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An Assessment of Microtox™ as a Biomonitoring Tool for Whole Effluent Testing for Los Alamos National Laboratory

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INTRODUCTION

The United States Environmental Protection Agency (USEPA) policy statement (Federal Register 49, 9016, March 9, 1984) for the development of water quality-based permit limitations includes toxicity testing of effluents as an important part of a water quality approach to controlling toxins (Pickering, 1988). The National Pollution Discharge Elimination System (NPDES) permit program was implemented by the USEPA to regulate the discharge toxics as well other water quality parameters into the nation's waters. In addition to regular testing of parameters such as five day biochemical oxygen demand (BOD_5), pH, and total suspended solids (TSS); it was proposed that more stringent testing be included that would test for all contaminants on the USEPA priority pollutant list. Besides the obvious high cost of such testing, other short comings include (Federal Register 49, 9016, March 9, 1984):

- the great number of toxic chemicals that may potentially be discharged to receiving waters and the difficulties in their analysis,
- the changes in the toxic effects of a chemical resulting from reactions with the matrix of constituents in which it exists, and
- the inability to predict the effects of exposure to combinations of chemicals.

To overcome some of these problems, the USEPA introduced the concept of whole effluent toxicity (WET) as an approach to measure the toxic impact of an effluent discharge on specified biota in receiving water. In conjunction with WET testing, basic parameters such as pH, ammonia, alkalinity, BOD_5, turbidity, coliform count, copper and chlorine would still need to be monitored. Basically, WET uses specific representative aquatic species to evaluate the potential toxicity of a given effluent sample. Four short-term tests which have been developed by the USEPA to provide the most favorable cost-benefit relationship and meet the NPDES permit

requirements (USEPA, March 1989). Two of these tests involve using fathead minnows (*Pimephales promelas*), one uses *Ceriodaphnia dubia* water fleas, and the final one uses green algae (*Selenastrum capricornutum*). Field studies were conducted to determine the validity of these test methods in predicting adverse ecological impacts of toxic discharges on various rivers throughout the United States (USEPA, March 1989).

Each of the four tests has an established standard operating procedure which must be followed. The regulated test conditions cover (EPRI, January, 1989): the test organisms and their age (participating laboratories either use larvae produced in-house, larvae from eggs produced by a commercial supplier, or larvae from eggs produced by EPA-Newtown); the food and feeding of the test organisms; the size and construction of the test chambers; test solution volume; test materials (reference toxicants or effluents); control conditions; dilution water; number of replicates per test concentration; aeration; lighting and photo period; test temperature; water quality parameters to be measured; toxicant or effluent renewal frequency; test duration; and experimental endpoints. The endpoints reported are the No Observed Effect Concentration (NOEC), which is the highest concentration tested that shows no observable adverse effects on the test organism, and the Lowest Observed Effect Concentration (LOEC) which is the lowest concentration that causes adverse effects on the organism.

Fathead Minnow Tests (*Pimephales promelas*): There are two fathead minnow tests regulated by the USEPA:

- The seven-day, sub-chronic, fathead minnow (*Pimephales promelas*), static renewal, larval survival and growth test.
- The seven-day, sub-chronic, fathead minnow (*Pimephales promelas*), static renewal, embryo-larval survival and teratogenicity test.

The larval survival and growth test involves exposing the fathead fry (less than 24 hours old) to different concentrations of effluent or receiving water in a static renewal system for seven days (USEPA , March 1989). At the end of the test period the fish are sacrificed and weighed. The survival and growth (based on mean dry weight) of the larvae are the parameters used to determine the NOEC and the LOEC. The embryo-larval survival and teratogenicity test uses fertilized eggs and larvae as the test organisms. The test parameters observed are mortality and gross morphological deformities (terata) (USEPA, March 1989).

Water Flea Test *(Ceriodaphnia Dubia)*: The EPA test using *Ceriodaphnia dubia* is called the three-brood, seven-day, chronic, cladoceran, static renewal, survival and reproduction test. This test involves exposing *Ceriodaphnia dubia* to different concentrations of effluent, or receiving water in a static renewal system until 60% of surviving control organisms have three broods of offspring, which occurs with in seven days (USEPA, March 1989). The LOEC and NOEC are based on survival and reproduction for each of the various concentrations used.

Algae-Based Test *(Selenastrum Capricornutum)*: This method is called the four-day, chronic algal, (*Selenastrum Capricornutum*), static, growth test. A *Selenastrum* population is exposed to a series of concentrations of effluent or to receiving water in a static system for 96 hours. The response of the population is measured in terms of changes in cell density (cell counts per ml), biomass, chlorophyll content, or absorbance (USEPA, March 1989).

Typically, the seven-day, sub-chronic, fathead minnow, static renewal, larval survival and growth test, and the three-brood, seven-day, chronic, cladoceran, static renewal, survival and reproduction test is run on discharged effluents to meet the NPDES permit requirements. These tests can be expensive and time consuming; usually results of toxicity screening analyses are not obtained less than three to four days.

Los Alamos National Laboratory (LANL) has special discharge problems relating to potential radioactive content of the effluent discharge waters (Zachritz, June 29, 1992). Because of this, all testing must be performed on-site and results must be rapidly determined. There is a need to examine the development of a real-time procedure for effluent biomonitoring to meet these site limitations. The Microtox™ unit for toxicity testing is a microbially-based test system that shows great promise to be used for WET testing. The overall goal of this study is to develop an acceptable protocol for operational biomonitoring using the Microtox™ toxicity test for LANL. The specific objectives include:

- Development of an appropriate toxicity testing protocol using the Microtox™™ toxicity test for whole effluent toxicity testing, and
- Evaluate the protocol based on factors such as sensitivity, response time, cost of analysis, and simplicity of operation.

MICROBIAL TOXICITY TESTS

A number of different organisms have been used for toxicity testing including algae, higher plants (duckweeds), yeast, bacteria, and fungi (Walker, 1990). These tests have been applied to sediment, water, wastewater, and soil samples. The precedence for using microorganisms for toxicity testing is well established (Walker, 1990) and a number of methods are available. A review of methods for toxicity testing indicates that there are many applicable methods available including:

- Microtox™, based on photoluminescence using *Photobacterium phosphoreum*, (Microbics, 1988),
- Motility inhibition based on *Spirilum volutans* (Goatcher et al., 1984),
- Tetrazolium-based activity (dehydrogenase assay) using mixed cultures (Logue et al., 1989),
- Oxidation of target substrates using *Nitrobacter* (Williamson and Johnson, 1980),
- Growth rate measurements using *Psuedomonas fluorescens* (Dutka and Kwan, 1983), and mixed cultures (Alsop et al., 1980; Adam and Kott, 1989),
- Radiolabeled C^{14} uptake using mixed cultures (Larson and Schaeffer, 1982),
- Respiration measurements using mixed cultures (Organization of European Community Development (OECD), 1987), and
- Respiration measurements using nitrifiers (Alleman, 1986; Arbucklye and Alleman, 1991).

Response time for these methods varies from about 5 to 30 minutes to over 12 hours. These tests have all shown a certain degree of sensitivity to contaminants and in most cases, results can be obtained more rapidly and easier than with the *Ceriodaphnia* or fathead minnow tests.

Comparison of test methods for toxicity characterization of wastewater effluents indicated that Microtox™ and *Mysidopsis bahia* were less sensitive than

Ceriodaphnia dubia (Goodfellow et al., 1989). Other studies by Arbuckle and Alleman (1992) comparing conventional *Ceriodaphnia dubia* testing with Microtox™ and an enriched nitrifier culture, indicated that the bacterial test were much easier to perform, but that neither bacterial-based test was consistently sensitive enough to detect acute *Ceriodaphnia* toxicity of treated municipal wastewaters. Interestingly, the 7-day chronic *Ceriodaphnia* method appeared more sensitive than the 48-hour *Ceriodaphnia* test. An earlier study by Blum and Speece (1991) examining the sensitivity of three bacterial groups (*Nitrosomonas*, aerobic heterotrophs, and methanogens), Microtox™, and fathead minnows to specific organic compounds, indicated that *Nitrosomonas*, Microtox™, and fathead minnows exhibited the same degree of sensitivity. These sensitivities were significantly greater than the other two groups of microorganisms tested. These findings do not necessarily limit the use of bacterial-based tests as an operational tool, but do indicate that in many cases bacterial tests have not been shown to be as consistently sensitive as the prescribed USEPA methods.

The Microtox™ Model 500 analyzer, marketed by Microbics Corporation of Carlsbad, California, is a toxicity test which involves using lyophilized (freeze dried) luminescent bacteria (*Photobacterium phosphoreum*). The instrument has 30 well incubators, a reagent well, and a measuring chamber which are all temperature controlled at 15°C. At this carefully selected temperature, the reagent is sensitive to the widest spectrum of toxicants (Microbics Corp., 1992).

