composite effluent. Each of these sub-samples was subjected to the Microtox assay. This resulted in the generation of three response values for each Microtox endpoint and only one from each of the four biomonitoring parameters. Samples were considered toxic to an organism when the NOEC or EC50 value was lower than 100% or the highest dilution tested.

Initial analysis of the data was done using a binary system: Toxic, non-toxic. We compared the percent agreement between the toxic response of MTX and the 7-day parameters. Samples were considered in agreement when the presence/absence of toxicity was confirmed by the MTX endpoint and the 7-day parameters. If the MTX endpoint indicated no toxicity was present and even one of the 7-day parameters indicated a toxic response, then the two tests were considered to disagree. If the MTX endpoint indicated toxicity, only one of the parameters needed to indicate toxicity in order for the tests to be considered in agreement.

Statistical anaylsis of data was performed using Systat version 5.02 (Systat

1993). Data were ranked by SYSTAT in the following categories: MTX EC50 subsample 1, 2, and 3; MTX EC50 low; MTX EC50 high; MTX NOEC subsample 1, 2, and 3; MTX NOEC low; MTX NOEC high; *P. promelas* survival; *P. promelas* growth; *C. dubia* survival; *C. dubia* reproduction; traditional organism low; and traditional organism high. Systat was then used to perform a Pearson correlation. Each reported correlation coefficient is significant at $p \leq 0.05$. When a NOEC value was reported as "less than", the value following the symbol was used for data analysis (e.g. < 10 was analyzed as 10).

RESULTS AND DISCUSSION

Percent Agreement/Disagreements

A total of 53 wastewater samples were analyzed using all three toxicity tests (Table 2). When analyzing the tests strictly for the presence of toxicity the results for MTX NOEC are encouraging and reflect values from the literature. The MTX NOEC percent agreements are very similar to those found by Bulich (1982) and Dutka and Kwan (1981) when studying MTX EC50 and acute toxicity of complex wastes. Bulich found a 78% agreement between fish and MTX and a 63% agreement between *Daphnia* and MTX. Dutka and Kwan reported MTX agreed to toxicity found in 81% of effluents toxic to *P. promelas* and agreed 62% with Daphnia.

Figure 1 represents comparisons between the two MTX endpoints and the higher organismal parameters. MTX EC50 agreed to the presence of toxicity in only 30% of the tests when compared simultaneously to *C. dubia* and *P. promelas* parameters. Individual comparisons between MTX EC50 and *C. dubia* or *P.*

promelas data yielded better agreements (43% and 50%, respectively). When the MTX NOEC was compared to that for the higher organisms, 79% of the tests agreed (62% vs. *C. dubia* and 79% vs. *P. promelas*). This large increase in agreement can be linked directly to the apparent increase in sensitivity of MTX as a result of using the NOEC.

The samples with which MTX EC50 disagreed were all nontoxic according to MTX EC50. However, all of these samples were toxic in some degree to at least one of the 7-day parameters. MTX EC50 was not sufficiently sensitive to predict toxicity for these effluents.

When MTX EC50 indicated a sample was toxic, toxicity was also observed with at least one of the 7-day parameters. When MTX EC50 indicated toxicity was present in the first of three subsamples, 48 hour toxicity (LC50 < 100%) was also observed with the *C. dubia* and/or *P. promelas* survival. However, not all acute toxicity observed with *C. dubia* and *P. promelas* corresponded with MTX EC50 toxicity. Therefore in several tests MTX EC50 was not sensitive enough, even to acute toxicity, to detect effects deleterious to the higher organisms.

Statistics

One major drawback to the use of the NOEC method is the limitation imposed on the results by the dilution /concentration scheme chosen. The EC50 allows for an extrapolation to the concentration at which 50% inhibition occurred. Since the NOEC simply compares each concentration to the control, it can only reflect values from the chosen concentrations. The tighter the dilution scheme chosen, the more closely the NOEC represents the concentration at which no significant adverse biological effects

occur.

