THE EFFECTS OF SINGLE AND JOINT TOXICITY OF ATRAZINE AND ALACHLOR ON THREE NON-TARGET AQUATIC ORGANISMS

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Robyn Ann Blackburn
M.S. Environmental Health Science

Abstract

The acute and chronic toxic effects of two commonly used herbicides were determined singularly and combined on three non-target aquatic organisms. Test organisms, Lemna minor (plant), Ceriodaphnia reticulata (invertebrate) and Pimephales promelas (fish) representing the different ecological trophic levels, were exposed to various concentrations of atrazine and alachlor to determine the EC50 or LC50 for each species and when possible determine the NOEC and LOEC values associated with each test. Short-term chronic and acute testing procedures utilizing static and static renewal test methods were employed throughout the study. Atrazine test results suggest that the EC50 for Lemna was under 200 ug/L. Acute LC50 for Ceriodaphnia was 7.8 mg/L; and approximately 15.0 mg/L for Pimephales larvae. The acute effects of alachlor were found to occur at 35.4 ug/L (EC50) for Lemna, 3.6 mg/L for Ceriodaphnia (LC50) and 4.9 mg/L for Pimephales (LC50). The combined effect of these herbicides was determined by joint tests using a 1:1 ratio of Toxic Unit concentrations demonstrated that a greater than additive effect occurred on Lemna and an additive or greater than additive effect was observed on the Pimephales larvae. Testing with Ceriodaphnia was discontinued after single testing due to problems in the stock culture maintenance.

Chronic effects were observed in all test species at concentrations significantly lower than the calculated LC50 or EC50 values. LOEC and NOEC values for Lemna indicate a chronic effect (frond development) occurs between 25 and 100 ug/L for atrazine and at or below 7.5 ug/L for alachlor. The lowest observable effect for Pimephales occurred at 3.0 mg/L for atrazine and 2.0 mg/L for alachlor.

Alachlor appears to be more toxic than atrazine to all organisms tested and is known to occur in the environment at levels found to be directly toxic in this study. The occurrence of a greater than additive effect is of concern since these herbicides commonly co-occur in aquatic environments.

Thesis Supervisor
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INTRODUCTION

Research Objective

The adverse effects of atrazine and alachlor can occur in the aquatic ecosystem by being directly toxic to the non-target species. The indirect effect can occur by altering or contaminating populations of organisms that serve as habitat or food for other wildlife. The objectives of this research were to identify and quantify the comparative acute and chronic toxicity of atrazine and alachlor; and to examine for the first time the joint toxicity of these herbicides to aquatic organisms. Three aquatic species Lemna minor (duckweed), Ceriodaphnia reticulata (water flea) and Pimephales promelas (fathead minnow) were chosen to represent different trophic levels within an aquatic system so that the toxic responses of the various components of a simple aquatic community could be compared. Laboratory toxicity testing procedures were utilized to establish estimated LC50 (or EC50) values; and the NOEC and LOEC levels of each chemical.

Background

The adverse effects of the thousands of chemicals and other anthropogenic compounds used in our daily lives is not a new concern. Efforts are continuously being made to examine the potential contamination to aquatic ecosystems through experimentation. Presently, the assessment of the toxicity of pollutants to aquatic

compounds. The effects may be expressed by quantifiable criteria such as number of organisms killed, percent egg hatchability, changes in length and weight, percent enzyme inhibition, number of skeletal abnormalities, and tumor incidence (Rand and Petrocelli, 1985).

In order to achieve the basic goals of the Federal Water Pollution Control Act Amendments of 1972 (PL 95-500) and the Clean Water Act of 1977 (PL 95-217) the biological monitoring of the nation's waters is needed. These Acts essentially call for the restoration and protection of the integrity of the nation's waters. The National Pollutant Discharge Elimination System (NPDES) permit program was initiated March 9, 1984 to help regulate and mitigate the impacts of discharged wastes on receiving waters. In part, the permit program requires that the waste be analyzed to determine the degree of toxicity and thus provide some measure of the adverse effects on aquatic systems. The required degree of toxicity control is only established by determining the anticipated adverse impacts on water quality. The single-species toxicity test has, thus, been adopted by the United States Environmental Protection Agency (USEPA) to standardize the approach to biological testing procedures.

