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A CHRONIC TOXICITY ASSESSMENT OF AN INDUSTRIAL EFFLUENT UTILIZING *PIMEPHALES PROMELAS* AND *CERIODAPHNIA SP*.

A Thesis

Presented to

the Faculty of the College of Arts and Sciences

Morehead State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

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by

Sharon J. Whitaker Fugate

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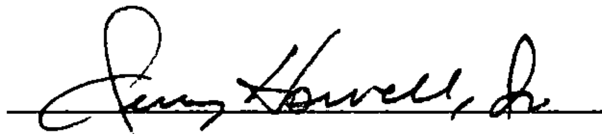
Accepted by the faculty of the College of Arts and Sciences, Morehead State University, in partial fulfillment of the requirements for the Master of Science Degree.



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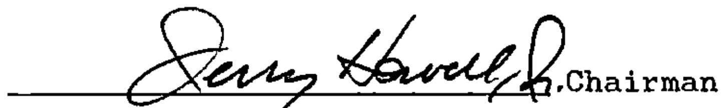
Master's Committee:  Chairman

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ABSTRACT

A CHRONIC TOXICITY ASSESSMENT OF AN INDUSTRIAL EFFLUENT UTILIZING PIMEPHALES PROMELAS AND CERIODAPHNIA SP.

Sharon J. Whitaker Fugate
Morehead State University, 1990

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A chronic seven-day toxicity assessment was performed on an industrial effluent, Outfall A. Test organisms were Ceriodaphnia sp. (water flea), less than 24 hours old, and nine-day old Pimephales promelas (fathead minnow). All testing and quality assurance procedures were done in accordance with EPA manual 600/4-85-014. The fathead minnow test was triplicated and the daphnid test was duplicated, utilizing a control of moderately hard synthetic water and the 100% concentrated effluent. A static renewal test was employed through monitoring Ceriodaphnia for survival and reproduction. Offspring were counted daily. Thirty fathead minnows per control and outfall A were monitored for survival, and finally sacrificed for weight analysis. Statistical tests employed were Fisher's Exact, the independant one-sided t test, and Probit Analysis. The t-test was performed on

minnow survival and growth and Ceriodaphnia reproduction The table t value (2 df, p=0.05) for fathead minnow survival was 2.920; the calculated t value was 3.57. The calcuated t for minnow growth was 0.769; the table t was 2.920. The table t (38 df, p=0.05) for Ceriodaphnia reproduction was 1.686, while the calculated t was -0.546. For Ceriodaphnia mortality data, the Fisher's Exact test was employed. The table value, calculated with EPA's SAS software, was 0.331, greater than 0.05 probability, so no significant difference existed. A significant difference was found in the minnow survival test between the effluent and the control. Other tests showed no significant differences. The minnow reference toxicant test began on April 10 and ended April 14, 1989. Concentrations used were 0.00 (control), 0.006 mg/l, 0.012 mg/l, 0.090 mg/l, 0.142 mg/l, and 0.180 mg/l cadmium chloride. The LC_{50} value (Probit Analysis) was 0.0093 mg/l. The range for Pimephales promelas for ninety six hours was 0.10 - 0.41 mg/l. The Ceriodaphnia reference toxicant used concentrations of 0.00 (control), 0.002 mg/l, 0.012 mg/l, 0.090 mg/l, and 0.180 mg/l cadmium chloride. The LC_{50} (Probit Analysis) was 0.1478 mg/l. The 48-hour ranges for related daphnid species were Daphnia magna, 0.01 - 0.09 mg/l and Daphnia pulex, 0.01 - 0.20 mg/l.

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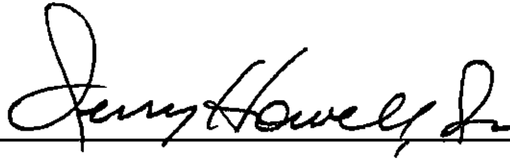

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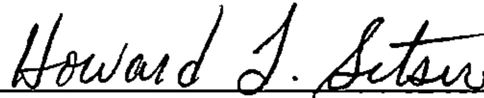

Dr. Howard Setser

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I also thank Florence Kessler for her aid in statistical analysis. She provided the Fisher Exact tables from EPA's SAS software.

My sincere thanks also go to my husband, Archie, who has endured my temperaments while working on the project and paper. His aid in typing has been greatly appreciated. Thanks to God who has blessed me, and to my parents, who taught me never to give up and always set goals, then work hard to achieve them.

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INTRODUCTION

Water pollution has intensified with increased population and industrial growth. Water treatment plants are being located closer to sewage treatment outlets and, with new chemical and substance introductions, there is an increased possibility of adding toxic substances to an already stressed aquatic environment. Kentucky and the United States Environmental Protection Agency (EPA) have initiated acute and chronic toxicity tests to identify problems and, to pinpoint toxicants, are using the "Toxicity Identification Evaluation" and "Toxicity Reduction Evaluation" (NPDES Seminar, 1988). This study involves a chronic seven-day static renewal toxicity test.

Polluted water contains some substance, or is characterized by some condition, to such a degree that it cannot be used for a specified purpose- "intended use" (Miller, 1975). Chemicals and compounds that must be prevented from entering streams in large amounts are radioisotopes, heavy metals, pesticides, PCBs, oil and sludges. While dilution does not protect all water, some short-lived toxic chemicals can be sufficiently diluted to render them harmless if stream flow is adequate, and total loads are not excessive.

River pollution problems are not as problematical as

those of lakes, primarily because rivers flow, permitting chemical pollutants to recombine and become diluted. However, some variables do control recovery time: size of the river, presence of dams, flow rate, and waste volume, particularly wastes that are non-biodegradable and substances easily biologically magnified. The United States Department of the Interior (DOI) reports approximately 400-500 fish kills each year; 50-75% of the kills result from industrial and muncipal activities. Insecticide runoff contributes to 10% of the fish kills (Miller, 1975).