Consistently, analysis of *P. promelas* survival and growth vs. MTX NOEC, second subsample (MTX $NOEC_2$), generated the highest correlations of all statistical comparisons (Table III). When all tested samples were ranked, the correlations between MTX $NOEC_2$ and *P. promelas* survival and growth were at their lowest (0.773 and 0.754, respectively). We were not satisfied that the dilution schemes chosen represented the most refined case possible. Therefore, we eliminated all samples with a MTX NOEC of 45% and a dilution scheme represented by a dilution factor of 2 resulting in dilutions of 91, 45, 22 and 11%. This indicated a potentially large gap between the derived NOEC and the concentration at which no significant adverse biological effects would occur in nature. The correlations between the MTX $NOEC_2$ and *P. promelas* survival and *P. promelas* growth increased to 0.799 and 0.795, respectively. Finally, we ranked only the tests from ID # 91044 to 91090 for which dilution schemes were specifically designed to be tight and got correlations of 0.848 and 0.845 (Table IV). This last step eliminated 7 of the 12 samples in which MTX NOEC and *P. promelas* did not agree concerning the presence of toxicity. The final total of 21 samples represented 15 refinery effluents, four municipal effluents, and two cooling tower waters. It is obvious from these statistics that the concentration scheme chosen plays a significant role in the utility of the NOEC method. Since no correlations were found between the municipal MTX EC50/MTX NOEC vs. municipal 7-day parameters, and the number of cooling tower effluents tested was insignificant, the high correlations were considered unique for refinery effluents.

There was an increased representation of refinery effluents in the final comparisons, the correlation coefficients for refinery samples alone were 0.757 and

0.733 for MTX $NOEC_2$ vs. *P. promelas* survival and growth (Table V). When the tighter dilution schemes were chosen within the refinery samples, the correlation values increased to 0.837 for both MTX $NOEC_2$ vs. *P. promelas* survival and vs. growth (Table VI). The WQRL has identified the major contaminants in these effluents as non-polar organics which can either be eliminated or significantly reduced by non-polar adsorbents such as activated carbon treatment (Helems 1993).

The MTX vs. *P. promelas* values were somewhat lower than the correlation value of 0.97 reported by Lebsack et al. (1981) for MTX EC50 versus 24-hour static *P. promelas* tests. However, Lebsack worked with oil shale retort waters that are generally more toxic than a final effluent from a secondary wastewater treatment system so that the decreased correlation would be expected, according to Munkittrick et al. (1991).

MTX NOEC indicated the correct toxic response of *C. dubia* in 62% of the samples; however, the degree of response within these samples was not strongly correlated. A total of 35 MTX NOEC test results compared with *C. dubia* parameters agreed with respect to the presence/absence of toxicity. When these 35 tests were analyzed, the highest correlation (0.614) was generated between MTX NOEC third subsample and *C. dubia* reproduction. Based on other statistical analyses very few correlations were found between MTX NOEC and *C. dubia* parameters and most of those were weak correlates (Tables III and IV).

When comparing MTX to the 7-day test results, it became evident that the relationship between MTX and *P. promelas* was more reliable for these samples than that between MTX and *C. dubia*. *P. promelas* parameters were found to be the most

sensitive measures of toxicity in this collection of samples. When all 53 samples were considered, *P. promelas* survival and growth correlated strongly (0.868 and 0.897, respectively) with the lowest values generated from all 7-day parameters.

In all statistical analyses of MTX and *P. promelas* parameters the MTX NOEC correlations were much higher than the MTX EC50. The highest value demonstrated by MTX EC50 (0.521) represents a comparison between MTX $EC50_1$ and *P. promelas* growth when all samples with MTX NOEC dilution schemes of 91, 45, 22 and 11% were removed from ranking. However, this would not be a legitimate statistical consideration for MTX EC50 since a refined dilution scheme would only directly affect MTX NOEC. When all data were considered (Table III), the highest MTX EC50 correlation (0.476) was a comparison between MTX EC50, of the first subsample, and *P. promelas* growth. Thus the MTX NOEC increased the apparent sensitivity of the system.

Despite the low correlations between MTX EC50 and the higher organismal parameters, they were still higher than correlations calculated for *C. dubia* and *P. promelas* parameters. When all samples were considered, a comparison of survival between the two species yielded a correlation of 0.305, and the comparison between the sublethal effects of reproduction and growth yielded a correlation of 0.410. These values did not alter significantly when samples were regrouped for statistical purposes.