Toxicity tests are often used by the USEPA and state NPDES programs to identify toxic discharges and as a self monitoring tool for NPDES permittees. The American Society for Testing and Materials (ASTM) was charged by the Federal Government with standardizing methods for toxicity testing using aquatic organisms. Standard methodologies for acute tests were first published in 1980 utilized

for use by regulatory agencies' and others in determining the level of toxicity of environmental pollutants.

Generally, acute toxicity tests utilized for aquatic organisms have commonly been used to determine the concentration of toxicant that produces mortality to a given species. Acute effects are those that occur rapidly as a result of short term exposure to a chemical (Parrish, 1985). A test is utilized to determine the amount of a chemical or effluent concentration, expressed as percent volume, that is lethal to 50 percent of the organisms within the prescribed period of time (LC50).

Most aquatic tests are conducted by exposing groups of organisms to several treatments in which the concentration of the material has been mixed with an acceptable prescribed dilution water. A common approach is to structure a test to elicit an all or nothing response: dead or alive. This quantel response produces the relationship between the concentration of the test material and the corresponding percent mortality of the organism. There are certain limitations that occur when using a laboratory to simulate the realism of the natural habitat. The tests examine only the responses of individuals which are averaged and given as mean responses for a particular test species (Cairns, 1981). A decade of single species testing has provided an experimentally reproducible measure of the toxicity of a test material. It has been found that a 96 hour exposure period in an acute test is generally sufficient to cover the period of lethal action (Parrish, 1985). Thus, acute lethality tests are useful to

rapidly estimate the concentration of a test material which will cause irreversible harm to a species (Macek et al, 1978).

Research over the past decade on partial and complete aquatic life cycle toxicity testing resulted in the realization that a quicker and less costly method was needed to meet the demand for more toxic data on an almost endless list of toxicants and chemical pollutants. An examination of existing fish life history tests revealed that certain developmental stages of most species of fish studied were more consistently sensitive than others. The chronic toxicity to fish can be predicted by the use of shorter tests with early developmental stages (ELS). Macek and Sleight (1977) found that exposure of critical lifestages of fish to toxicants provide estimates of chronically safe concentrations similar to the results from full life-cycle toxicity tests. They reported that "for a great majority of toxicants, the concentration which will not be acutely toxic to the most sensitive life stages is the chronically safe concentration for fish, and the most sensitive life stages are the embryos and fry." McKim (1977) evaluated data from 32 full life-cycle tests on fathead minnows and found the embryo-larval and early juvenile life-stages were the most sensitive stages. This research resulted in the establishment of ELS testing of fish as an acceptable method of testing for chronic effects (Horning and Weber, 1985). The chronic test typically measures parameters such as reproduction and/or growth (weight and length). The "no effect" or "safe "

concentration can be estimated from the quantified biological data produced by such a test.

Data from chronic tests are typically examined statistically to estimate the threshold concentration that produces significant deleterious effect. This level is commonly expressed as the maximum acceptable toxicant concentration (MATC) (Mount and Stephan, 1967). The highest concentration of toxicant to which organisms are exposed which causes no statistically significant adverse effect on the observed parameters is the No Observable Effect Concentration (NOEC). The lowest concentration of toxicant to which organisms are exposed which causes a statistically significant adverse effect on the observed parameters is termed the Lowest Observable Effect Concentration (LOEC). The MATC value is commonly expressed as that value that lies between the NOEC and LOEC. By comparing the MATC and the estimated environmental concentration of a chemical, an evaluation of the potential hazard to aquatic organisms is possible.

Many factors influence the effects of toxic materials in aquatic systems. The susceptibility of an aquatic environment to chemical toxicity often depends on the characteristics of the living components occurring in each ecological niche. In addition the ecosystem's physical and chemical properties can modify the impact of chemical pollutants. For example, some ecosystems may possess the ability to pH buffer an adverse impact or the natural flushing of a system (e.g. tidal action) may provide for removal or dilution of a chemical. Often physical-chemical processes (e.g. hydrolysis) which

chemical pollutants undergo in the environment may ameliorate toxic properties of the same contaminants. Goodman (1984) outlined several functional properties of a chemical that help predict its environmental behavior: (i) persistence in the environment. (ii) environmental mobility and (iii) failure to form inert compounds. From these general properties of a chemical substance a prediction of potential hazard can be assessed according to its solubility in water, dissociation constants, formation of chemical complexes, volatilization and other specific characteristics. However, knowing how a particular chemical substance will react or transform in one aquatic medium may not be enough to predict how the toxicity of the same chemical will be expressed in a similar type of ecosystem. Minor differences in biota can result in the fate and effect of the same chemical substance to be unpredictable.