Bioluminescent bacteria have been used as assay organisms for studies on drugs, temperature and pressure, anesthetics, assessment of toxicity for air, industrial pollutants, and effluent discharges (Hinwood, et. al.). Three major strains have been identified: *Photobacterium fischeri*, *Photobacterium phosphoreum (P. phosphoreum)* and *Photobacterium harveyi* (Hinwood, et. al., Hastings and Nealson, 1977), all of which are marine organisms. The *P. phosphoreum* strain of

bioluminescent bacteria emits a blue-green color of light. The light emission is the result of a reaction catalyzed by luciferase which involves the luminescent oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain aliphatic aldehyde (probably tetradecanal) by molecular oxygen, which is expressed as (Krieg, 1984):

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{Luciferase}} h\nu + FMN + RCOOH$$

Due to the fact that this reaction is linked to the electron transport system it is indicative of the bacteria's state of health and can be used to measure acute toxic responses.

MATERIALS AND METHODS

Microtox™ Basic Test Protocol: Tests were conducted using the Microtox™ Model 500 toxicity analyzer, lyophilized luminescent bacteria, and the microtox reconstitution solution (distilled water), all obtained from Microbics Corporation. The 2% NaCl microtox diluent and 22% NaCl microtox osmotic adjustment solution (MOAS) were either purchased from Microbics, or prepared on-site using distilled water followed by autoclaving. The Microtox™ protocol for the basic test was followed (Microbics Corp. 1992), which consisted of one control group and four serial dilutions. All assays were conducted at 15°C and the EC50's for 5, 15, and 30 minute contact periods were recorded.

Microtox™ Sucrose Protocol. Tests were conducted using sucrose in place of the NaCl solutions. Test procedures were followed as described by Microtox™ Information leaflet, M-127 Sucrose-Based Microtox™ Assay Procedure, which is identical to the basic test procedure except that all salt solutions are replaced with sucrose. Similar to the basic test protocol, all assays were conducted at 15°C and the EC50's for 5, 15, and 30 minute contact periods were recorded.

Microtox™ Data Analysis. All statistical analysis to determine the EC50 was performed by the Microbics Corporation data reduction software. The analysis involved converting the results into terms of gamma which is the ratio of the light lost at time t to the light remaining at time t for a given sample concentration:

$$G_t = (R_t I_o - I_t)/I_t = (R_t I_o)/I_t - 1$$

where G_t is gamma at time t, R_t is a correction factor obtained by dividing the light output of the control (blank) at time t by the initial light output of the control, I_o is the initial light output before sample is added, and I_t is the light output at time t for

a given sample. A statistical regression can then be run based on the rate theory that biological inhibition predicts a simple mathematical relationship between the concentration of a toxicant and the response of a susceptible organism when the response is measured in terms of gamma values (Microbics Corp., 1992). This can be expressed as:

$$\log C = b \log G + \log a$$

where C is the concentration, gamma is the light reduction value (or light increase value if negative), b is the slope, and $\log a$ is the intercept.

The EC50 is the sample concentration that reduces the reagent light output by 50%. This value occurs at the point where gamma equals 1.0 on the dose-response curve. Once the EC50 value has been determined standard statistics are used to determine the 95% confidence interval.

Biomonitoring Split Samples. Split samples were obtained from the Las Cruces Wastewater Treatment Plant (LCWWTP) which the city collects as part of their NPDES biomonitoring requirements. Once a month over a one week period three composite samples were collected over a 24-hour period. An ISCO sampler was used to collect an aliquot of effluent every hour in a polypropylene bottle and was stored at 4°C. Collection for all three samples began at 1:30 pm on the Sunday, Tuesday, and Thursday of the sample week and continued until 12:30 pm the following day. A one gallon flow-weighted composite sample was then compiled for each collection period. This sample was then split and a portion was shipped to the biomonitoring lab via Federal-Express that evening (at 6:00 pm) in a cooler with blue ice. The other portion was transported to the NMSU laboratories for Microtox analysis. All procedures were performed as defined by USEPA sampling and analysis methodology (Keith, Mueller, and Smith,1992).

Biomonitoring Laboratory Protocol. The seven-day, sub-chronic, fathead minnow, static renewal, larval survival and growth test (See Appendix I), and the three-brood, seven-day, chronic, cladoceran, static renewal, survival and reproduction test (See Appendix II) were the NPDES compliance tests conducted. The second and third composite samples are used for renewal during the seven day tests. All samples were required to be analyzed within 36 hours of collecting the last aliquot.

The results wee then analyzed by a battery of statistical tests such as: the Shapiro-Wilk's test, the Bartlett's test, the Dunnett's multiple comparison test, the Chi-Square test, and the Bonferroni T-test. From this data analysis the NOEC and LOEC were determined and the city was sent a monthly report of these results, as well as information on other parameters of the effluent such as alkalinity, hardness, pH, conductivity, total residual chlorine, and ammonia content of the samples.

Target Toxin Testing. The LCWWTP NPDES permit stipulates that if biomonitoring studies indicate chronic lethality in 95% effluent, then a series of studies called Toxicity Reduction Evaluations (TREs) are required (ENSR, Jan. 13, 1993). A TRE is a series of studies designed to identify and control sources of effluent toxicity such that permit limitations can be met (ENSR, Jan. 13, 1993). The 95% effluent concentration was based on a calculation of the theoretical concentration of effluent in the Rio Grande during low flow conditions (ENSR, 1993).

Based on the results from these TREs it was determined that ammonia and diazinon are the most problematic toxins found in the wastewater effluent. There is no concentration limit set for these toxins, they must only meet the required effluent biomonitoring standards as stipulated in the NPDES permit. In addition to ammonia and diazinon, copper was also selected as a target chemical, because it was found to exceed the NPDES permit level of 0.026 mg/L per day, or a 0.021 mg/L

monthly average. Target chemicals were tested individually and in pairs at known concentrations using both the Microtox™ basic procedure and the sucrose-based assay.

Ammonia. Ammonia is a common problematic toxin in municipal wastewater treatment plants across the country. Some controversy in the regulation of ammonia has developed because ammonia is part of the nitrogen cycle and to some extent occurs naturally in water bodies due to animal excretion, fixation of atmospheric nitrogen, and ammonification of organic nitrogen. No concentration limits have been set by the USEPA for ammonia and it is only regulated via biomonitoring (Garcia, 1994).

Ammonia can be found in the ionized (NH_4^+) and un-ionized (NH_3) form based on pH and temperature.

$$NH_3 + nH_2O \longrightarrow NH_4^+ + OH^- + (n\text{-}1)H_2O$$

The percent of un-ionized ammonia can be determined by the equation (Yake and James, 1983):

$$NH_3 = 100/[1 + \text{antilog}(pK_a - pH)]$$

where the pK_a for ammonia is 9.3. Ammonia can be used directly by green plants resulting in eutrophication or it can be oxidized to nitrites (NO_2) and nitrates (NO_3), a reaction which depletes the dissolved oxygen content in a body of water. In wastewater treatment plants breakpoint chlorination is commonly used to oxidize ammonia to nitrogen gas:

$$3Cl_2 + 2NH_4^+ \longrightarrow N_2 + 6HCl + 2H^+$$

This reaction produces an acid which must then be neutralized greatly increasing the total dissolved solids in the effluent (USEPA, 1975).

All tests were run using ammonium sulfate ($(NH_4)_2SO_4$) expressed as total ammonia at a pH between 6.5 and 7.5. At this pH NH_4^+ is the predominant form; only at pH's above 9.3 does NH_3 become predominant.

Diazinon. Diazinon, an organophosphorous compound, is a commercial pesticide used to control flies, fleas, cockroaches, lice, and insect pests of ornamental plants and food crops. Although diazinon can be found in many forms, it is typically found as an amber-to brown-colored liquid with a sweet odor. Its molecular formula and molecular weight are $C_{12}H_{21}N_2O_3PS$ and 304.3. It has a boiling point of 83°C at 0.002 mm Hg, a vapor pressure of 1.4×10^{-4} and a density of 1.12 g/ml at 20°C. Diazinon degrades rapidly in plants, with half-time persistence usually less than 14 days; however, persistence increases as temperatures and moisture decrease, and alkalinity increases (Eisler, 1986). After being converted to its oxygen analog diazoxon, diazinon exerts its toxicity by binding to the neuronal enzyme acetylcholinesterase, resulting in asphyxia caused by respiratory failure in animals.

The LCWWTP uses USEPA's Sampling and Analysis Method 8140 to determine the presence of diazinon in wastewater samples. Ortho-Diazinon PLUS™ (Chevron Chemical Company) insect spray (25% diazinon, 75% inert ingredients) was used when making up all test solutions and mixed with refrigerated distilled water to reduce its rate of degradation. All diazinon solutions were prepared daily.