Ecosystem rebounding abilities vary from site to site. State or local regulatory authorities may set pollutant limits based on site-specific areas (Ambrose, et al., 1988).

The Federal Water Pollution Control Act Amendment of 1977 (P1-95-217, Section 101a3) states. "It is the national policy that the discharge of pollutants in toxic amounts be prohibited." Current toxic discharge controls are based on individual chemical effluent limitations (Peltier and Weber, 1985). Levine (1988), quoting EPF science associate David Fanning, lists more than 60,000 toxic chemicals used in American agriculture and industry.

On July 29, 1985, EPA produced final ambient water quality criteria for seven toxic pollutants: ammonia,

arsenic, cadmium, copper, cyanide, lead and mercury. Acute treshhold and no-effect concentrations for each toxicant were specified, and tolerable duration and exposure frequency for two concentrations were given (Ambrose, et al., 1988). In 1985, the first toxic pollutant controls were administered. The controls were aimed principally at electroplating companies, jewelry makers and automobile producers. The Clean Water Act mandates, and eventually requires, industry to reduce toxic pollution by 96%. These mandates are primarily enforced by the states (Anonymous, 1986). States are expected to develop their own water-quality-based effluent limits, when violations are either identified or projected (NPDES Seminar, 1988).

Toxic-effect severity is based on pollutant strength and exposure duration. Brief exposure to a high concentration may not be as harmful as long term exposure to a low concentration (Ambrose, et al., 1988).

Health effects of toxic pollutants fall into two categories: (1) carcinogenic or mutagenic (genotoxic) effects, for which exposure levels exist; (2) all other health effects classed as target organ effects (systemic toxicity), for which it is presumed there are safe exposure levels (EPA, 1985).

With genotoxicity, somatic cell DNA damage can lead

to cell proliferation and cancer. There are two carcinogenic pollutant classes: (1) electrophilic, and (2) those metabolized in vivo from electrophilic reactants that may ultimately interact with cellular DNA. This interaction is thought to be the initiating, and critical, step for genotoxic carcinogens. The usual approach to detecting systemic toxicity is the conduction of whole-animal testing for subchronic or chronic effects. Systemic toxicity is usually affected through oral exposure. Two examples of systemic toxicity are hepatatoxicity (liver and/or renal toxicity) and cardiovascular toxicity (heart and blood vessels) (EPA, 1985).

Toxicity testing has been used for more than 50 years, but it has recently become important and more commonly practiced by industrial and municipal water treatment operators. In March 1984, EPA issued a directive governing the use of aquatic toxicity limitations in the National Pollution Discharge Elimination System (NPDES) permitting process, under which all discharge permits are issued, and the Clean Water Act of 1987 specifically addresses toxics management (Preconference, 1988).

Many "non-priority" pollutants are toxic, and because they can act additively, synergetically and/or

antagonistically in mixtures, their control has shifted to a whole-effluent approach. This approach also considers "non-conventional toxics," such as pH extremes and low dissolved oxygen.

Water quality criteria are not intended to offer the same degree of safety for survival and reproduction at all times to all organisms; they are intended to protect aquatic life and direct water users. Indicator organisms usually represent, or exhibit, responses expected of other associated organisms (EPA, 1976).

Aquatic organisms are useful indicators of water quality and conditions, especially after disturbances (Davidson and Hellenthal, 1986). Fish are good water quality indicators because they occupy the highest trophic level in their food chain. What alters the fish population also alters the balance of periphyton, plankton and microinvertebrates. Microinvertebrates should also be tested because they have differing reactions than fish to toxins (AWWA, 1985).

Fish kill causes vary from natural occurrence to the result of human activity. Human-caused fish kills may be attributed to industrial or muncipal wastes, and agricultural and water control activities (AWWA, 1985).

The most noticeable problem indicator is the disappearance of entire fish populations. This has

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occurred in some lakes and rivers in Norway and Sweden, and in more than 100 lakes in New York's Adirondack Mountains and the La Cloche Mountain lake district of Ontario (Cole, 1983). Fish kill causes may be complex: "Some materials may have multiple effects; for example, an iron salt may not be toxic; an iron floc or gel may be an irritant or clog fish gills to effect asphyxiation; iron at low concentrations can be a toxicant. Materials also can affect organisms if their metabolic byproducts cannot be excreted. Unless otherwise stated, criteria are based on the total concentration of the substance because an ecosystem can produce chemical, physical, and biological changes that may be detrimental to organisms living in or using the water" (EPA, 1976).

Planktonic and benthic organisms reflect clearly the opportunities and constraints associated with life in aquatic environments. Populations decrease during stressful periods and rise when favorable conditions reoccur. Organisms with higher reproductive rates recover more quickly (Mann, 1980).

Living systems need to maintain chemical regulation for both the long and short terms. Foreign chemicals in living systems can affect enzymes, immune systems, hormones, and the nervous system (Ford, 1977). Inorganic chemicals and minerals (an array of acids, salts and

finely divided metals or metal compounds) may increase the acidity, salinity, and toxicity of water. There are approximately 10,000 different organic chemicals currently in use, and 300-500 new compounds are introduced each year (Miller, 1975).

Organismal stress causes internal imbalance, leading to attempts at system regulation. Biofeedback mechanisms, including responses to pH levels, prevent extreme reactions (Ford, 1977). However, longer food chains develop in many stable environments, while highly unstable environments show fewer specialized higher organisms (Mann, 1980). Upsetting mechanisms, prompted by pollutants, can cause irreversible disruptions (Cole, 1983).

These disruptions can be assessed through biomonitoring, a test subjecting organisms such as fathead minnows (*Pimephales promelas* Rafinesque) and water fleas (*Daphnia spp*. or *Ceriodaphnia spp*.) to discharge water from industries and sewage treatment plants. If the organisms live, die, become deformed, or exhibit stunted growth, the water is considered at least somewhat toxic (Martin, 1988).

In biomonitoring, aquatic organisms are exposed to discharges to detect and measure the presence and/or adverse effect of a substance alone, or in synergy.