The contact or exposure an aquatic organism has to a chemical in the aquatic medium is inescapable. Chemicals may be present in the water, sediment or in the food items ingested by the organism. Both water soluble chemicals, which are readily available to aquatic organisms, and water insoluble chemicals, which gradually dissociate from adsorbed surface provide for many routes of exposure. The exposure may affect the kinetic factors of the organism such as absorption, distribution, biotransformation, and excretion which ultimately determines the toxicity of a chemical (Pritchard, 1978).

Many of the abiotic and biotic characteristics that modify the toxicity of a pollutant have been studied. The abiotic conditions

are generally thought of as the physiochemical characteristics of the water surrounding the organism. The temperature, pH, dissolved oxygen concentration, salinity and hardness can all modify toxicity to an organism. Some biotic factors include the type of organism being exposed, life stage, size of individual, health status and degree of acclimation to the pollutant. These factors and their impact on test results must be considered in any testing procedure and standardized approaches when testing for the toxicity to aquatic organisms have been adopted (Standard Methods, 1985; Horning & Weber, 1985; Peltier & Weber, 1985).

The Federal Water Pollution Control Act of 1972 provided dramatic results in the control of conventional water pollution. One of the most controversial issues of the act was that it required the national goal of eliminating the discharge of all pollutants, or zero discharge, into America's waters by 1985. The act did not recognize that some wastes are toxic and do require zero discharge and other wastes can be tolerated or broken down by natural systems. With this realization the 1972 Act was amended with the Clean Water Act of 1977. This act divides water pollution into three categories: (1) toxic - chemicals with standards to be set by USEPA. (2) non-conventional - some pesticides and metal compounds and (3) conventional - organic wastes and sewage. The USEPA's approach to water pollution control basically focused on conventional pollutants with concentration on such parameters as pH, dissolved oxygen, biological oxygen demand and total suspended solids. The amendment

forced the EPA to develop a standardized procedure for evaluation the toxicity of water pollutants (Peltier & Weber, 1985; Horning and Weber, 1985).

The standardized acute and chronic toxicity testing procedures adopted by USEPA employ three basic techniques:

1) Static Test. This test is conducted by adding the test material to dilution water to produce the desired concentration. The organisms are exposed to the various concentrations and control in test containers which are kept at a controlled constant temperature by means of an environmental chamber room or water bath. The test solution is added once at the beginning of the test and organisms are exposed throughout the desired test period in the still water.

2) Static Renewal Test. A test procedure similar to the static test except the solution is renewed with a fresh solution of the same concentration every 24 hours. This method is preferred because a toxicant may absorb onto test chambers and the uptake by the organisms may change the degree of toxicity. A more steady exposure to a prepared concentration is achieved by renewing the test concentrations daily.

3) Flow Through Test. In this test water flow is maintained into and out of the chambers in which the organisms are kept. A flow through test provides a steady flow of the proper proportion of the test material and dilution water by means of a meter pump or diluter. Often cited as providing a more sensitive measure of toxicity than the static tests, it is most often be used in long term testing of

substances that may be degraded under static conditions. The flow through test requires a large volume of dilution water and is considered too costly and impractical to conduct off-site in a laboratory facility.

These methods were designed specifically to determine the presence of toxic material in effluent produced from point source such as manufacturing plants and hazardous waste sites, but are based on sound toxicological principles making them applicable to any toxicity test regime.