MTX NOEC second subsample results consistently correlated most strongly with *P. promelas* parameters. Although these values did not tend to be much higher than the correlations with subsample 1, the correlations with subsample 3 were always

the lowest values. Perhaps *P. promelas*, at a vulnerable stage, became sensitized by the first subsample so that the effect of the second subsample was intensified. The lower correlations with the third subsample simply show that the quality of this subsample was not as significant to the health of the organism as the second subsample. This relationship was not seen between MTX EC50 and 7-day parameters.

It is noteworthy that in several tests, MTX indicated toxicity in only one of the three sub-samples for an effluent that resulted in toxicity in the 7-day parameters. Seven-day test results have very little power to discriminate between the individual toxic effects of subsamples.

CONCLUSIONS

The binary system of toxicity analysis resulted in mostly consistent comparisons between MTX NOEC and *P. promelas* parameters (79% of samples in agreement) and *C. dubia* parameters (62% of samples in agreement). These numbers are adequate if the only concern is the existence of toxicity for further consideration. However, the binary system did not allow exploration as to the degree of toxicity present. This was done by statistical analysis at a significance level of $p \leq 0.05$.

C. dubia and *P. promelas* do not respond with the same sensitivity to all complex mixtures. Likewise, Microtox does not respond with the same sensitivity as other organisms to all complex mixtures. When MTX EC50 values were used, the highest correlation between MTX and the 7-day test results, represented by *P. promelas* growth, was 0.476. However, when MTX NOEC values were used, the highest correlation between MTX and the 7-day NOEC values, represented by *P.*

promelas survival, was 0.848. MTX NOEC increased the apparent sensitivity of the assay while retaining all the benefits of the traditional Microtox assay. Only minor changes in assay procedure were necessary and the data could be analyzed by the same statistical procedure currently used with the *C. dubia* and *P. promelas* 7-day tests.

The addition of the NOEC method to the MTX system increases the utility of the assay. This method may minimize the need to concentrate toxicants in order to initiate bioluminescent inhibition as has been suggested by previous research (Dutka et al. 1986, 1988a, b, and Ribo et al. 1985). However, the dilution scheme must be chosen carefully in order to get as accurate a biological NOEC as possible. We recommend the WQRL screening chart (Table I) for initial work, which may be further tailored to individual needs.

Our data confirm the importance of multi-species tests as no one species can predict all possible toxicants. Therefore, MTX NOEC can be a valuable complement to standard biomonitoring. The rapid, easy, cost efficient assay can be used to screen effluents prior to more lengthy and costly tests, as well as allowing analysis of samples which otherwise could not be run due to number or volume constraints.

The MTX NOEC method used by WQRL requires stringent pipetting practices and may be unsuitable for some laboratories. Microbics has recently developed a MTX NOEC protocol (Microbics 1992b) that is appropriate for technicians uncomfortable with the rigorous demands of small volume pipettors.

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TABLE I

MICROTOX SCREENING REFERENCE TABLE[a]

Avg. Light Value of 91%	Initial Concentration	Number of Dilutions[b]	Dilution Factor
80 - > 100[c]	98	3	1.5
58 -79	91	4	1.5
46 - 57	98	6	2
34 - 45	91	8	2
21 - 33	45	4	2
8 - 20	22	6	2
0 - 7	Screen at 1.8%		

[a]This table is designed to obtain EC50 and NOEC values concurrently.

[b]Effluents with slopes > 1 may not require as many dilutions. Effluents with slopes < 1 may require more dilutions.

[c]EC50 values will be > 100% effluent.