Standard procedures used include bioassay and toxicity testing (AWWA, 1985).

Bioassay is used to evaluate a chemical's relative potency by comparing its effects on a living organism with the effects of a standard preparation on the same species. Bioassays are frequently used in the pharmaceutical industry to evaluate vitamin and drug potency.

The toxicity test serves many useful purposes, particularly the assessment of favorable and unfavorable concentrations of dissolved oxygen, pH, temperature, salinity, and turbidity. Other uses include determining (1) the effect of environmental factors on toxicity, (2) toxicity to a particular species, (3) relative sensitivity of species to toxicants, (4) the amount of waste treatment necessary to meet pollution control requirements, (5) the effectiveness of waste treatment, and (6) compliance with regulations (Standard Methods, 1985).

Analytical variability must also be considered when examining wastewaters for specific components, such as heavy metals, and in testing total organic carbon (TOC) and biochemical oxygen demand (BOD), both potential contributors to toxicity (Preconference, 1988). Variability in toxicity tests may result from the selection of species used - the strain or source of test

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organisms, and their condition or health. Test conditions, such as temperature, dissolved oxygen, food and water quality, may also affect results (NPDES Seminar, 1988). Variability may be related to the laboratory conducting the test, with greater variability observed when more than one laboratory conducts replicate tests. Bias may result from using organisms that may be stressed, have parasites or diseases, or be in poor physical condition (Preconference, 1988).

Fish have been more widely used for toxicity tests than any other group of aquatic organisms (Standard Methods, 1985). Davidson and Hellenthal (1986) relate that fish are sensitive to environmental changes, and have life spans and an appropriate number of generations for use in assessments. Fish and invertebrates have differing susceptibilities to certain toxicants (AWWA, 1985).

The three organisms used most often in toxicity tests, P. promelas, Daphnia spp., and Ceriodaphnia spp., are utilized because they are considered to be more tolerant to toxicants than many other species. If these species are adversely affected, then other species are more likely to be the first adversely affected. Therefore, the more tolerant organisms are recognized as concrete problem indicators. However, depending on the ultimate discharge point and governmental regulations,

various other organisms are used in toxicity tests. Other commonly-used species are *Daphnia magna* Straus, *Daphnia pulex* Leydig, various species of grass and mysis shrimp, *Cyprinodon variegatus* Lacepede (sheepshead minnow), *Salvelinus fontinalis* Mitchell (brook trout), *Lepomis macrochirus* Rafinesque (bluegill sunfish) and *Ictalurus punctatus* Rafinesque (channel catfish). These species were originally selected because they represent components of various freshwater habitats from fast flowing streams to stagnant lakes and ponds (AWWA, 1985).

The permitting agency, such as EPA, selects which test species and what life stage will be used. Two different organisms are used because different senstivities exist (Preconference, 1988).

If more than 20% of the control organisms die during a chronic test, the toxicity test is considered invalid, and the test must be repeated (Roth and Westerman, 1987).

Fathead minnows and water fleas are used because (1) they are easily cultured in the laboratory, (2) they reach sexual maturity quickly, (3) historical data exist for the toxic limits of these species, (4) they are used as EPA national test organisms, (5) adequate sources of healthy individuals are readily available, and (6) the species are native to Kentucky (Roth and Westerman, 1987).

Typical chronic effects of toxicants are reproductive

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[illegible] the [illegible]

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impairment, decrease in competition resulting from physiological stress, and gradual population decline and/or absence from an area. Bioaccumulation of materials may cause chronic toxicity to ultimate consumers (EPA, 1976).

Assumptions regarding the use of aquatic species as indicators of environmental quality can be made. There are no fish in water of the lowest quality; invertebrates typically present in this turbid and dark water are Culex (mosquito larva), Eristalis (rat-tailed maggot of the Syrphus fly), and Tubifex (sewage worm). Algae present include Oscillatoria (blue green algae), Sphaeotilus (bacteria), and Melosira (diatom). Water of poor quality contains the same genera as water of the lowest quality, except that Paramecium (ciliate protozoa), Beggiatoa (sewage bacteria), and Stentor (protozoa) are also usually present in water of poor quality. Poor-quality water contains tolerant fish species and invertebrates, such as Chironomus (fly larva) and Simulium (black fly larva). Plankton present are Pandorina (colonial protozoa), Spriogyra (green algae), and Euglena (mastigophoran protozoa). In water of good quality, there exists a "normal" fish population of game, food, and forage fish. Dominant invertebrates include several genera of caddisflies and stoneflies. Plankton are typically

Navicula (diatom), Oedongonium (green algae), and Dinobryon (colonial protozoan) (Odum, 1971).

The fathead minnow can tolerate high temperature, extreme turbidity, low oxygen, and is well-suited for survival in stagnant pools (Pflieger, 1975). It is found principally in shallow water over, or near, sandy bottoms, although it sometimes frequents small muddy streams. The fathead minnow is primarily a fish of upland ponds and streams; it is common in bog ponds and sluggish streams. But, near stream mouths where numerous lowland forms are competitors, it is usually rare or absent. It feeds on organic matter in mud, filamentous algae and vegetable debris, small insect adults and larvae, crustaceans, worms, and fish eggs (Schrenkeisen, 1939).

Cladocerans have blood with the red respiratory pigment haemoglobin; the blood is remarkably similiar to that of humans. Pale in well-aerated water, the blood may bc bright red in poorly-aerated water, because of increased haemoglobin. There are over 4000 species in eight families (Larousse Encyclopedia, 1967). They live in a pH range of 6.5-8.5. Magnesium may act as a limiting factor by inhibiting reproduction (Pennak, 1978).

Daphnia have compound eyes, excrete by antennial glands, respire through gills or the body surface, are dioecious, have larval stages and are cosmopolitan

(freeliving or parasitic). Daphnia's subclass (Branchiopoda) cohorts have a carapace. Parthenogenesis is common (Hickman, 1967).