Toxicity testing is an important tool used by the USEPA to determine the potential toxicity of the more than 243,000 chemical substances recorded in the Inventory Candidate List for the U.S. Toxic Substance Control Act. The federal government also attempts to control 43,000 different pesticides produced by 7400 manufacturers, whose output is estimated at one billion tons of toxins (Cornaby, 1981). The chemical control of pests is achieved by applying the pesticide to adversely affect a specific group of organisms (target organism). Current mono-crop farming practices in the U.S. requires major applications of pesticides in order to produce the expected crop yield. Varying concentrations of the pesticides ultimately enter the aquatic environment through run off, over application and improper disposal practices. In the aqueous medium they may undergo transformation by photochemical and chemical reactions. The presence of pesticides and their by-products in aquatic systems can produce undesirable acute and chronic effect on organisms not originally

intended for control (non-target organisms). Toxicity tests can evaluate the effect of pesticides on populations of organisms by creating a direct toxic effect on aquatic species (Nimmo, 1985). Ridgeway et al. (1978) estimated world production of pesticides at 3.7 billion pounds of which the U.S. produced half. About 70 percent of the new chemicals produced in the United States are used in agriculture and insecticides, herbicides and fungicides comprise about 90 percent of all pesticides in agriculture. Of the herbicides used each year in the United States, 40 percent is applied to corn and 17 percent to soybeans (USDA, 1980).

The importance of studying the affects of herbicides is of special concern in the central United States where the country's leading field crop, corn, is grown. The primary herbicides used are alachlor, atrazine and 2,4 - D over a decade ago, atrazine and alachlor together account for 26.5 percent of the herbicide and 22 percent of the overall pesticide in the United States (von Rumker et al, 1974). Current use figures are difficult to be obtained, but overall use of these two herbicides has probably increased.

The Natural Resource Economics Division 1982 Staff Report estimated that atrazine was applied on corn crops at the rate of 37.3 million pounds per year. Atrazine (2 chloro-4-ethylaminol-6-isopropylamino-1,3,5-triazine) acts on non-tolerant weed species through electron transport inhibition. It inhibits photosynthesis by interrupting electron flow within a portion of the photosynthetic mechanism known as photo system II (Rao, 1976). Species such as

corn and sorghum are resistant to the affects of atrazine and thus provides an effective means of weed control for these crops. Atrazine production began in the late 1950's and quickly gained major recognition in agriculture. It's non-persistent nature and selective herbicidal properties lead to its extensive use with over 100 million pounds being applied annually to agriculture lands in the U.S. (Hall et al. 1972). Research with atrazine has demonstrated that direct toxicity to aquatic animals is questionable in contaminated habitats (Saunders, 1970; Tooby, et al., 1975; Macek, et al., 1976; deNoyelles, et al., 1982; Dewey, 1986). The estimated LC50 value of several species do not appear to occur unless relatively high concentrations of atrazine are present (Table 1). Currently, the direct toxicity of atrazine to non-target organisms appears to be of little concern because the actual concentrations of atrazine that occur in the environment are below most estimated LC50 values and the 95 percent confidence limits (Table 2).

The extensive use of atrazine and the lack of information concerning the long-term, subtle effects on aquatic prompted studies to determine the affects of continuous chronic exposure to fish and invertebrates (Macek et al., 1976). The maximum allowable concentration (MATC) was determined for certain fish and fish-food organisms to establish a more realistic and meaningful water quality criteria and standards. There have been extensive studies which examined the indirect toxicity of atrazine. When experimental ponds were treated with varying concentrations of atrazine, inhibition of

TABLE 1: Summary of lethal concentration (LC50) values for freshwater organisms caused by atrazine and alachlor exposure

Organism	LC50 (mg/L) Atrazine	Alachlor	Reference
Lepomis macrochirus (bluegill)	6.7	3.2	Macek et al. 1976
Pimephales promelas (fathead minnow)	15.0	5.0	Macek et al. 1976
Salmo gairdneri (rainbow trout)	-	1.4	Macek et al. 1978
Ictalurus punctatus (Channel catfish)	-	6.5	Birge et al. 1980
Rana pipiens (leopard frog)	7.6	-	Birge et al. 1980
Rana catesbeiana (bullfrog)	0.4	-	Birge et al. 1980
Chironomus tentans (midge)	0.7	-	Macek et al. 1976
Daphnia magna (water flea)	6.9	-	Macek et al. 1976

TABLE 2: Aquatic environment concentration values (aqueous phase) for alachlor and atrazine based on data retrieved from USEPA's Storet System for Iowa, Kansas, Nebraska and Missouri (EPA Region VII) during the period of record (1977-1984). Only those sample sites for which both alachlor and atrazine were analyzed have been included.