Copper. Copper is a heavy metal found naturally in the groundwater at concentrations less than 1.0-3.0 ppb (Dragun, 1988). Copper sulfate is reported to be used by many people to control root infestation in sewer pipes. This may explain the presence of copper in wastewater effluents in many New Mexico communities.

It has a molecular weight of 63.6 with a pK_a value of 6.8. At pH's less than 6.5, copper is predominantly found as free copper (Cu^{2+}) and in the pH range of 6.5 to 9.5, the major copper containing species is $CuCO_3$ (Snoeyink and Jenkins, 1980). Alkalinity has a profound effect on free copper which explains the fact that soft water is more toxic to fish than hard water due to complexation occuring in hard water which reduces its toxicity (Snoeyink and Jenkins, 1980).

Tests were run using solutions of Cupric Sulfate ($CuSO_4 \cdot 5H_2O$) expressed as mg/L of copper at pH's between 6.5 and 7.5.

RESULTS AND DISCUSSION

Specific Toxin Testing. The three main problematic toxins found in LCWWTP's effluent (ammonia, copper, and diazinon) were tested individually and in pairs to determine the potential synergistic effects between the toxins. Results for the individual tests on copper, ammonia, and diazinon after 5, 15, and 30 minute contact times using both the Microtox™ basic protocol and the sucrose-based assay can be seen in Table 1. As the contact periods for the tests increase from 5 minutes to 30 minutes, the bioluminescent bacteria becomes about three times more sensitive to copper for both the basic and the sucrose-based assay. A slight decrease can be seen with diazinon, which may be due to the tendency of diazinon to break down rapidly in moisture (Eisler, 1986). Using the Microtox™ basic protocol, there was no significant difference in the results for the three contact periods for ammonia.

The sucrose-based test was found to make the bioluminescent microbes about 36 times more sensitive to ammonia, 2.6 times more sensitive to copper, and 1.6 times less sensitive to diazinon. Ankley, et al, (1990) also found that in comparison to the basic test protocol, the sucrose-based assay's sensitivity varied based on the toxicant. The percent difference between the EC50s for the basic test protocol and the sucrose-based protocol can be seen in Table 2. Hinwood, McCormick, and McCormick (undated), believe that the Microtox™'s insensitivity to ammonia is caused by changes in sodium chloride concentration that results in changes in the ionized (NH_4^+) and un-ionized (NH_3) ratio. The NH_3:NH_4^+ ratio is pH and temperature dependent, with the un-ionized fraction being the primary toxicant of the total ammonia content (Yake and James, 1983). It is postulated that the use of

Table 1. Ammonia, Copper, and Diazinon EC50s and 95% Confidence Intervals for Both the Microtox™ Basic Protocol and the Sucrose-Based Assay.

	TEST	TIME (min)	mg/L	
			EC50	95% C.I.
Ammonia	Basic	5	2,220	± 300
		15	1,760	± 180
		30	1,570	± 150
	Sucrose	5	54	± 13
		15	42	± 16
		30	62	± 39
Diazinon	Basic	5	0.226	± 0.024
		15	0.255	± 0.073
		30	0.285	± 0.034
	Sucrose	5	0.347	± 0.175
		15	0.384	± 0.213
		30	0.462	± 0.259
Copper	Basic	5	0.265	± 0.065
		15	0.167	± 0.047
		30	0.086	± 0.013
	Sucrose	5	0.107	± 0.046
		15	0.059	± 0.027
		30	0.034	± 0.019

sucrose allows freshwaters to be analyzed without detoxifying the sample while providing the osmotic protection needed by the bacteria (Hinwood, McCormick, and McCormick, undated).

While the sucrose-based assay increased the sensitivity of the Microtox™ procedures to all toxicants tested in this study, some concern was noted with the

Table 2. Percent Increase in Sensitivity Using the Microtox™ Sucrose-Based Assay Over the Microtox™ Basic Test Protocol.

	Time		
	5 Min	15 Min	30 Min
Ammonia	98	99	99
Diazinon	-35*	-34*	-38*
Copper	60	65	61

*The sucrose-based assay showed less sensitivity.

significant increase in the values for the resulting 95% confidence intervals (Table 3). The confidence interval for the basic test ranges from 10 to 28% of the EC50, while the sucrose-based assay ranged from 24% all the way up to 63% of the EC50. It may be that the bioluminescent bacteria are not in their naturally occurring saline habitat, thereby stressing the bacteria, which resulted in higher variance in the toxicants EC50s. That the sucrose osmotically adjusted control groups decreased their light output at a rapid rate in comparison to those groups adjusted with saline supports this theory. This may also explain the increased sensitivity to ammonia using sucrose: the bacteria were already stressed to a point that it takes very little toxicant to elicit a toxic response. Microtox™ shows a decreased sensitivity to organics that have poor solubility and to chemicals that do not penetrate the cell walls easily (E.V.S. Consultants, March 1989). It is also possible that sucrose somehow makes it easier for the ammonia and copper to penetrate the *P. phosphoreums* cell wall and more difficult for diazinon.

Table 3. The Confidence Interval Percentage of the EC50 for the Microtox™ Basic Test Protocol and Sucrose-Based Assay.

	Time (min)	Basic Test Protocol	Sucrose Based Assay
Ammonia	5	14	24
	15	10	38
	30	10	63
Diazinon	5	11	50
	15	19	56
	30	12	56
Copper	5	25	43
	15	28	46
	30	15	56

Table 4 compares the reported LD50 (lethal dose to 50% of the test organisms) values for *Daphnia Magna*, fathead minnows, and EC50s for the Microtox™ for ammonia, diazinon, and copper. Information on the fathead minnows LD50 for ammonia was unavailable; thus, the LD50 for the next most representative specie available, the rainbow trout, was reported. Due to the lack of information for LD50's for *Ceriodaphnia* for the three target toxicants, values for *Daphnia Magna* were reported. For the Microtox™ basic test protocol the bioluminescent bacteria EC50s were less sensitive than the *Daphnia Magna* for all three toxicants. When the sucrose-based Microtox™ assay was implemented, the bioluminescent bacteria EC50s were more sensitive to ammonia than the *Daphnia Magna*. When comparing these results to LD50s for fathead minnows, the Microtox EC50s were more sensitive to diazinon, just as sensitive to copper, and less sensitive to ammonia for both osmotic adjusters. These results support similar findings by other researchers (Microtox™ Info. M-116). Overall the Microtox™ toxicity analyzer

Table 4. Daphnia Magna and Various Fish LD50s, and Microtox™ EC50s for Ammonia, Copper, and Diazinon.

	Daphnia Magna	Fish	Microtox™ (mg/L) 5,15 and 30 min	
	LD50 (mg/L)	LD50 (mg/L)	Saline	Sucrose
Ammonia	129[a]	17[c,d]	2220 1760 1570	54 42 62
Diazinon	0.0013[b]	5.1 - 15[b,e]	0.226 0.225 0.285	0.347 0.384 0.462
Copper	0.01 - 0.06[a]	0.02 - 0.10[a,e]	0.265 0.167 0.009	0.107 0.059 0.034

[a] Munkittrick, Power, and Sergy, 1990
[b] Eisler, 1986
[c] Hinwood, McCormick, and McCormick, undated
[d] Rainbow Trout LD50
[e] Fathead Minnow LD50

typically correlates well to fathead minnows except for when the primary effluent toxicant is ammonia. The analyzer did not correlate as well to results obtained with *Ceriodaphnia* water fleas, especially for inorganics and pesticides (Munkittrick, Power, and Sergy, 1990).

Paired Toxin Testing. Toxicants can occur in almost any concentration or combination in wastewater discharges. The combinations can result in a greater (synergism) or lessor (antagonism) toxic effect depending on the interactions of the specific toxicants with the given water chemistry. The determination of these interactions becomes very complex and little work has been done to examine the effect of multiple toxicants because of the vast number of potential environmental interactions. A few specific toxicants have been repeatedly identified in municipal wastewater treatment plants using WET tests and TRE procedures. Three of these

toxicants were selected to be evaluated in a series of paired tests. These included ammonia, diazinon, and copper; nitrate was excluded because preliminary testing resulted in no toxicity at concentrations up to 17,300 mg/L.

Figures 1 through 6 show the combined concentrations required to produce an EC50 for various toxicant pairs at 5, 15, and 30 minute contact times. The line representing the theoretical simple additive effect (TSAE) of the two toxicants is based on an assumption of a linear relationship between the concentration of the two. If the data falls to the left of this line, the effects are synergistic; if it falls to the right, the effects are antagonistic. Linear regression analysis of the EC50 data for the toxicant pairs was used to determine if the effects of the combined toxicants were synergistic or antagonistic.