"Water fleas", including Ceriodaphnia and Daphnia are about 0.15 to 3.00 mm in length, and there about 425-450 species. The carapace encloses the trunk and trunk appendages, but leaves the head and antennae free; the exoskeleton is a hard limy chitin (Hickman, 1967).

Daphnia feed by filtering microscopic-sized plants from the water; other cladocerans have lost this ability (Larousse Encyclopedia, 1967). They swim by way of affecting jerky movements using their antennae, facilitating oxygen and food particle consumption. They reproduce parthenogenetically, until affected by adverse environmental conditions, and live 30-60 days. They are common in aquatic vegetation along pond, lake and river margins, both in temporary and permanent pools. They generally are eurythermal, but some are restricted to warm waters. Their food consists of protozoans, bacteria, algae and organic deitrus (Hickman, 1967).

Males do not appear until adverse environmental conditions arise; they then mate with the females to produce ephippa or sexual eggs. Ephippa are resistant to freezing and drying and hatch when environmental conditions improve. (Larousse Encyclopedia, 1967).

Peltier and Weber (1985) report that males in a laboratory culture will not appear unless stock cultures are neglected, or the culture experiences environmental stress.

The life cycle of Daphnia is dependent on the species involved and environmental conditions. The life span increases as temperature decreases, due to lowered metabolic activity. For instance, the average life span for D. magna is 40 days at 25°C. At 20°C, it is 56 days. The four periods in the life cycle are egg, juvenile, adolescent, and adult. The clutch of eggs, usually six to ten in a brood chamber, is released in approximately two days when the female molts (Peltier and Weber, 1985).

Daphnia have been used for over a century in tolerance studies. Laboratory food for Daphnia includes bacteria, algae, and yeast, together with soil extracts and organic materials. They will eat cottonseed meal, herring meal, enriched trout fry granules, and powdered dried grass (AWWA, 1985).

Neonates (first instar young, about 0.8-1.0 mm long) are less tolerant of many substances than other instars (and many other animals). Daphnia are more susceptible to most substances at ecdysis than between molts. In general, they are less tolerant of toxic substances than are fish (AWWA, 1985).

Daphnia is a very common test organism because of size, sensitivity, and low cost. Ceriodaphnia is increasingly being used as a test organism because of its ability to reproduce quickly. A seven-day test can result in three separate broods of eggs, thus a chronic endpoint (reproductive success) can be determined for about the same cost as an acute test (NPDES Seminar, 1988).

Almost all Ceriodaphnia cultures from EPA's Newtown, Ohio facility and Duluth laboratories in Minnesota are C. reticulata Jurine or C. dubia Herrick. These species may be able to hybridize (Berner, 1987). Their general characteristics are very similiar to Daphnia, and generalizations made regarding Daphnia are also applicable to Ceriodaphnia.

Extra care should be exercised when handling Daphnia. Exposure to air can cause a bubble to be trapped under the carapace, preventing normal swimming and leading to death (Roth and Westerman, 1987).

Toxicity affects crustaceans through causing erratic swimming, loss of reflex, discoloration, changes in behavior, excess mucus production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannabalism (AWWA, 1985).

This study presents the results of a chronic seven-day static renewal toxicity test on the effluent outfall

of a rubber company. Effects measured are the survival and reproduction of *Ceriodaphnia sp*. and the survival and growth (biomass) of *P. promelas*. This study utilized a quantal toxicity test - the organisms show a dichotomous response; individual organisms either live or die. The test estimates the concentration of test solution causing a lethal response in 50% of the test solution (LC_{50}). A lethal response involves immobilization, swimming fatique, avoidance reaction, and ultimately, death. Ninety five percent confidence limits were determined for the LC_{50} reference toxicant. The outfall was tested at 100% effluent concentration against a control (AWWA, 1985). Potential sources of toxicity were also examined.

MATERIALS AND METHODS

Materials

The following materials were used to conduct the toxicity tests:

1). Reference weights; class S.

2). Dissecting scope; Fisher Scientific Stereomaster II, Model SPT 1H.

3). Balance; Mettler, Model H31AR, accurate to 0.0001 g.

4). pH meter; Fisher Accumet, model 825-MP.

5). Conductivity meter; YSI model 33.

6). Dissolved oxygen probe; Orion, model 97-08-00, with Fisher Accumet model 610-A pH meter.

7). Pyrex beakers; for fathead minnow tests. Solo cups; P-35A, for Ceriodaphnia sp. tests. For reference toxicant test, disposable plastic beakers were used and discarded.

8). Aquariums, pumps, hose, glass eyedroppers, nets, and other miscellaneous equipment, as outlined in EPA manual 600/4-85-014.

Methods

One outfall from a rubber company was analyzed and labeled "discharge A". A seven-day chronic static renewal test was performed according to guidelines outlined in EPA manual 600/4-85-014, "Short Term Methods for Estimating

[illegible]

laboratory tools.

1) Reference weights [illegible]

2.) Dissecting scope, Fisher Scientific [illegible] Model III.

3 Balance, Mettler, Model H51AR [illegible]

4) pH meter, Fisher Accumet model 825 MP

5) Conductivity meter; YSI model [illegible]

6 [illegible]

7) [illegible]

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Methods

One or [illegible]

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Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms". Procedural deviations were kept to a minimum.

Twenty-four hour composite samples were taken daily over a period of seven days (April 9-15, 1989). The sample jugs were transported on blue ice in a cooler and refrigerated at 4°C. Each sample was prepared 24 hours after collection. Table 1 describes water parameters for the field samples.

Table 1. Field Sample Water Quality Parameters.

Sample Date	Temperature (°C)	pH	Dissolved Oxygen (ppm)
April 09, 1989	5.0	7.45	4.8
April 10, 1989	7.2	7.11	6.0
April 11, 1989	1.1	7.15	5.2
April 12, 1989	4.4	7.21	5.1
April 13, 1989	7.8	7.12	5.2
April 14, 1989	5.0	7.28	5.4
April 15, 1989	3.3	7.17	6.0

Sample jug contents were composited in 1000 ml. beakers and gradually acclimated to 25°C in a warm water bath and aerated prior to set up.