	Atrazine concentration (ug/L)	Alachlor concentration (ug/L)	Alachlor & Atrazine Co-Occurrence	Atrazine Only (No. of observations)	Alachlor & Atrazine Absent	Percent Co-Occurrence
Total Observ. (n=536)	3.20	3.86	479	54	3	90
Lentic (n=132)	1.23	0.29	130	2	0	98
Lotic (n=404)	3.85	5.02	349	52	3	87
Jun,Jul Aug (n=292)	4.51	6.11	287	4	1	99
Sep,Oct Nov (n=122)	0.48	0.08	102	20	0	84
Dec,Jan Feb (n=22)	0.04	0.31	8	14	0	57
Mar,Apr May (n=100)	3.39	2.68	82	16	2	84

Percent total observations for Iowa, 90%: Kansas, 9%: Nebraska, .08%: Missouri, .02%

photosynthesis within the phytoplankton community occurred at concentrations as low as 20 ug/L, a concentration often recorded in natural waters within agricultural watersheds (deNoyelles et al., 1982). It followed that the animal communities in the ponds were also affected by the change in plant communities. Dewey, 1986, found overall species richness, species equitability and total emergence also declined in the presence of 20 ug/L of atrazine.

It is now a common practice to mix and apply combinations of herbicides to produce the desired pest control. Another commonly used herbicide, alachlor, is often tank mixed with atrazine and then applied to crops. Alachlor [2-chloro-2',6-diethyl-N-methoxymethyl) acetanilide] is one of the most widely used herbicides of the chloracetamide group and currently is the most heavily used herbicide and insecticide in the United States (USDA, 1983). EPA estimates 90 to 95 million pounds are applied each year in the United States, primarily by corn and soybean farmers.

The mode of action of this non-ionic, highly water soluble (242 mg/L) compound has only recently been determined. Most studies suggest chloracetamide herbicides (i.e. alachlor) are inhibitors of protein synthesis (Jaworski, 1956; Mann, Jordan and Day 1965; Ashton, 1968; Rao and Duke, 1976). It has also been suggested the herbicidal action of alachlor is due to inhibition of ion absorption (Balke, 1979) and alteration of membrane permeability (Truelove et al. 1979).

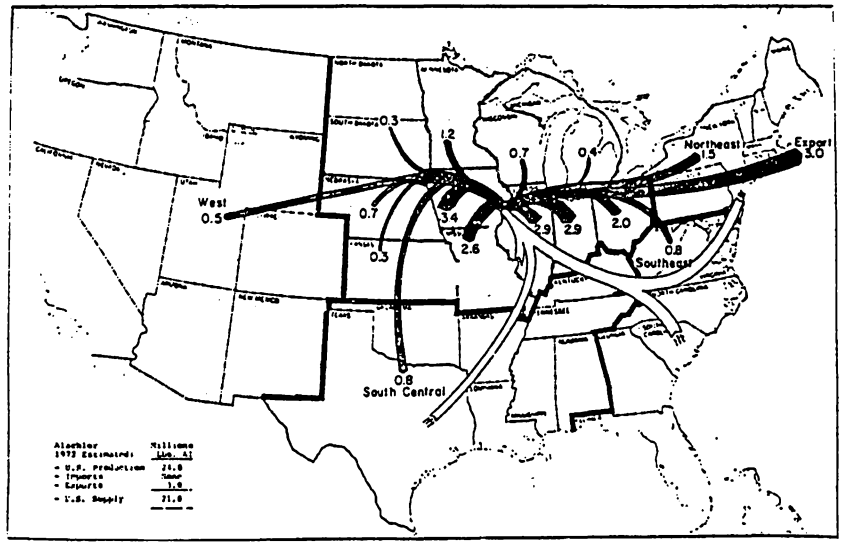
Recently the USEPA has temporarily imposed tighter restrictions on the use of alachlor as a result of tests conducted by the

manufacturer, The Monsanto Company, where laboratory animals fed alachlor developed cancer. The agency has categorized alachlor as a "probable human carcinogen" and is primarily concerned about potential hazards to the 1.3 million people in the farm communities who handle the chemical. The USEPA is also evaluating the potential risk the general public is exposed to from drinking alachlor-contaminated water. Alachlor has been detected, mostly at low concentrations, in surface and ground waters in several states (Science, 1986). Based on the behavior of other leaching type herbicides, concentrations will increase the longer it is used. Atrazine, which has been in use since 1959, took a long period of time to reach groundwater but is now commonly detected. Currently, Canada and the state of Massachusetts have banned the use of alachlor based on the Monsanto cancer studies and the detection of alachlor in water supplies (U.S. Water News, 1987).