Figures 1 through 3 shows the results of tests conducted using the Microtox™ basic test protocol. The R^2 values are typically very low for the 5 minute readings and tend to increase with increased contact time. The TSAE of the toxicants and the linear regression run on the actual data points tend to converge for the toxicants paired with copper with increased contact time, and diverge for the ammonia and diazinon combination. For the thirty minute time plot, diazinon and copper combined toxicants have statistically significant R^2 values of 0.82 and the data points tend to lie on the left side of the TSAE line indicating a slight synergistic effect between the toxicants. Those toxicants paired with ammonia resulted data scattered to the left of the TSAE of the toxicants and show an antagonistic effect.

The results from the paired toxicant testing using sucrose as the osmotic adjuster are shown in Figures 4 through 6. The R^2 values for ammonia pairs are extremely low, ranging from 0.01 to 0.35. These tests showed no significant improvement over time and for these toxicant combinations there appeared to be no linear relationship between the EC50 data and the plot of simple additive effects

of toxicants. Some other relationship may be inherent to the data, but no further attempts to made to fit the data. The copper and diazinon combination did to show improvement over time with R^2 values progressing from 0.60 to 0.97. The linear regression of the data lies to the right of the TSAE of the toxicants and exhibits a strong antagonistic effect.

In comparison to the basic test protocol, these low R^2 values for the sucrose-based assay correspond to the excessive increase in the 95% confidence interval for the individual toxin testing when using sucrose. One explanation for the high variance in results may be due faulty technique. A single 10 µl pipet is used to transfer the Microtox™ reagent into the cuvettes containing the sucrose solution. With each transfer, sucrose is introduced into the reagent well. Sucrose may be toxic to the bioluminescent bacteria based on the rapid decrease in light output by the control organisms when sucrose is used as the osmotic adjuster rather than saline. Further tests need to be conducted using a repeat pipetter which would ensure that no sucrose contaminates the reagent well. Comparing these results to that of a 10 µl pipette would help determine if the error was due to the microorganisms toxic reaction to the sucrose or if it was due to human error.

Even with this high range of variability using the sucrose method, it can seen that the increase in sensitivity to ammonia and copper is so great that the method is still worth conducting in conjunction with the basic test protocol. As with the individual toxicant tests, the sensitivity to diazinon decreased using sucrose as the osmotic adjuster rather than saline.

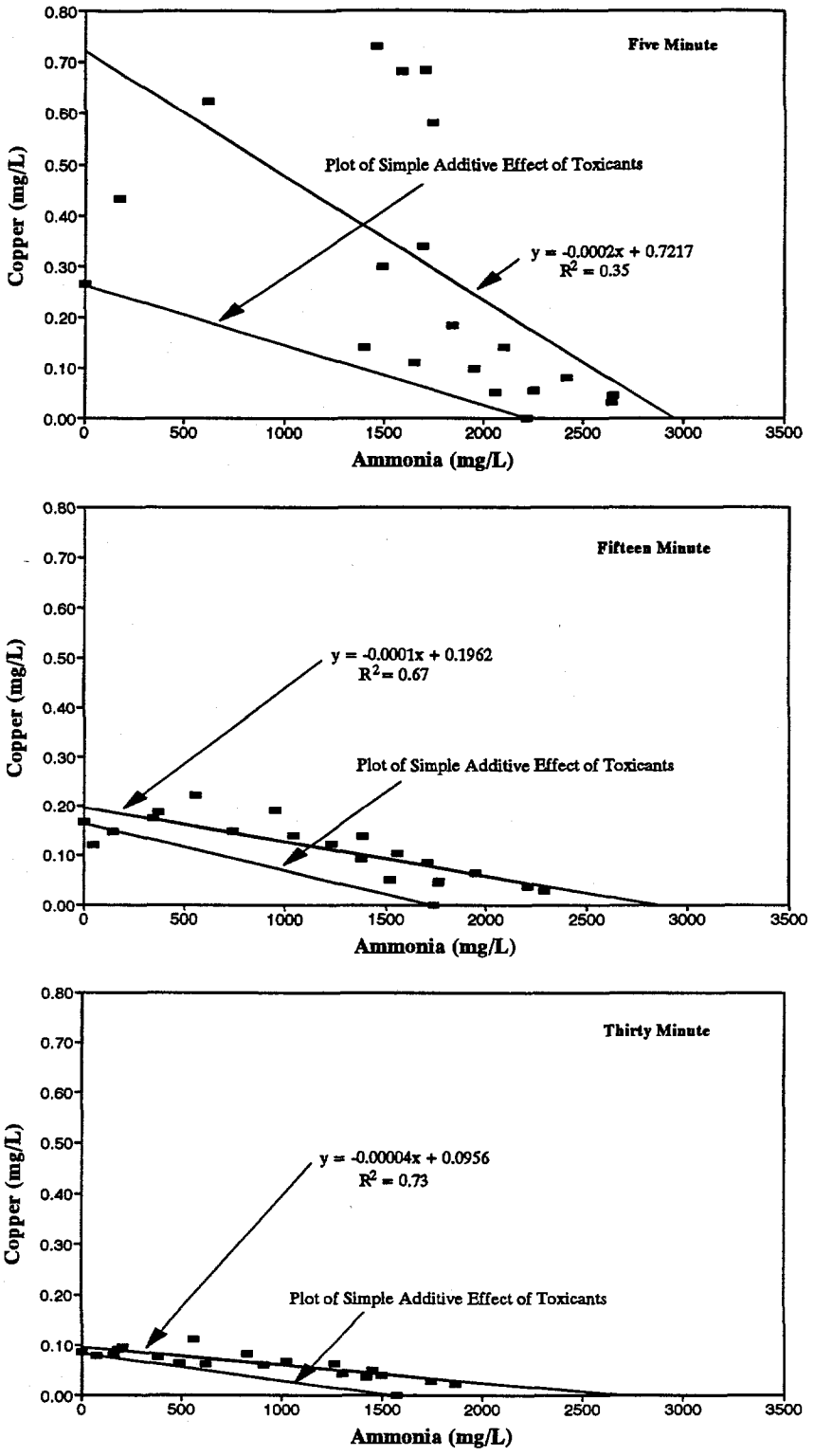

Figure 1. Five, Fifteen, and Thirty Minute EC50's for Ammonia and Copper Using the Microtox™ Basic Test Protocol.