The test organisms, purchased from Aquatic Research Organisms (ARO) in New Hampshire and shipped in coolers

through Federal Express, were Pimephales promelas and Ceriodaphnia sp. On receipt (April 7, 1989), the cultures were checked and acclimation to dilution water temperature (25 $\pm$ 1°C) was performed. Excess water was siphoned off, and then replaced with moderately hard synthetic water, made according to EPA manual 600/4-85-014, and prepared on April 6, 1989. Thirty eight liters of deionized water were placed in a cleaned 10-gallon aquarium. 2.28 grams of magnesium sulfate, 3.648 grams of sodium bicarbonate, and 1.52 grams of potassium chloride were added to the aquarium and stirred well. 2.28 grams of calcium sulfate dihydrate were added to one liter of deionized water in a separate flask and placed on a magnetic stirrer until dissolved. This solution was added to the aquarium and stirred well. The aquarium water was aerated vigorously for 24 hours. The moderately hard synthetic water had a pH range of 7.4-7.8, a hardness of 80-100 ppm, and an alkalinity of 60-80 ppm.

Organisms were observed over a 24-hour period for mortality occurrence. To obtain neonates, adult Ceriodaphnia were placed into individual solo cups with dilution water on April 9, 1989. Ceriodaphnia culture organisms and those in cups were fed Cerophyl (yeast trout chow). Those in cups received 0.1 ml of Cerophyl (2 drops dispensed by an eyedropper). Fish were acclimated and

placed in an aquarium, then fed finely ground flake food provided by ARO. During all phases of the procedure, all test organisms were kept in separate containers.

Water chemical analyses methods followed EPA manual 600/4-79-020, "Methods for Chemical Analysis of Water and Wastes"(1983), and the sixteenth edition of Standard Methods (1985).

The pH method (method 150.1) involved a three-point calibration on buffers of pH 4.0, 7.0, and 10.0. Calibration was performed daily with fresh buffers.

Hardness (method 309) was performed using the EDTA titrimetric technique, and method 310.1 was used for alkalinity.

Specific conductance was performed according to method 120.1, measured in µmhos at 25°C. The conductivity meter was calibrated daily with fresh KCl solution.

Dissolved oxygen was performed with an Orion oxygen electrode and a pH meter with digital readout, while temperature was strictly monitored to assure a temperature of 25 ± 1°C.

Test method 1000.0 for the chronic seven-day static renewal test for fathead minnows was used and tests were performed in triplicate with 10 organisms and 1000 ml of sample in each beaker. Direct and indirect effects measured were death or survival, locomotor activity, gill

ventilation rate, heart rate, blood chemistry, enzyme activity, histopathology, olfactory function and terata. However, due to laboratory limitations, only mortality and growth were definitively measured.

Each day a new sample was prepared ± two hours from the initial set up time. Live fish in each beaker were counted and observed for normal and erratic behavior. Dead fish were noted and discarded. Uneaten food and old test solutions were siphoned off until about one inch of water remained with the fish in the beaker. To prevent organismal stress during solution renewal, each new sample was carefully poured. Fish were then fed once a day, after solution renewal, with one pinch of dry, finely-ground tetramin staple fish food in each beaker.

Discharge A was set up in triplicate at 100% effluent concentration, and a control in triplicate was prepared with moderately hard synthetic water. Organisms used were nine days old. Analyses performed daily on the ending solutions were dissolved oxygen and temperature. On each new sample, pH, specific conductance, temperature, dissolved oxygen, alkalinity, and hardness tests were performed.

On the seventh day of testing, the remaining fish larvae were counted, preserved in four percent formalin, and labeled. Later, larvae lengths were individually

measured, and the fish rinsed in deionized water. The control and reference toxicant fish and fish subjected to discharge A were put in a tared weighing boat and baked for a minimum of two hours at 100°C. After baking, the weighing boats were placed in a desiccator until individual fish could be weighed. Documented observations included minimum total length, maximum total length, and the average total length for each concentration. Minimum weight, maximum weight, and average weight was recorded for each concentration. Survival data and weight (biomass) data were used in the statistical analyses.

The test method used for Ceriodaphnia sp. was 1002.0 (EPA Manual 600/4-85-014). Neonates (<24 hours old) were used and procedural deviations were minimized. Discharge A was prepared in duplicate at 100% effluent concentration and a control in duplicate was prepared with moderately hard synthetic water, using 50 ml into each of 20 solo cups. 100% effluent test solutions were prepared in the same manner. Single continually-submersed neonates were placed into each cup. After the initial set up, individual Ceriodaphnia were fed two drops of Cerophyl. Dissolved oxygen and temperature were recorded daily on the ending solutions. On each new sample, pH, specific conductance, temperature, dissolved oxygen, alkalinity, and hardness tests were performed.

A new sample was prepared for each of the remaining days of the test. Adult Ceriodaphnia were transferred via glass eyedropper to each of the new solo cups containing the new sample, and were then fed. A permanent marker was used to label each cup. Dead adults were counted and that cup was discarded after all counting was completed. After all adults were transferred to the new sample, offspring were counted and the contents of the old solo cup were discarded into a BOD bottle and saved for dissolved oxygen measurements for both the 100% effluent and the control. On new solutions, pH, specific conductance, temperature, dissolved oxygen, alkalinity and hardness tests were performed on each of the seven testing days.

Each day a new sample was prepared ± two hours from the initial set up time. On the seventh day, the live adults were counted and recorded. Offspring were counted and discarded with the solo cup contents into BOD bottles for recording the dissolved oxygen. During the tests, organismal behavior was noted.

Due to laboratory limitations, the only effects that could be accurately measured and readily seen were mortality and reproduction. Survival and reproductive data were used in statistical analyses.

Quality assurance has two aspects: quality verification and quality control. Quality verification

shows a proposed study plan was followed and involves work documentation. It includes chain of custody procedures, objective of the study, problem at the outset, work assignments, and log books. Quality control involves procedures dealing with the number of samples to be taken, the mode of collection, and standard operating modes for analysis and spiking protocol (Horning and Weber, 1985). Both aspects of quality assurance were incorporated and followed in these tests.