TABLE II

ORGANISMAL RESPONSE TO COMPLEX EFFLUENT SAMPLES MONITORED
FROM NOVEMBER 1990 TO OCTOBER 1991

Type	ID #	Microtox EC50 (%)			Microtox NOEC (%)			Bioassay NOEC (%)			
		#1	#2	#3	#1	#2	#3	FHS	FHG	CS	CR
R	90131	>100	>100	>100	11	<30	12	43	43	95	63
N	90135	>100	>100	>100	11	22	22	73	30	100	85
R	90139	>100	>100	>100	22	11	45	43	43	95	95
E	90140	>100	>100	>100	<11	11	22	100	100	100	100
N	90141	>100	>100	>100	45	45	45	85	73	100	100
R	90142	68	45	37	5.5	<3.8	2.8	12.5	<12.5	48	12.5
R	91006	>100	>100	>100	91	91	91	100	100	100	100
R	91007	8.4	11	12	<2.8	<2.8	<2.8	10	<10	<10	<10
M	91012	>100	>100	>100	91	91	91	100	100	100	100
M	91013	>100	>100	9.7	91	91	<2.8	100	100	10	10
M	91014	>100	>100	>100	91	91	91	100	100	74	74
R	91016	>100	>100	>100	22	45	45	43	<43	95*	52
R	91017	33	>100	>100	91	91	91	100	100	100	77
R	91020	75	61	71	2.8	2.8	<2.8	<10	<10	48	10
R	91021	>100	>100	>100	91	91	91	100	25	100	25
M	91023	>100	>100	>100	91	91	91	100	100	100	100
M	91024	>100	>100	>100	91	91	91	100	100	100	100
M	91025	>100	>100	>100	91	91	91	100	100	50	50
R	91026	>100	I	>100	22	45	11	40	40	100	100
R	91027	>100	>100	>100	22	45	22	52	43	95*	77
R	91028	>100	>100	>100	91	91	91	100	100	100	100
R	91030	92	91	>100	5.6	2.8	11	12.5	<10	48	25
R	91031	>100	I	>100	91	91	91	100	100	65	48

R=Refinery, M=Municipality, E=Electric generating facility cooling tower, N=Industry, I=Insufficient data.

TABLE II CONTINUED

Type	ID #	Microtox EC50(%)			Mtx NOEC (%)			Bioassay NOEC (%)			
		#1	#2	#3	#1	#2	#3	FHS	FHG	CS	CR
M	91032	>100	>100	>100	91	45	45	100	100	50	50
M	91035	>100	>100	>100	91	91	91	100	100	69	69
M	91036	>100	>100	>100	91	22	91	100	100	75	75
M	91037	>100	>100	>100	91	91	91	100	100	100	100
R	91038	86	72	>100	11	2.8	22	10	10	63	43
R	91039	>100	>100	>100	91	45	91	2.4	2.4	50	25
R	91040	>100	>100	>100	91	91	91	50	50	25	25
R	91042	I	I	>100	22	45	22	25	12.5	65	25
R	91044	>100	>100	>100	45	45	60	40	40	100	100
R	91048	>100	>100	>100	11	22	22	12.5	12.5	48	25
R	91049	87	71	71	7.9	7.9	<5.3	10	10	100	100
R	91054	>100	>100	>100	27	40	27	55	40	100	70
E	91055	>100	>100	>100	98	98	90	100	100	100	30
R	91057	>100	>100	>100	27	<27	27	10	10	100	100
E	91058	>100	>100	>100	98	98	65	100	100	100	100
M	91059	>100	>100	>100	44	98	98	100	100	75	75
R	91061	>100	>100	>100	27	60	18	25	25	65	65
M	91063	>100	>100	>100	98	98	98	60	41	60	60
R	91064	96	I	82	1.4	11	2.8	10	10	100	63
M	91065	>100	>100	>100	98	98	67	100	100	50	50
R	91067	>100	99	64	11	11	2.8	<40	<40	100	55
R	91073	>100	>100	>100	13	11	27	25	25	65	25
R	91074	>100	>100	>100	11	2.8	5.6	10	10	95	77
R	91075	>100	>100	>100	5.6	5.6	2.8	10	10	<10	<10
R	91077	>100	>100	>100	27	40	27	12.5	12.5	65	25
M	91079	>100	>100	>100	98	65	98	100	100	75	75
R	91082	>100	>100	>100	27	5.6	27	10	10	95	43
R	91086	>100	>100	>100	19	29	19	25	25	48	25
R	91089	>100	>100	>100	27	27	27	10	10	95	95
R	91090	>100	>100	>100	18	27	27	12.5	12.5	65	48

R=Refinery, M=Municipality, E=Electric generating facility cooling tower, N=Industry, I=Insufficient data.