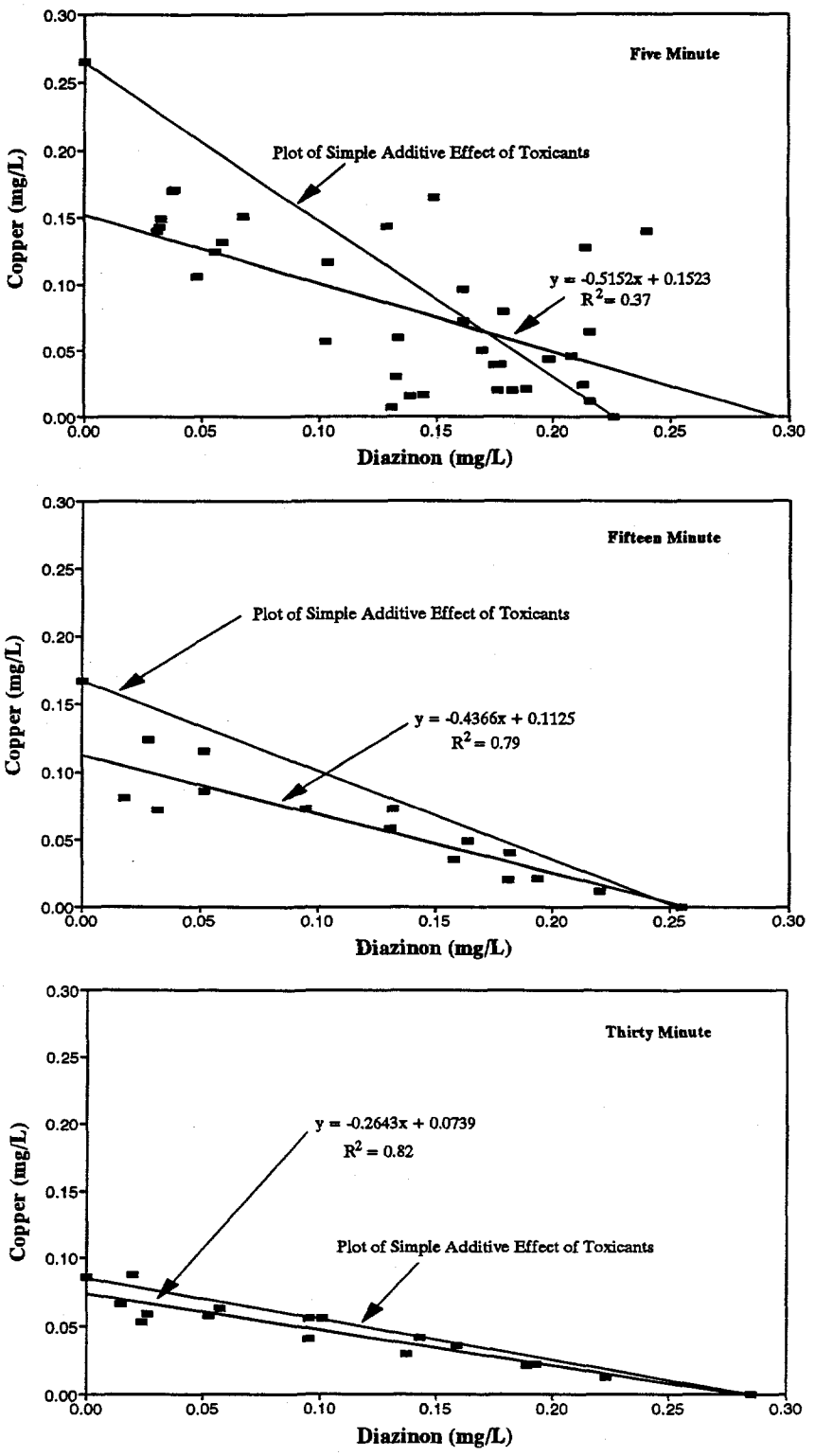

Figure 2. Five, Fifteen, and Thirty Minute EC50's for Diazinon and Copper Using the Microtox™ Basic Test Protocol.

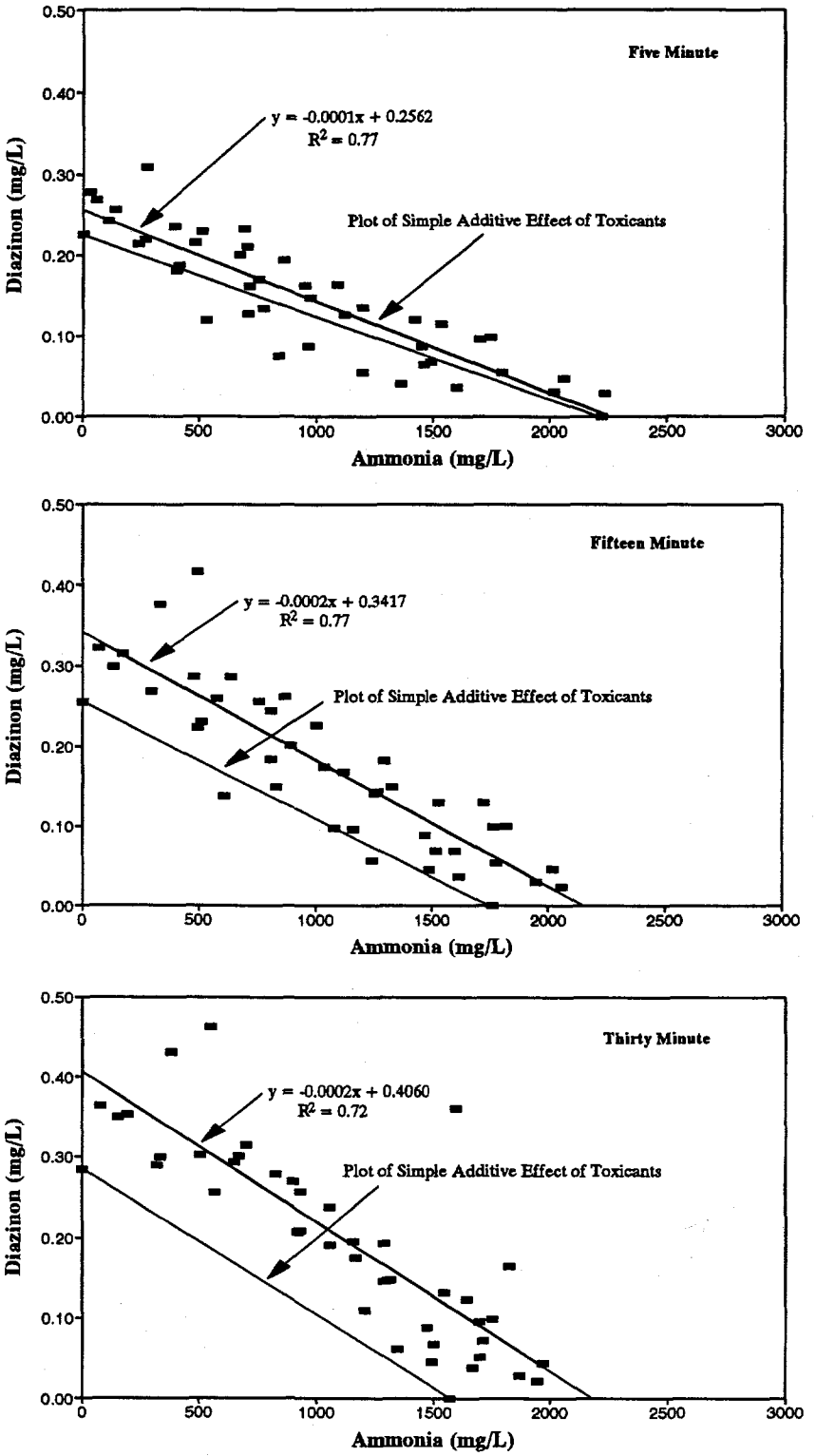

Figure 3. Five, Fifteen, and Thirty Minute EC50's for Ammonia and Diazinon Using the Microtox™ Basic Test Protocol.

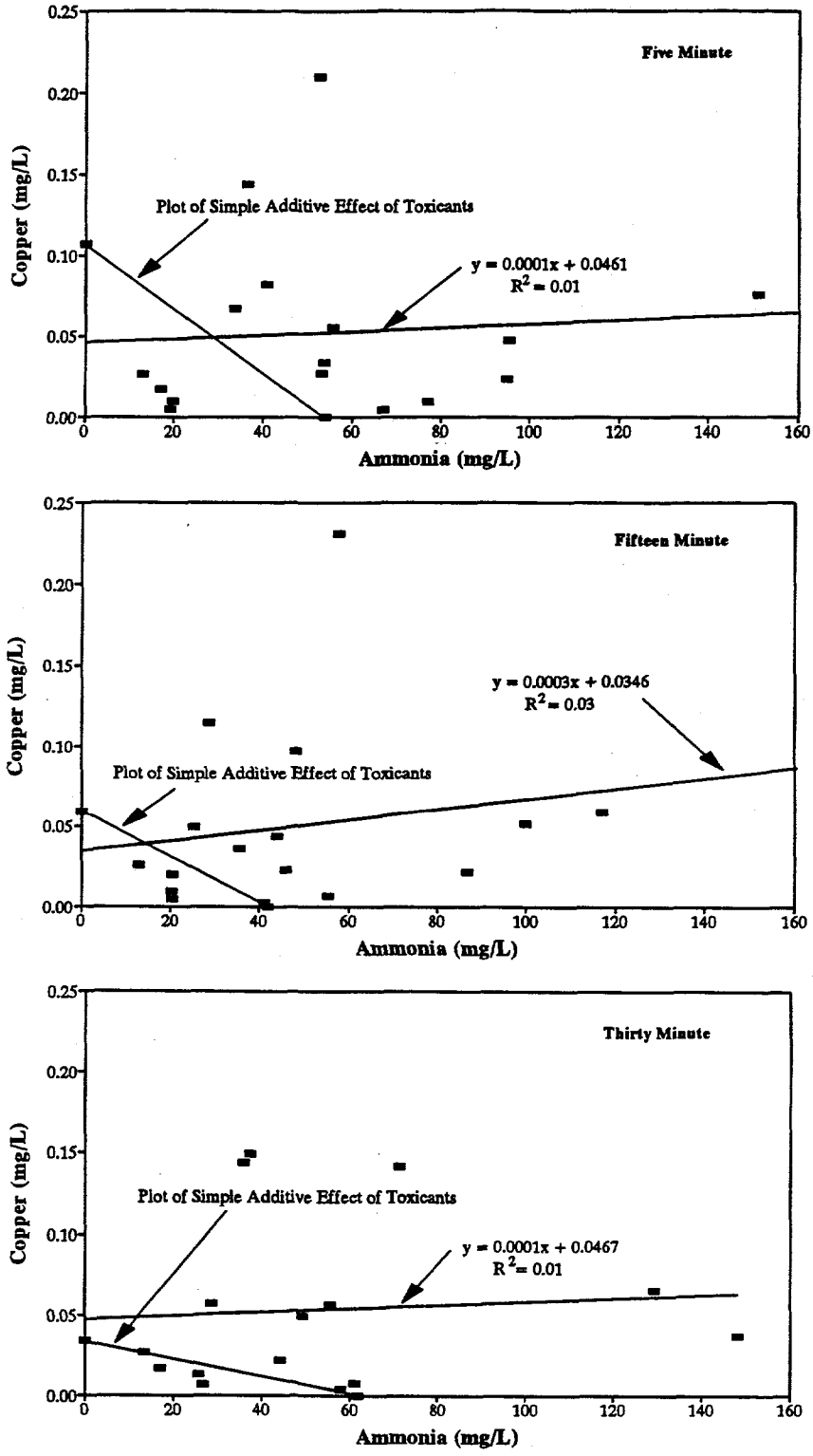

Figure 4. Five, Fifteen, and Thirty Minute EC50's for Ammonia and Copper Using the Microtox™ Sucrose-Based Assay.