All instrumentation was calibrated prior to its usage, and routine maintenance was performed and noted. When a new chemical was received and/or opened, the date was noted on the bottle. If no labelled expiration date was given, it was assumed to be six months. After this time, it was disposed of properly. The balances were serviced and cleaned under a semi-annual maintenance contract; they were also routinely checked with a set of class S weights.

Test beakers were cleaned to assure against chemical carry-over. Glassware cleaning was performed according to EPA manual 600/4-84-014. The glassware was washed in an automatic dishwasher, rinsed twice, then rinsed with 20% nitric acid to remove scale, metals, and bases. Then, it was rinsed with tap water twice and once with full strength acetone to remove organic compounds. The

glassware was then rinsed well with tap water and, finally with dilution water.

Testing conditions involved lighting at 50-100 footcandles (adequate light), with 16 hours of light and eight hours of darkness. The maintained temperature was 25 ± 1°C.

The chronic seven-day static renewal test measures both quantal and quantitative responses. Responses for quantal tests are death and mutagenicity; quantitative responses are growth and reproduction (AWWA, 1985).

Traits noted during testing were actual deaths, and concomitant progressive steps in its logical sequence. In fish, death is defined as no gill movement. For daphnids, there is no reaction when the organism is gently prodded. Stress clues include erratic swimming, loss of reflex, discoloration, opaque eyes, curved spine, hemorrhaging, molting and cannibalism (Peltier and Weber, 1985).

Control survival must be 80% or better before results are considered valid. Replication and test sensitivity depend on replicates, probability level selected and type of statistical analyses performed.

The effect measured in this test was decreased survival in the sample as compared to the control for the Pimephales promelas and Ceriodaphnia sp. An independent one-sided "T" test, with a probability level of 0.05, was

used for each test organism group because only one concentration (100%), was compared with the control. For <u>Ceriodaphnia</u> reproduction, a one-sided independent "T" test was used, and for <u>Ceriodaphnia</u> survival, a Fisher's Exact test was used.

The reference toxicant used was cadmium chloride, ordered for consistency from the EPA laboratories in Cincinnati. Moderately hard synthetic water was used in diluting to various concentrations in volumetric flasks. A reference toxicant was tested with <u>P</u>. <u>promelas</u> and <u>Ceriodaphnia</u> <u>sp</u>. The purpose of a reference toxicant was to confirm the validity of the test organism deaths. Factors affecting accuracy are age, condition of organisms, sensitivity, and water temperature.

The LC_{50} (lethal concentration: the concentration at which 50% of test organisms die) was found by plotting concentrations used versus the number of living organisms. Smaller LC_{50} values on the concentration axis indicated higher toxicity.

Containers used in the reference toxicant analyses were used once and discarded. For fathead minnows, plastic, disposable beakers were used and for <u>Ceriodaphnia</u> <u>sp</u>., solo cups were used. Twenty organisms were used for each concentration.

During the test, solutions were not renewed and

organisms were not fed. Duplicates for each concentration for each organism were also performed. Instantaneous changes in pH, specific conductance, osmotic strength and dissolved oxygen were avoided. Each new batch of organisms was evaluated with a reference toxicant preceding or concurrent with the test, following procedures of Peltier and Weber (1985).

A 48-hour reference toxicant test was performed on *Ceriodaphnia sp.* and a 96-hour reference toxicant was ran on fathead minnows. The LC_{50} for fathead minnows was 0.0093 mg/l. The LC_{50} for *Ceriodaphnia sp.* was 0.1478 mg/l.

Individual neonates were put into twenty cups for each test concentration and each control. Organisms were observed over a 48-hour period for mortality. pH, specific conductance, temperature, dissolved oxygen, hardness, and alkalinity were recorded on the beginning and ending solutions.

Concentrations used in reference toxicant tests for *Ceriodaphnia sp.* were control, 0.002 mg/l, 0.012 mg/l, 0.090 mg/l, and 0.180 mg/l. The LC_{50} was 0.1478 mg/l. For the 48-hour test, the range for cadmium chloride for *Daphnia magna* was 0.01-0.09 mg/l. For *Daphnia pulex*, the range was 0.11-0.20 mg/l. *Ceriodaphnia sp.* was used in this reference toxicant test.

Concentrations used for the fathead minnow reference toxicant test were control, 0.006 mg/l, 0.012 mg/l, 0.090 mg/l, 0.142 mg/l, and 0.180 mg/l. The LC_{50} was 0.0093 mg/l. All concentrations were performed in duplicate and organisms were observed 96 hours to assess mortality. Physiochemical tests were performed on the beginning and ending solutions, as they were for the _Ceriodaphnia sp._ solutions. Ten _Ceriodaphnia sp._ per plastic container were tested and all concentrations were done in duplicate.

For the 96-hour test, the range for cadmium chloride toxicity to _P. promelas_ was 0.10-0.41 mg/l, according to the EPA sheet accompanying the toxicant.

Temperature was monitored to assure that the room was consistently 25 $\pm$ 1°C. A thermometer, calibrated by a National Bureau of Standards thermometer, was kept immersed in deionized water.

Appendices A, B, and C include dissolved oxygen meter calibration, pH meter calibration, fish length and weight analysis respectively.

RESULTS

The chronic seven-day tests for fathead minnow and *Ceriodaphnia sp*. began on April 10, 1989 and were terminated on April 18, 1989. The fathead minnow test was triplicated; the daphnid test duplicated. Tables 2 and 3 describe the physiochemical test data for both species.

Table 2. Physiochemical Test Data for *Ceriodaphnia* (At 25°C).