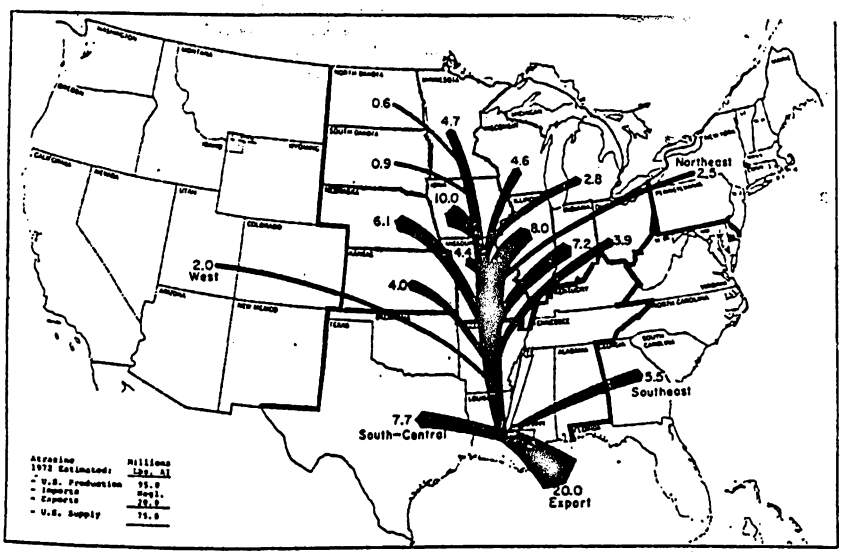
The acute toxicity of alachlor to aquatic organisms has only been estimated for a few species. The few studies which have been done indicate that the acute toxic responses of aquatic animals were relatively low compared to those of atrazine (Table 1). Even fewer tests have been done to examine the chronic response of aquatic organisms. The fact that low concentrations of alachlor produce acute toxicity to aquatic organisms is of concern. Its acute toxicity further accentuates the need to evaluate the more sensitive measure of chronic toxicity.

The need to identify the toxic effects of the combination of atrazine and alachlor is especially relevant in the agricultural regions of the United States. The materials flow diagrams (Figure 1) of von Rumker, et al. (1974) show the region of most heavy usage. Tank mixing and occurrence of atrazine and alachlor treated fields within watersheds probable accounts for the high percentage of co-occurrence of these two herbicides in aquatic environments (Table 2). It has been determined that soil surface applied herbicides (i.e. atrazine and alachlor) are lost to the environment at a rate of about five percent due to runoff of surface waters draining agricultural lands (Wachope, 1978). Accordingly, as much as 1.8 millon pounds of atrazine and 4.5 millon pounds of alachlor could have been lost to the environment in 1985.

It is evident that these two herbicides rarely occur singularly in the aquatic environments of the central U.S. yet the impact of the effects of potential joint action caused by the combination of these chemicals and other pesticides found in the environment has been seldom examined. Multicontaminant pollution of the aquatic ecosystems has been confirmed by insitu chemical monitoring. The simultaneous existence of chemical mixtures are found in both the ambient environment and in tissues of organisms (FAO, 1972; Kerr and Vass, 1973). This presents the problem of exposing organisms to two or more pollutants which may subsequently produce a new or different toxic threshold. Anderson and d'Apollonia (1978) defined a particularly hazardous joint toxicity producing an effect greater



Alachlor



Atrazine

FIGURE 1: Material Flow Diagram for Atrazine and Alachlor from von Rumker, et al., 1974

than that predicted on the basis of the potency of each component of a mixture. The complex nature of predicting and analyzing the reactions of chemicals resulting and joint toxicity created the need to develop a common measurement and terminology.