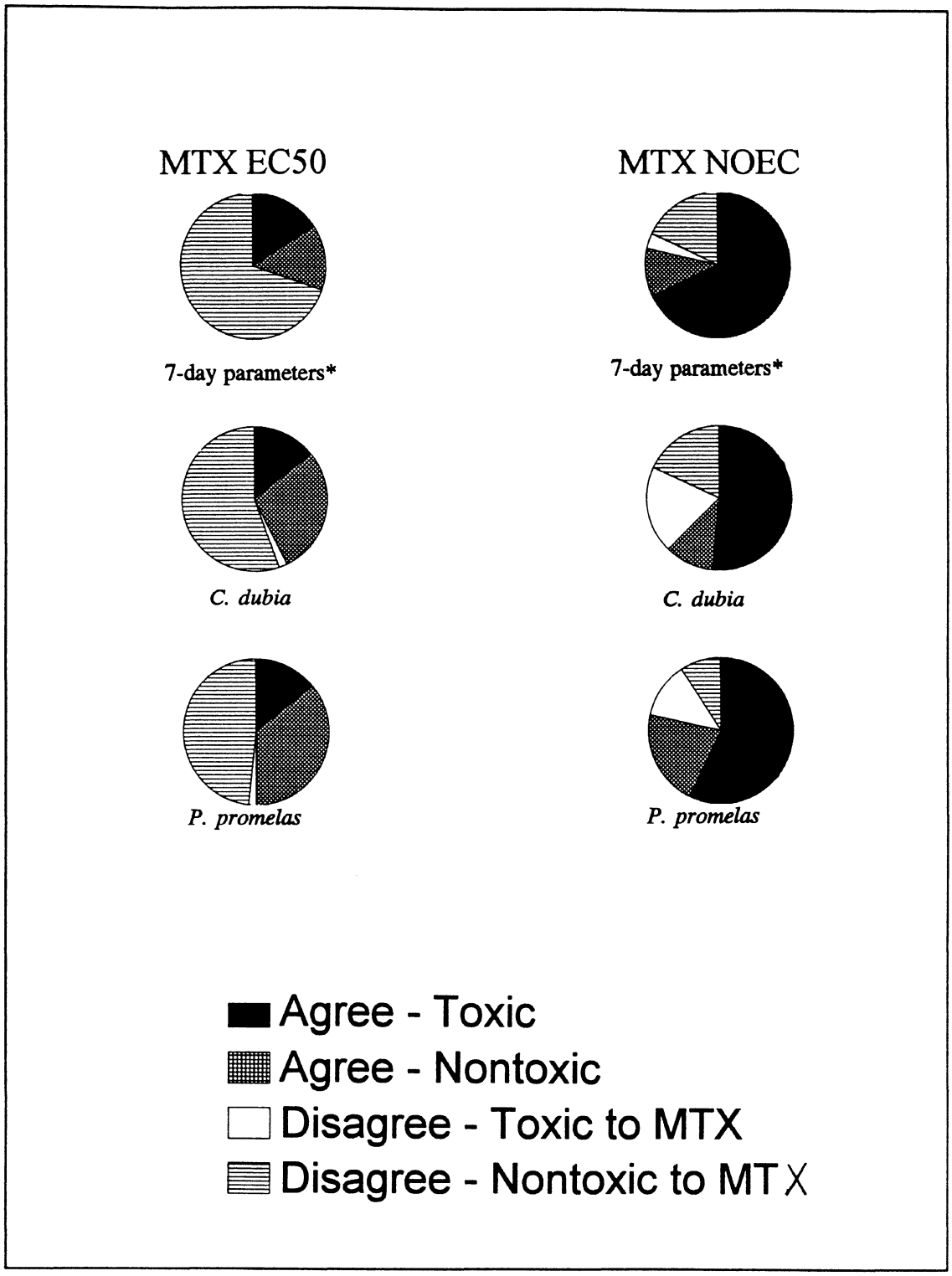

Figure 2. Percent agreement/disagreement between Microtox endpoints and 7-day parameters. *Includes both *C. dubia* and *P. promelas* tests.