Date	ID	Sp. Cond. (µmhos)	pH	Alkalinity (ppm $CaCO_3$)	Hardness (ppm)	D.O. (ppm) Begin	D.O. (ppm) End
4/10/89	A	335	7.31	80	20	6.73	7.93
	C	335	7.41	60	70	8.32	7.80
4/11/89	A	305	8.00	80	37	7.90	6.60
	C	300	7.86	80	85	7.90	6.40
4/12/89	A	160	7.81	110	57	6.80	6.34
	C	250	7.56	60	94	7.80	7.39
4/13/89	A	180	7.75	95	37	7.34	6.69
	C	320	7.58	65	79	7.90	7.39
4/14/89	A	215	7.83	110	57	8.07	7.52
	C	280	7.46	70	94	8.07	6.45
4/15/89	A	195	7.63	90	33	7.92	6.45
	C	290	7.47	70	90	6.82	6.78
4/16/89	A	215	8.07	120	32	8.02	7.43
	C	290	7.61	60	88	8.10	7.86

A = 100% Outfall A
C = Control

Table 3. Physiochemical Test Data for Fathead Minnow (At 25°C).

Date	ID	Sp. Cond. (µmhos)	pH	Alkalinity (ppm CaCO3)	Hardness (ppm)	D.O. (ppm) Begin	D.O. (ppm) End
	A	335	7.31	80	20	6.73	5.64
4/10/89	C	335	7.41	60	70	8.32	7.89
	A	305	8.00	80	37	7.90	4.90
4/11/89	C	300	7.86	80	85	7.90	6.20
	A	160	7.81	110	57	6.80	6.61
4/12/89	C	250	7.56	60	94	7.80	6.42
	A	180	7.75	95	37	7.98	7.34
4/13/89	C	320	7.58	65	79	7.90	7.18
	A	215	7.83	110	57	8.07	6.48
4/14/89	C	280	7.46	70	94	8.07	7.36
	A	195	7.63	90	33	7.92	6.47
4/15/89	C	290	7.47	70	90	6.82	6.22
	A	215	8.07	120	32	8.02	7.58
4/16/89	C	290	7.61	60	88	8.10	7.10

A = 100% Outfall A
C = Control

Fathead minnows were tested for survival and growth (increase in biomass); Ceriodaphnia sp. were tested for survival and reproduction. The independent one-sided t-test was used for fathead minnow survival and growth. Table 4 summarizes fathead minnow survival results.

Table 4. Fathead Minnow Survival.

Sample ID	Number Alive	Calculated t Value	Table t Value	Conclusion
A	22			
Control	29			
Results		3.57	*2.920	Null hypothesis rejected

*Tabular t value with 2 degrees of freedom and P = 0.05.

A = Outfall A

The table t value (2 d.f., $p \geq 0.05$) for fathead minnow survival was 2.920; the calculated t value was 3.57. Because the calculated t value was greater than the table t value, there was a significant difference in fathead minnow survival between the control and outfall A. The control had one death in thirty fish; outfall A had eight deaths in thirty fish.

The average weight in the fathead minnow control group was 0.167 mg., while the Outfall A group averaged 0.157 mg. Table 5 summarizes fathead minnow growth data.

Table 5. Fathead Minnow Growth Data (in mg. Dry Weight).

Sample ID	Mean Dry Weight (mg)	Calculated t Value	Table t Value	Conclusion
A	0.157			
Control	0.167			
Results		0.769	*2.920	Null hypothesis Accepted

*Tabular t value with 2 degrees of freedom and P = 0.05.

A = Outfall A

The calculated t value for fathead minnow growth was 0.769; the table t was 2.920. There was no significant difference in fathead minnow growth between the control and outfall A because the calculated t value was less than the table t value.

Fisher's Exact Test was used to measure significance in Ceriodaphnia sp. mortality. Table 6 summarizes the Ceriodaphnia mortality.

Table 6. Ceriodaphnia Mortality.

Sample ID	Number of Organisms Alive	Dead	Total	Probability	t	Conclusion
A	16	4	20			
Control	18	2	20			
Totals	34	6	40	*0.05	0.331	A = C

*Tabulated probability levels obtained from Florence Kessler (EPA, Cincinnati) on February 12, 1989. Tabulated values generated from SAS software.

A = Outfall A

Two deaths in 20 organisms were observed in the control, while four of 20 organisms in outfall A died. Probability tables were used at an alpha level of 0.05, 20 organisms per concentration with two deaths in the control. Since the calculated t value of 0.331 is greater than 0.05, the null hypothesis is not rejected. There is no significant difference in survival between the control and outfall A.

For Ceriodaphnia sp. reproduction statistical analysis, an independent t test was used. The offspring of twenty organisms are used for the analysis. Table 7 summarizes Ceriodaphnia reproduction data.

Table 7. Ceriodaphnia Reproduction.

Sample ID	Calculated t Value	Table t Value	Conclusion
A Control			
Results	-0.546	*1.686	Null hypothesis accepted

*Tabular t value with 38 degrees of freedom and P = 0.05.

A = Outfall A

The table t value (38 d.f., $p \geq 0.05$) was 1.686. The calculated t value was -0.546, indicating there was no significant difference in reproduction between the control and outfall A.

Cadmium chloride, the reference toxicant used for the fathead minnows, originated at EPA in Cincinnati. The time period for testing was four days (April 10-14, 1989).

The concentrations used were control (0.000 mg/l), 0.006 mg/l, 0.012 mg/l, 0.090 mg/l, 0.142 mg/l, and 0.180 mg/l cadmium chloride. A Probit Analysis was used and the LC_{50} was 0.0093 mg/l. The 24-hour range for Pimephales promelas was 0.13 - 0.78 mg/l; the 96-hour range was 0.10 - 0.41 mg/l. The analysis indicated that the organisms used were more sensitive than expected.