Over the past decade, the British Water Pollution Research Laboratory in Stevenage, has developed and tested a system where simultaneous effects of several pollutants are expressed as a single factor. Toxic Unit was developed to serve as the common factor when describing chemical mixtures' toxicity. The term, Toxic Unit (TU), originally was translated from the term °giftenhet' as used to compare relative toxicities of Swedish pulp mill wastes (Bergstrom and Vallin, 1937). More recently the development of the TU system began with Lloyd (1961b) who showed it would work for mixtures of copper and zinc salts.

The system is based on the incipient LC50's derived from single toxicant bioassay procedures on fish. The strength of a given toxicant as measured in any suitable chemical unit (e.g. ug/L) is expressed as a fraction or proportion of its lethal threshold concentration (measured in the same units) (Brown, 1968). The incipient LC50 is set to equal 1.0 Toxic Unit. When two toxicants are added and the number produced is greater than 1.0, half the fish will be killed. If the added number is less than 1.0, less than half the fish will be killed (Sprague and Ramsay, 1965). The illustration of results produced in bioassays are simplified and more easily

understood when analyzed by the method of toxic units (Sprague, 1969).

There has been confusion and ambiguity associated with the use of the terms synergism and antagonism when used to describe the effect caused by mixing two or more chemicals. The literature on mixture toxicity reflects the inconsistency in terminology and Sprague (1970) suggested the terms be avoided since different authors use the terms differently. Marking (1985) reported that the terms were non-quantitative and a more quantitative system was needed. In accordance to Sprague's 1970 interpretation, Calamari and Alabaster (1980) thought that the ideal model should describe the effects of mixtures of chemicals as simply additive, more than additive or less than additive while recognizing the possibility of synergism and antagonism to exist.

Gaddum (1948) defined the joint action of chemical agents which act simultaneously. Sprague (1969) adapted Gaddum's scheme to fit toxicity in an aquatic medium. The joint action is broken down into the three special cases as additive, less than additive and more than additive. The nature of joint toxicity of two components can be defined by exposing the organism to one half of the concentration of toxicant A (0.5 TU) which was needed to produce a given response (i.e. LC50) and one half of the concentration of toxicant B (0.5 TU) that produced the same result. Thus, the toxic units added together are equal to 1.0 TU. If this combination produces the same response (LC50) the action of A and B are exactly "additive" (Figure 2). If