TABLE III

PEARSON'S R VALUES FOR CORRELATIONS* OF MICROTOX ENDPOINTS VERSUS 7-DAY PARAMETERS USING ALL SAMPLES

7-DAY PARAMETER	Subsample	MTX EC50	MTX NOEC
P. promelas survival	1	0.463	0.734
	2	0.461	0.773
	3	#	0.668
P. promelas growth	1	0.476	0.715
	2	0.465	0.754
	3	#	0.641
C. dubia survival	1	0.337	#
	2	#	#
	3	#	#
C. dubia reproduction	1	0.337	0.271
	2	0.328	0.273
	3	0.338	0.344

#No correlation found

*All correlation coefficients are significant ($p \leq 0.05$)

TABLE IV

PEARSON'S R VALUES FOR CORRELATIONS* OF MICROTOX NOEC VERSUS *P. promelas* PARAMETERS USING SAMPLES REPRESENTING TIGHT DILUTION SCHEMES

7-DAY PARAMETER	Subsample	MTX NOEC
P. promelas survival	1	0.748
	2	0.848
	3	0.713
P. promelas growth	1	0.746
	2	0.845
	3	0.710

*All correlation coefficients are significant ($p \leq 0.05$). No correlations were found between MTX EC50 and the 7-day parameters nor MTX NOEC vs. *C. dubia* parameters.

TABLE V

PEARSON'S R VALUES FOR CORRELATIONS* OF MICROTOX ENDPOINTS VERSUS 7-DAY PARAMETERS USING ALL REFINERY SAMPLES

7-DAY PARAMETER	Subsample	MTX EC50	MTX NOEC
P. promelas survival	1	0.431	0.587
	2	0.392	0.757
	3	#	0.576
P. promelas growth	1	0.460	0.556
	2	0.410	0.733
	3	#	0.550
C. dubia survival	1	0.395	0.342
	2	#	0.355
	3	#	#
C. dubia reproduction	1	0.373	0.386
	2	#	0.349
	3	#	#

#No correlation found

*All correlation coefficients are significant ($p \leq 0.05$)

TABLE VI

PEARSON'S R VALUES FOR CORRELATIONS* OF MICROTOX ENDPOINTS VERSUS 7-DAY PARAMETERS USING REFINERY SAMPLES WITH TIGHT DILUTION SCHEMES

7-DAY PARAMETER	Subsample	MTX EC50	MTX NOEC
P. promelas survival	1	0.469	0.731
	2	0.421	0.837
	3	#	0.689
P. promelas growth	1	0.506	0.721
	2	0.444	0.837
	3	#	0.662
C. dubia survival	1	0.412	0.393
	2	#	0.408
	3	#	#
C. dubia reproduction	1	0.389	0.468
	2	0.364	0.419
	3	#	0.409

#No correlation found

*All correlation coefficients are significant ($p \leq 0.05$)

CHAPTER 5

RESULTS AND DISCUSSION

Munkittrick et al. (1991) expressed concern over the number of studies whose sole analysis was based on a binary system of toxic/nontoxic comparisons. However, a binary approach does offer important information in the initial screening or prescreening of samples. Therefore, we chose to use the binary approach in conjunction with statistical analysis. The results of this study have shown that no correlaton exists between MTX NOEC and *C. dubia* NOEC. This does not negate the objective of the study as *C. dubia* was less sensitive than *P. promelas* to this particular group of effluents.

When MTX EC50 values were used for evaluation, the highest correlation between MTX and the 7-day test results, represented by *P. promelas* growth, was 0.476. Therefore MTX EC50 could not be considered an adequate measure of toxicity for these effluents. When MTX NOEC values were used, the highest correlation between MTX and the 7-day NOEC values, represented by *P. promelas* survival, was 0.848. MTX NOEC increased the apparent sensitivity of the assay while retaining all the benefits of the traditional MTX assay.