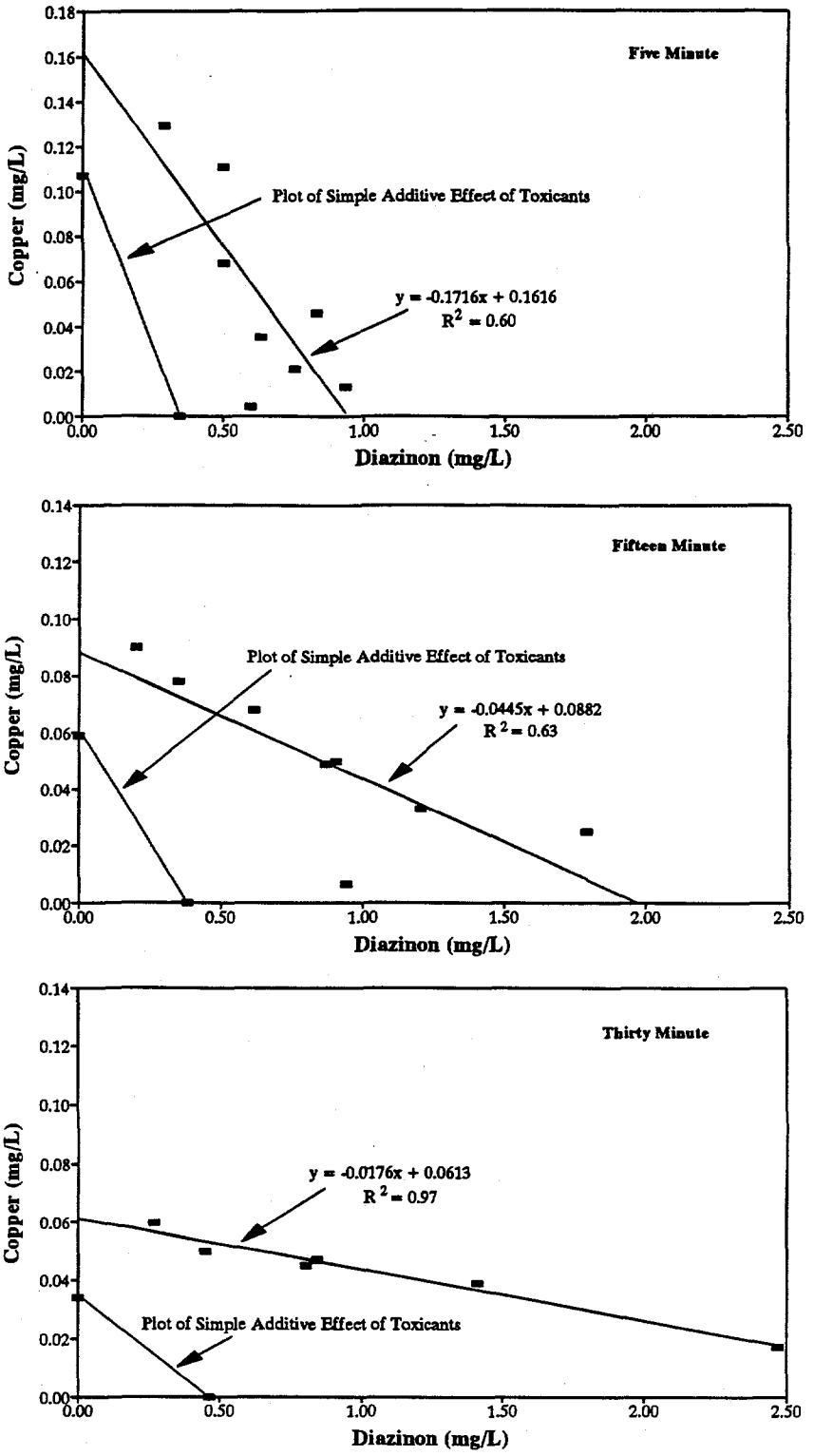

Figure 5. Five, Fifteen, and Thirty Minute EC50's for Copper and Diazinon Using the Microtox™ Sucrose-Based Assay.

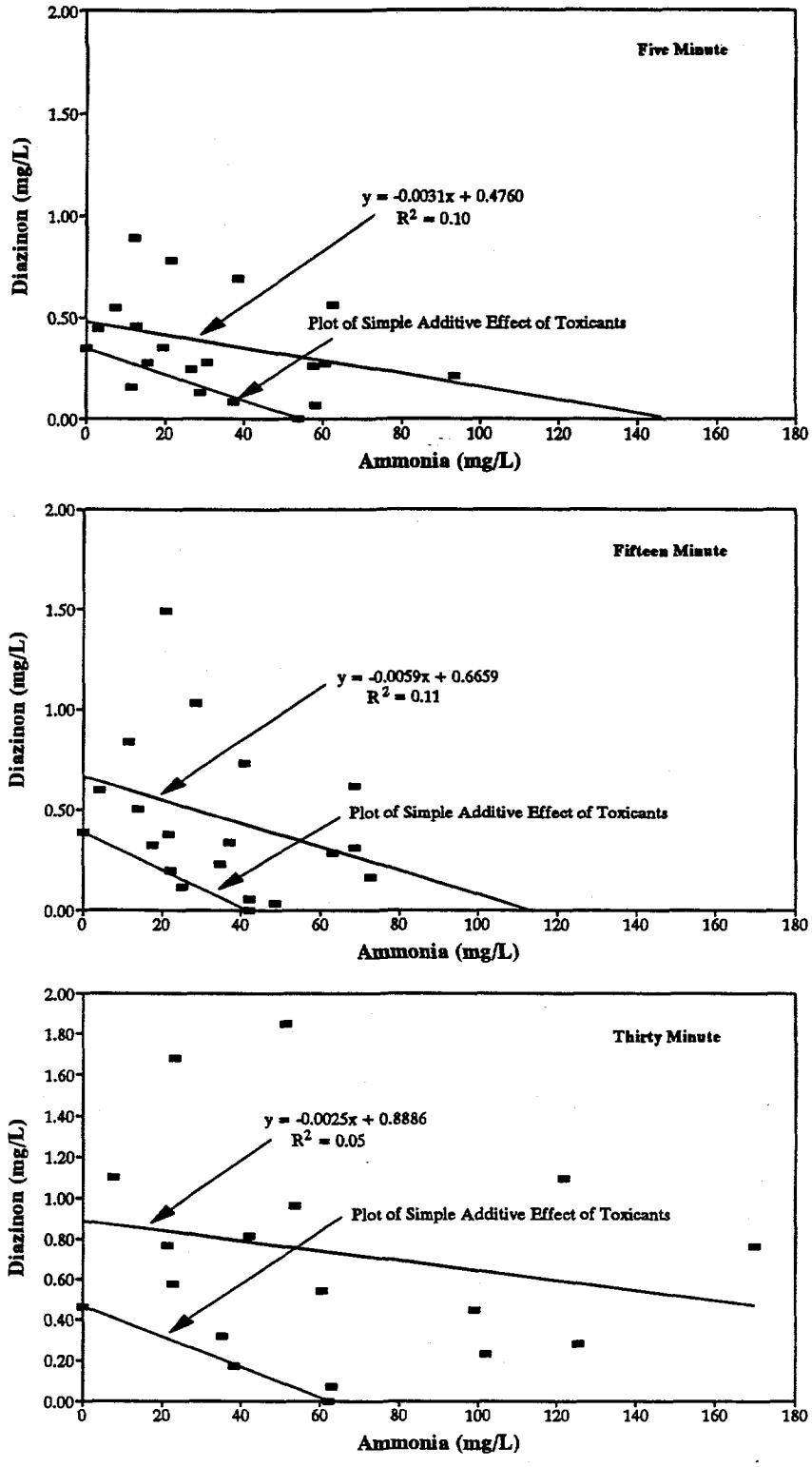

Figure 6. Five, Fifteen, and Thirty Minute EC50's for Ammonia and Diazinon Using the Microtox™ Sucrose-Based Assay.