The cadmium chloride reference toxicant testing period for Ceriodaphnia sp. was 48 hours (April 10-13, 1989). Concentrations used were control (0.000 mg/l), 0.002 mg/l, 0.012 mg/l, 0.090 mg/l, and 0.180 mg/l. A Probit Analysis was used and the LC_{50} was 0.1478 mg/l. The given EPA tolerance range for the cadmium chloride ampule was for related daphnid species, but not Ceriodaphnia sp. Daphnia magna had a given range of 0.03 - 0.23 mg/l (for 24 hour exposure), while the range was 0.01 - 0.09 mg/l for 48 hours. Daphnia pulex had a given range of 0.14 - 0.70 mg/l for 24 hours and 0.11 - 0.20 mg/l for a 48-hour period. This indicated that the LC_{50} found for Ceriodaphnia sp. more closely fell into the range given for Daphnia pulex. These daphnids were not overly sensitive and probably not stressed before testing.

DISCUSSION AND CONCLUSIONS

Biomonitoring is a leading laboratory method for assessing aquatic toxicity. It can be used to lessen future disturbances of the aquatic environment; its limitation is that toxicity is often discovered after the fact.

The usefulness of toxicity testing occurs in TIEs (Toxicity Identification Evaluations) and TREs (Toxicity Reduction Evaluations), methods in which causative agents are identified and preventative industrial procedures are devised for lowering further toxicity. The time to assess the toxin varies. It is an elimination process, can be laborious, and requires professional experience.

Some chemicals act synergistically or additively, so there may be more than one causative agent.

The purposes of a TIE and TRE are to pinpoint pollutant-causing toxicity, identify the source, and evaluate treatment options. Characteristics studied in the first phase are solubility, volatility, and filterability. In the second phase, ammonia, metals, oxidants, cationic metals, volatiles, nonpolar organics, and metal chelates are specifically identified. The most economic and viable treatment designed to render some toxics inactive is an activated carbon adsorption or chemical oxidation (NPDES Seminar, 1988).

Cadmium chloride, synthesized and sealed by EPA's Cincinnati laboratory, was used as a reference toxicant. Hall (1989) describes cadmium's mode of action as a cumulative toxicant, mostly binding to red blood cells. About one-half the total cadmium adsorbed accumulates in the kidney and liver in humans. Cadmium binds to metallothionein, a protein of high sulfhydryl content with a capacity for binding zinc, copper and cadmium (Hall, 1989).

Certain invertebrates and fish are sensitive to low levels of cadmium. The most sensitive organisms are salmonids and cladocerans. Decreased toxicity of cadmium in freshwater acute toxicity tests is linked to increased hardness and/or alkalinity (EPA, 1976).

Outfall A passed three out of four tests performed on the two species (see Results). However, the fathead minnow survival test showed a significant difference between the control and Outfall A. The next logical step would be to perform a definitive test on the fathead minnow to assess an LC_{50} or a median lethal concentration. The sample would be subjected to the following five concentrations of Outfall A (100%, 50%, 25%, 12.5%, and 6.25%) with testing initiated within 96 hours after toxicity was discovered.

Limitations of this paper are the lack of a TIE and

the definitive toxicity test. However, the testing performed does indicate that the fathead minnow was the more sensitive of the two species to Outfall A, although this may not be true of the next sample discharge because many factors influence toxicity.

Shortcomings of toxicity testing are that there may be spikes of toxicity, between tests and some materials may take longer than seven days to accumulate for effect. The fathead minnow and Ceriodaphnia are used because they are hardy species, and they are cosmpolitan in the mainland United States. However, a one-species test is not valid enough to predict long term effects, yet a more sensitive species cannot be adequately assessed without much accumulated data over an extended period of time.

Another limitation is that moderately hard test water may not mimic the effluent because it may be soft water or vice versa. Metals are more toxic in soft water than hard water, for example. Also, other chemical properties of the riverbed may be present in the receiving stream, but not present in the laboratory-created water.

I believe the best method of assessing toxicity is a flow-through test in situ at the discharge site or at the immediate stream entry point. But this is expensive and sometimes impractical (it is not always 25°C in nature).

For the present, biomonitoring is the best technique to assess stream toxicity.

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APPENDICES

APPENDIX A

Dissolved Oxygen Meter Calibration

Date	Temperature (°C)	Expected D.O. (ppm)	Actual D.O. (ppm)	Battery
4/10/89	25	8.10	8.10	13.49
4/11/89	25	8.10	8.10	13.49
4/12/89	25	8.10	8.10	13.49
4/13/89	26	7.96	7.97	13.49
4/14/89	25	8.10	8.10	13.49
4/15/89	26	7.96	7.97	13.49
4/16/89	25	8.10	8.11	13.48
4/17/89	25	8.10	8.09	13.48

APPENDIX B

pH Meter Calibration

Date	Temperature (°C)	Buffer 1 pH 4.000	Buffer 2 pH 7.000	Buffer 3 pH 10.000
4/10/89	25.0	4.003	6.978	9.981
4/11/89	27.5	4.016	7.045	10.042
4/12/89	25.0	3.998	6.980	10.010
4/13/89	22.5	3.996	6.995	9.978
4/14/89	25.0	4.000	6.982	9.979
4/15/89	25.0	3.995	7.010	9.983
4/16/89	25.0	3.996	7.001	9.998
4/17/89	25.0	3.998	7.003	10.007

APPENDIX C

Fish Length and Weight Analysis

Sample ID	Average Length (mm)	Min/Max Length (mm)	Average Weight (mg)	Min/Max Weight (mg)
Control				
A	5.30		0.170	
B	5.20		0.180	
C	5.40		0.150	
$\bar{X}$ Control	5.30	4.0 - 6.0	0.167	0.10 - 0.20
Outfall A				
A	5.30		0.160	
B	5.10		0.160	
C	5.00		0.150	
$\bar{X}$ Outfall A	5.13	4.0 - 6.0	0.157	0.10 - 0.20
Ref. Tox. ($CdCl_2$)				
0.060 mg/l	4.35		0.100	
0.012 mg/l	4.25		0.100	
0.090 mg/l	4.40		0.100	
0.142 mg/l	4.00		0.100	
0.180 mg/l	4.30		0.100	
$\bar{X}$ Ref. Tox.	4.26	3.0 - 5.0	0.100	0.10 - 0.10