Disadvantages

It was noted during our study that when mean values generated by Toxstat

were used to calculate the MTX EC50 the CIs were slightly larger than when the raw light values were directly entered into the MTX software. This coupled with an increased CI due to the use of the 100% method (Tarkpea and Hansson 1988) may prove to weaken the value of the EC50 generated from the proposed testing. Microbics (1992a) stresses that a tight CI may be maintained with strict attention to pipetting practices. The 100% method relies upon pipetting of very small volumes (10ul) which might account for the loss of confidence between the two methods. Microbics has recently developed a MTX NOEC protocol (Microbics 1992b) that is appropriate for technicians uncomfortable with the rigorous demands of small volume pipettors. Unfortunately, the increased number of pipette transfers involved in the Microbics NOEC method may prove to introduce just as much error as the small volume WQRL NOEC method.

Advantages

It is noteworthy that in several tests, MTX indicated toxicity in only one of the three sub-samples for an effluent that resulted in toxicity in the 7-day parameters. Seven-day test results have very little power to discriminate between the individual toxic effects of subsamples.

MTX has been widely used because it is a rapid, cost-efficient biomonitoring assay that requires only small volumes of sample. The organism on which MTX depends is lyophilized so that there is no maintenance of living organisms between testing periods. The addition of the MTX NOEC method increases the usefulness and therefore, benefits of the assay.

Recommendations for Future Research

Before the MTX NOEC can be used in decision making, comparisons need to be performed between MTX NOEC and the NOECs of *P. promelas* and *C. dubia* of a particular effluent. Since MTX relies on a marine bacterium, a relationship must be documented between MTX sensitivity and the sensitivity of standard biomonitoring organisms before decisions can be made based upon MTX response to freshwater effluents. There is no way of knowing how the addition of ionic substances are going to affect the toxicity of chemicals present in the effluent. Therefore, we do not recommend that MTX be considered as a substitute for the standard organismal bioassays. We do recommend that it be used as a complement to the standard bioassays or for screening when further work is hindered by time or sample numbers (as in Toxicity Reduction Evaluation (TRE) work).

We recommend that future MTX NOEC work concentrate on defining tight dilution schemes for all organisms. Software programs should be used independently of each other unless Microbics develops software for analysis of EC50 and NOEC jointly.

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APPENDICES

APPENDIX A

MICROTOX DATA COLLECTION SHEET

MICROTOX DATA SHEET

Microtox File Name__________
Toxstat File Name__________

Company____________________
Sample #____________________
Replications________________

Sample I.D.____________________
Date Sample Received____________
Date Sample Run________________

PARAMETERS:
Number of Dilutions:_______
Initial Concentration:_____
Dilution Factor:___________

Units:_________________________
Ionic Adjustment:______________
Procedure:_____________________