Biomonitoring Split Samples. The Microtox™ toxicity analyzer never showed any toxic response to any of the LCWWTP biomonitoring samples for either the basic test protocol or the sucrose-based assay. In fact, there was typically an increase in light output by the bacteria. Table 5 shows the results from the biomonitoring laboratory for effluent split samples from January through December of 1993, as well as the pH, and ammonia and diazinon content of the three samples taken each month. A toxic response by the *Ceriodaphnia* was observed for the months of January and March when relatively high concentrations of diazinon were found in the effluent. The fathead minnows show toxic response to the January and May effluent samples which have a very high ammonia content. The toxic response to the August samples by *Ceriodaphnia,* and September samples by the fathead minnows is probably due to the presence of another undetected toxicant since the levels of diazinon and ammonia were low during these months.

These responses correspond well to the LD50 data (Table 4) that indicates that *Ceriodaphnia* are very sensitive to low concentrations of diazinon and fathead minnows are sensitive to low concentrations of ammonia. All toxicant concentrations found in the wastewater effluent were much lower than any of the EC50s found for the bioluminescent bacteria for either osmotically adjusted assays.

A problem in comparing the Microtox™ results to the biomonitoring data is that the Microtox™ uses point estimation methods to determine toxicity of the effluent, and the biomonitoring data is based on statistical hypothesis testing methods. Point estimations are based on effect concentrations (ECs or LDs) and use linear interpretations of the biological response of the organism. Hypothesis testing (NOEC's and LOEC's) conversely, has a magnitude of statistically distinguishable adverse effects which are greatly influenced by the design of the toxicity test and the natural variability of responses between organisms exposed to the same test conditions (Berger and Ellgas, 1992).

Table 5. Las Cruces Wastewater Treatment Plant Biomonitoring Split Sample Data.

Month & Sample Data	pH	Ammonia mg/L	Diazinon µg/L	% Effluent							
				Ceriodaphnia				Fathead Minnow			
				Survival		Reproduction		Survival		Growth	
				LOEC	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC	NOEC
1-12-93	7.6	19.2	0.08								
1-14-93	7.9	15.8	0.07	48	24	48	24	95	48	6	6
1-16-93	7.8	21.2	0.31								
2-23-93	7.5	7.5	0.07								
2-25-93	7.6	5.6	0.06	100	95	100	95	100	95	100	95
2-27-93	7.6	6.0	0.06								
3-09-93	7.4	5.2	0.08								
3-11-93	8.4	6.7	0.10	24	12	6	6	100	95	100	95
3-13-93	7.8	6.0	0.81								
4-15-93	NA	5.9	0.10								
4-17-93	NA	6.3	0.05	95	48	95	48	100	95	100	95
4-21-93	NA	7.0	0.09								
5-18-93	7.8	16.4	0.11								
5-20-93	7.8	15.8	0.06	100	95	48	24	100	95	95	48
5-22-93	7.8	9.5	0.10								
6-14-93	7.4	3.4	0.08								
6-16-93	7.8	2.3	0.14	95	48	95	48	100	95	100	95
6-18-93	7.7	1.6	0.09								
7-27-93	7.8	6.1	0.12								
7-29-93	7.8	5.7	0.13	95	48	95	48	100	95	100	95
7-31-93	7.7	5.1	0.11								
8-17-93	7.4	3.5	0.12								
8-19-93	7.7	4.7	0.08	100	95	48	24	100	95	100	95
8-21-93	7.7	4.7	0.06								
9-14-93	7.6	6.3	0.24								
9-16-93	7.6	7.0	0.07	100	95	95	48	100	95	6	6
9-18-93	7.6	5.6	0.07								
10-19-93	7.7	2.4	0.08								
10-21-93	8.1	4.3	0.11	95	48	95	48	100	95	100	95
10-23-93	7.7	2.4	0.16								
11-16-93	7.6	5.1	0.04								
11-18-93	7.7	3.5	----	100	95	95	48	100	95	100	95
11-20-93	7.6	3.3	0.03								
12-6-93	7.6	9.4	0.10								
12-8-93	7.8	8.8	0.06	100	95	95	48	100	95	95	48
12-10-93	7.6	7.9	----								

Some of the advantages of using point estimation techniques over traditional hypothesis testing methods include (Berger and Ellgas, 1992):

- They provide an explicit, unambiguous biological criterion for evaluating toxicity test results and determining compliance with permit toxicity limits.
- Conclusions are less affected by experimental design and the natural variability of biological responses, and more accurately reflect the inherent toxicity of the effluent that is being tested.
- The inherent precision of the toxicity test and a laboratory's ability to perform the test can be calculated.

Although some of the toxicity reported by the biomonitoring lab may be due to hypothesis testing defects, performing hypothesis tests on the Microtox™ results would not improve the correlation between the procedures due to the fact that the light output from the bacteria increased consistently for all tests conducted with the biomonitoring split samples.

SUMMARY

The results of this study indicate that a Microtox™-based protocol using a combination of the sucrose-based and standard assays can be effective for detecting the individual target toxicants examined in this report. The sucrose-based assay was found to dramatically increase the toxicity tests sensitivity to copper and ammonia, reduce toxicity sensitivity to diazinon, but resulted in an overall increase in statistical variation of the data. This increase could be an anomaly of the actual procedure or could be improved with modifications in laboratory technique. Further testing in this area to pinpoint this source of variation in the sucrose-based tests is recommended.

The results of the whole effluent testing (WET) comparisons indicated that the conventional biomonitoring species were still more sensitive to toxicants such as ammonia than Microtox™-based procedures. In twelve months of comparison testing of Las Cruces, New Mexico wastewater discharges, conventional biomonitoring detected several toxic events while the Microtox™ procedures failed to detect these events. These results are predictable because the concentration of toxicants observed for these occurrences were well below the detection levels determined for the individual toxicants on the Microtox™ system. The direct substitution of Microtox™ procedures for conventional biomonitoring in many municipal treatment plants does not appear likely unless refinements to the concept of whole effluent testing can be determined.

To develop a protocol for biomonitoring wastewater discharges from the LANL facility, a number of issues would need to be addressed. Because of the unique characteristics of the LANL effluent, a similar study to the one done with the Las Cruces wastewater treatment plant would have to be performed. Split samples could be analyzed both offsite at a biomonitoring laboratory and in-house via

Microtox™ procedures for a period of a year This work would need to identify specific toxicants in the wastewater and match these to potential toxic responses from both the conventional tests and the bacterial-based tests. The Microtox™ procedures could be used to identify toxic hotspots and target specific contributing effluents with high toxicity. These effluents would then become targets for further process modification efforts to reduce toxicity. This overall effort to reduce toxicity would help establish relationships between the Microtox™ responses and the resulting downstream reduction of toxicity measured via conventional biomonitoring. Based on our preliminary work with the Las Cruces wastewater treatment plant, a minimum of a two tier test system using both osmotic adjusters would be needed. Establishing these relationships could give strong leverage to LANL to avoid standard biomonitoring protocol and allow the use of alternative onsite biomonitoring methods.

The advantage of the Microtox™ system or other microbial-based test systems over conventional biomonitoring methods is that tests take about 2-hours, can be conducted on-site making results immediately available, and is cost effective. Conventional Biomonitoring methods take up to 7 days, are typically off-site, and results may not be received until weeks or months later.

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APPENDIX I

1.0 INTRODUCTION

1.1 Objective

To determine if the effluent is chronically toxic to *Ceriodaphnia dubia* under static renewal test conditions.

1.2 Test Effluent

Three 24-hour composite effluent samples will be collected by the sponsor. The samples will be collected in disposable cubitainers, placed on ice, and delivered to ENSR via overnight delivery. The sample will not be dechlorinated in the laboratory; dechlorination facilities are now in operation.

2.0 MATERIALS AND METHODS

2.1 Basis

This protocol is designed to comply with USEPA method 1002.0, *Ceriodaphnia dubia* Survival and Reproduction Test (USEPA 1989).

2.2 Test Organism

1. Species - *Ceriodaphnia dubia*
2. Age - *Ceriodaphnia dubia* will be <24 hours old (all within 8 hours of the same age) at test initiation.
3. Source - *Ceriodaphnia dubia* will be obtained from ENSR's in-house culture.
4. Feeding - *Ceriodaphnia dubia* will be fed 0.1 ml of a yeast-trout chow-Cerophyl suspension (YCT, USEPA 1989) and 0.1 ml of *Selenastrum capricornutum* suspension (3.0×10^7 to 3.5×10^7 cells/ml) per 15-ml exposure chamber daily.

3.0 TEST SYSTEM

3.1 Dilution Water

Dilution water will be laboratory reconstituted water prepared to match (±15 percent) the pH, hardness, and alkalinity of the receiving stream.

3.2 Temperature

Test temperature will be 25 ± 1°C. Testing will be conducted in an environmental chamber or a temperature controlled water bath.

3.3 Test Containers

Test containers will be 30-ml plastic beakers containing 15 ml of test solution.

3.4 Photoperiod

The photoperiod will be 16-hours light and 8-hours dark.

3.5 Dissolved Oxygen Concentrations

Dissolved oxygen concentrations will be maintained ≥40 percent of saturation. If the dissolved oxygen concentration in the effluent and/or dilution water is low, the effluent and/or dilution water will be aerated prior to preparing the test solutions.

4.0 TEST DESIGN

4.1 Test Concentrations

The test concentrations will be 6, 12, 24, 48, and 95 percent effluent (V:V, effluent:dilution water). A dilution water control (0 percent effluent) will be conducted concurrently.

4.2 Number of Test Organisms

One *Ceriodaphnia dubia* will be randomly assigned to each test chamber and ten replicates will be tested per treatment.

4.3 Test Initiation/Renewal Frequency

Testing will be initiated within 36 hours of sample collection. Test chambers will be renewed on a daily basis with freshly prepared dilutions of the most recently received effluent sample.

4.4 Chemical and Physical Monitoring

At a minimum, the following measurements will be made:

1. Dissolved oxygen, temperature, and pH will be measured in each treatment at the beginning and end of each 24-hour exposure period.
2. Conductivity will be measured in each treatment at the beginning of each 24-hour exposure period.
3. Hardness, alkalinity, total ammonia, total residual chlorine, pH, and conductivity will be measured in the effluent and the dilution water on each day of sample receipt.

4.5 Biological Monitoring

Observations of mortality and reproduction in each test chamber will be made daily.

4.6 Test Duration

The test will be terminated when 60 percent of the control organisms have produced three broods.

4.7 Calculations

Fisher's exact test will be used to test for a significant difference in the survival of the various effluent concentrations and the control. Reproduction data will be compared from only those effluent concentrations where the mortality was not significantly different from the controls. Normality and homogeneity assumptions of reproduction data will be evaluated by the Shapiro-Wilk's test (or chi-square test depending upon the number of test organisms at test termination) and Bartlett's test, respectively ($p \leq 0.01$). If the data meet the assumptions, Dunnett's procedure will be used to make the comparison ($p \leq 0.05$). If the data do not meet the assumptions, Steel's many-one rank test will be used to make the comparison ($p \leq 0.05$). Reproduction will be determined by the number of young per original female. If the presence of males is visually confirmed at test termination, these organisms will not be used in the reproduction comparison. The no observable effect concentration (NOEC), lowest observable effect concentration (LOEC), and ChV (the geometric mean of the NOEC and LOEC) will be calculated on the basis of survival and/or reproduction.

4.8 Quality Criterion

The test will not be considered valid if control mortality exceeds 20 percent or average control reproduction is less than 15 young per original female.

5.0 TEST REPORT

The report will be a typed document describing the results of the test and will be signed by the Study Director and Quality Assurance Unit. The report will include, but not be limited to, the following:

- A USEPA Region VI summary sheet.
- A copy of all raw data.
- Name of test, Study Director, and laboratory.
- A description of the experimental design and the test chambers. the number of test organisms, replicates per treatment, and the lighting.
- Test organism scientific name, age, and diet.

APPENDIX II

1.0 INTRODUCTION

1.1 Objective

To determine if the effluent exhibits short-term chronic toxicity to the fathead minnow (*Pimephales promelas*) under static renewal test conditions.

1.2 Test Effluent

Three 24-hour composite effluent samples will be collected by the sponsor. The samples will be collected in disposable cubitainers, placed on ice, and delivered to ENSR via overnight delivery. The sample will not be dechlorinated in the laboratory; dechlorination facilities are now in operation.

2.0 MATERIALS AND METHODS

2.1 Basis

This protocol is designed to comply with USEPA method 1000.0, Fathead Minnow Larval Survival and Growth Test (USEPA 1989).

2.2 Test Organism

1. Species - *Pimephales promelas*
2. Age - Fathead minnows will be <24 hours old.
3. Source - Fathead minnows will be obtained from ENSR's in-house culture or a commercial supplier.
4. Feeding - Fathead minnows will be fed 0.1 ml of a concentrated suspension of newly hatched brine shrimp nauplii per test chamber three times daily.

3.0 TEST SYSTEM

3.1 Dilution Water

Dilution water will be laboratory reconstituted laboratory water prepared to match (±15 percent) the hardness, alkalinity, and pH of the receiving stream.

3.2 Temperature

Test temperature will be 25 ± 1°C. Testing will be conducted in an environmental chamber or a temperature controlled water bath.

3.3 Test Containers

Test containers will be 500 ml or 1-L beakers containing 250 ml of test solution.

3.4 Photoperiod

The photoperiod will be 16-hours light and 8-hours dark.

3.5 Dissolved Oxygen Concentrations

AE 4-30-93

Dissolved oxygen concentrations will be maintained ≥ 40 percent of saturation. If the dissolved oxygen concentration in any test chamber approaches this level, all test chambers will be aerated moderately.

4.0 TEST DESIGN

4.1 Test Concentrations

The test concentrations will be 6, 12, 24, 48, and 95 percent effluent (V:V, effluent:dilution water). A dilution water control will be conducted concurrently.

4.2 Number of Test Organisms

Ten fathead minnows will be randomly assigned to each test chamber and four replicates will be tested per treatment.

4.3 Test Initiation/Renewal Frequency

Testing will be initiated within 36 hours of sample collection. Test chambers will be renewed on a daily basis with freshly prepared dilutions of the most recently received effluent sample.

4.4 Chemical and Physical Monitoring

At a minimum, the following measurements will be made:

1. Dissolved oxygen, temperature, and pH will be measured in each treatment at the beginning and end of each 24 hour exposure period.
2. Conductivity will be measured in each treatment at the beginning of each 24 hour exposure period.
3. Hardness, alkalinity, total ammonia, total residual chlorine, pH, and conductivity will be measured in the effluent and the dilution water on each day of sample receipt.

4.5 Biological Monitoring

Observations of mortality in each test chamber will be made daily.

4.6 Test Duration

The test duration will be 7 days. At test termination the larvae in each test chamber will be counted and preserved in 4 percent formalin as a group for later dry weight analysis (if they cannot be weighed immediately). The preserved larvae will be rinsed with distilled water prior to dry weight analysis. The group of rinsed larvae from each test chamber will be transferred to a tared weighing boat and dried at 100°C for a minimum of 2 hours. Immediately after removal from the drying oven, the weigh boats will be placed in a desiccator to prevent absorption of moisture from the air, until weighed. The weights will be measured to the nearest 0.1 mg.

4.7 Calculations

Survival data will be transformed by arcsine squareroot. Growth in each replicate will be determined by the mean dry weight per surviving fish. Normality and homogeneity assumptions of survival and growth data will be evaluated by the Shapiro-Wilk's test and Bartlett's test, respectively ($p \leq 0.01$). If the data meet the assumptions, Dunnett's procedure will be used to make the comparison ($p \leq 0.05$). If the data do not meet the assumptions, Steel's many-one rank test will be used to make the comparison ($p \leq 0.05$). The no observable effect concentration (NOEC), lowest observable effect concentration (LOEC), and ChV (the geometric mean of the NOEC and LOEC) will be calculated on the basis of survival and/or growth.

4.8 Quality Criterion

The test will not be considered valid if control mortality exceeds 20 percent or if mean dry weight per surviving control fish is <0.25 mg.

5.0 TEST REPORT

The report will be a typed document describing the results of the test and will be signed by the Study Director and Quality Assurance Unit. The report will include, but not be limited to, the following:

- A USEPA Region VI summary sheet.
- A copy of all raw data.
- Name of test, Study Director, and laboratory.
- A description of the experimental design and the test chambers, the number of test organisms, replicates per treatment, and the lighting.
- Test organism scientific name, age, and diet.
- A detailed description of the effluent including its source, time of collection, composition, known physical and chemical properties, and any information that appears on the sample container or has been provided by the Sponsor.
- The source and characterization of the control water, and a description of any pretreatment.
- A description of any aeration performed on test solutions before or during the test.
- Percentage of test organisms that died in all treatments.
- The minimum dissolved oxygen concentration, range in test temperature and pH, and all visual observations of test solutions.
- Any deviations from protocol.
- Copies of chain of custody records.

6.0 LITERATURE CITED

USEPA. 1989. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Second Edition. EPA/600/4-89/001.

7.0 PROCEDURAL COMPLIANCE

All test procedures, documentation, records, and reports will comply with USEPA (1989) general guidance on quality assurance related to effluent toxicity testing. To this end, random audits of the test may be scheduled while the test is in progress. The raw data will be checked and compared to protocol requirements and Standard Operating Procedures, and the final report will be audited for accuracy and signed, if satisfactory, by the Study Director and an individual from the Quality Assurance Unit.