It_______

Dil.	Blank							
Rep.	1	2	3	4	5	6	7	8
A								
B								
C								
D								

It_______

Dil.	Blank							
Rep.	1	2	3	4	5	6	7	8
A								
B								
C								
D								

It_______

Dil.	Blank							
Rep.	1	2	3	4	5	6	7	8
A								
B								
C								
D								

APPENDIX B

ACUTE ORGANISMAL RESPONSE TO COMPLEX EFFLUENT SAMPLES MONITORED FROM NOVEMBER 1990 TO OCTOBER 1991

Type	ID #	ID #	48hrLC50 FS	48hrLC50 CS	24hrLC50 FS	24hrLC50 CS
R	90131		>100	>100	>100	>100
N	90135		86	>100	>100	>100
R	90139		>100	>100	>100	>100
E	90140		>100	>100	>100	>100
N	90141		>100	>100	>100	>100
R	90142		35	55	64	58
R	91006		>100	>100	>100	>100
R	91007		32	<10	34	16
M	91012		>100	>100	>100	>100
M	91013		>100	>100	>100	>100
M	91014		>100	>100	>100	>100
R	91016		>100	>100	>100	>100
R	91017		>100	>100	>100	>100
R	91020		56	57	74	>100
R	91021		>100	>100	>100	>100
M	91023		>100	>100	>100	>100
M	91024		>100	>100	>100	>100
M	91025		>100	82	>100	>100
R	91026		>100	>100	>100	>100
R	91027		86	>100	>100	>100
R	91028		>100	>100	>100	>100
R	91030		66	56	76	56
R	91031		>100	82	>100	82
M	91032		>100	75	>100	92
M	91035		>100	>100	>100	>100
M	91036		>100	>100	>100	>100
M	91037		>100	>100	>100	>100
R	91038		40	80	54	>100
R	91039		>100	74	>100	>100
R	91040		94	35	>100	35
R	91042		76	70	80	70
R	91044		>100	>100	>100	>100
R	91048		54	62	60	62
R	91049		60	>100	62	>100
R	91054		>100	>100	>100	>100
E	91055		>100	>100	>100	>100
R	91057		>100	>100	>100	>100
E	91058		>100	>100	>100	>100
M	91059		>100	>100	>100	>100
R	91061		76	86	94	94
M	91063		>100	>100	>100	>100
R	91064		58	>100	64	>100
M	91065		>100	84	>100	100
R	91067		>100	>100	>100	>100
R	91073		>100	90	>100	>100
R	91074		>100	>100	>100	>100
R	91075		93	82	>100	>100
R	91077		>100	82	>100	>100
M	91079		>100	>100	>100	>100
R	91082		95	>100	>100	>100
R	91086		80	60	86	70
R	91089		>100	>100	>100	>100
R	91090		>100	80	>100	80

R=Refinery, M=Municipality, E=Electric generating facility cooling tower, N=industry, I=insufficient data.

APPENDIX C

PEARSON'S R VALUES FOR CORRELATIONS* OF MICROTOX ENDPOINTS VERSUS 7-DAY PARAMETERS USING SAMPLES WITHOUT DILUTION FACTOR OF 2 AND NOEC OF 45%

7-DAY PARAMETER	Subsample	MTX EC50	MTX NOEC
P. promelas survival	1	0.504	0.789
	2	0.500	0.799
	3	#	0.715
P. promelas growth	1	0.521	0.781
	2	0.504	0.795
	3	#	0.692
C. dubia survival	1	0.354	#
	2	#	#
	3	#	#
C. dubia reproduction	1	0.349	0.334
	2	0.341	0.317
	3	0.356	0.436

#No correlation found

*All correlation coefficients are significant ($p \leq 0.05$)

APPENDIX D

PEARSON'S R VALUES FOR CORRELATIONS* OF MICROTOX NOEC VERSUS *P. promelas* PARAMETERS THAT AGREE TO PRESENCE/ABSCENCE OF TOXICITY

7-DAY PARAMETER	Subsample	MTX NOEC
P. promelas survival	1	0.728
	2	0.835
	3	0.718
P. promelas growth	1	0.732
	2	0.841
	3	0.732

#No correlation found

*All correlation coefficients are significant ($p \leq 0.05$)

APPENDIX E

PEARSON'S R VALUES FOR CORRELATIONS* OF MICROTOX NOEC VERSUS *C. dubia* PARAMETERS THAT AGREE TO PRESENCE/ABSCENCE OF TOXICITY

7-DAY PARAMETER	Subsample	MTX NOEC
C. dubia survival	1	#
	2	0.364
	3	0.444
C. dubia reproduction	1	0.501
	2	0.542
	3	0.614

#No correlation found

*All correlation coefficients are significant ($p \leq 0.05$)

APPENDIX F

PEARSON'S R VALUES FOR CORRELATIONS* OF MICROTOX ENDPOINTS VERSUS 7-DAY PARAMETERS THAT AGREE TO PRESENCE/ ABSCENCE OF TOXICITY

7-DAY PARAMETER	Subsample	MTX EC50	MTX NOEC
P. promelas survival	1	0.458	0.695
	2	0.440	0.762
	3	#	0.602
P. promelas growth	1	0.479	0.707
	2	0.454	0.774
	3	#	0.608
C. dubia survival	1	0.370	#
	2	#	0.309
	3	#	0.353
C. dubia reproduction	1	0.368	0.446
	2	0.360	0.448
	3	0.376	0.519

#No correlation found

*All correlation coefficients are significant ($p \leq 0.05$)

VITA

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