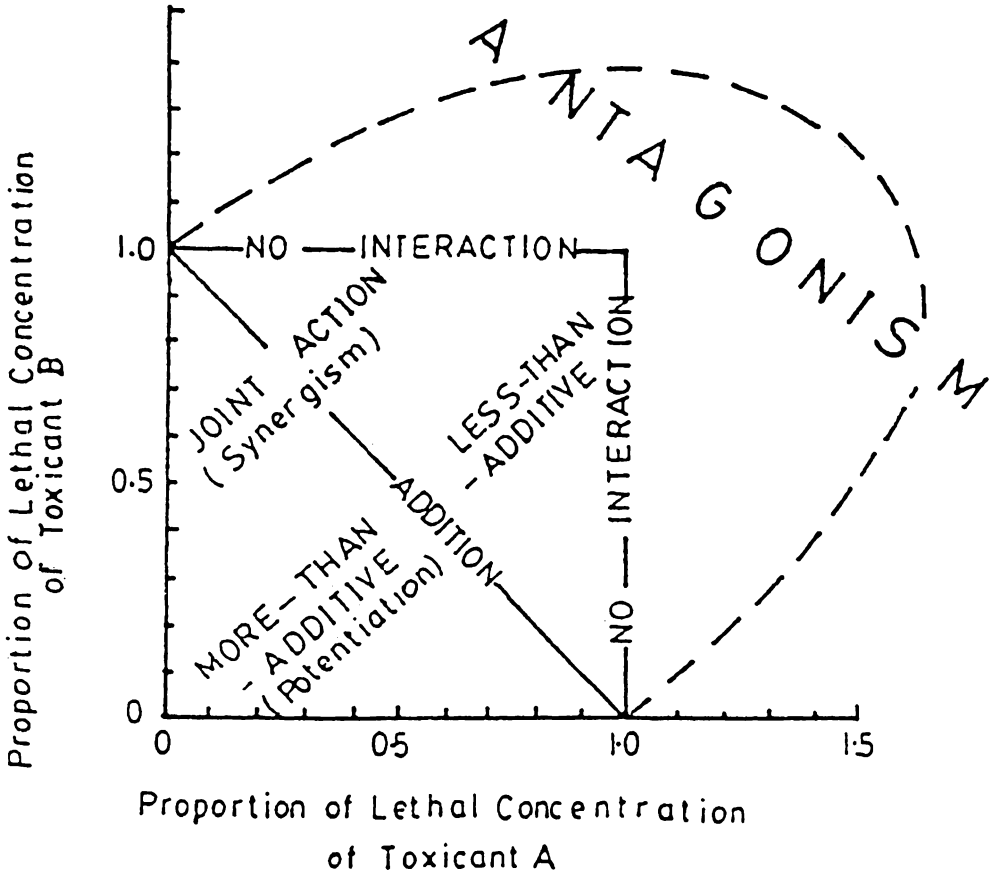


FIGURE 2: Types of Joint Action Between Two Toxicants in a Mixture. (from Alabaster & Lloyd, 1982)

the same combination was added together and it produced greater than the expected response then the observed toxicity is termed "more than additive". Figure 2 also diagrams an area to represent when the combination of the toxicants do not cause the given response (less than additive), show no interaction or are antagonistic. No theoretical basis has been claimed for this system. The various toxicants no doubt have different lethal mechanisms, but nevertheless the empirical observation is that sublethal effects sum within the fish and kill it. The lethal threshold is simply additive and therefore predictable (Herburt and Shurben, 1964). Sprague (197?) states that the strength of any toxicant may be calculated by the following equation:

$$\text{Toxic Unit} = \frac{\text{Actual Concentration}}{\text{Lethal Threshold of Concentration}}$$

This method may be used to calculate the number of toxic units for each component pollutant of a chemical mixture.

Test Organisms

The common duckweed, Lemna minor a widely distributed and often abundant aquatic macrophyte, was chosen as a test species because of its small size and rapid growth rate. These free floating perennial plants with a single root have been proposed as the typical aquatic macrophyte suitable for laboratory toxicity testing (Bishop and Perry, 1981). A typical Lemna plant is composed of a number of green, oval shaped, leaflike structures (fronds) and vegetative growth is accomplished through increases in the size and number of plant fronds. An estimate of growth inhibition of was determined by comparing the number of fronds produced in a given concentration to the growth in the control.

The original culture stock of Lemna was collected from a spring-fed stream located below the dam of Milford Reservoir in Geary County, Kansas. A clone culture was developed by isolating one healthy plant (two fronds) into a sterile three gallon plastic vessel containing approximately 1.5 gallons of NESA water. Nutrients were provided by adding an appropriate amount of Hutner's Solution (Appendix 1) to the NESA water and light levels were maintained at or near 400 foot candles in the environmental chamber in all the toxicity tests. This culture was held at 25 C and periodically thinned of dead or dying plants resulting from natural senescence. Culturing problems with the original Lemna stock made it necessary to conduct the joint toxicity tests and second set of single herbicide tests with Lemna minor plants from a different population.

Additional Lemna plants were obtained from a small oxbow lake located adjacent to the Kansas River near Lawrence, Kansas.

Approximately 500 Lemna plants were isolated from the stock clone culture one day prior to the initiation of each test. Only healthy appearing plants composed of two fronds of approximately equal size were selected as test organisms.

Ceriodaphnia, an invertebrate, is of the Cladoceran family. The Cladoceran are preferred food for fish larvae and are of great importance as food for the early life stages of development of various species (Filatov, 1972). Cladocerans are cosmopolitan in distribution and are commonly found in both the littoral and lemnetic zones of the United States (Pennak, 1953). Recently, Ceriodaphnia sp. have been recommended as a test species for acute and chronic toxicity testing (Mount & Norberg, 1985). Mortality was used to determine acute toxicity (LC50) and the chronic response (NOEC/LOEC) was measured by the rate of reproduction in various concentrations as compared to the control. Generally, control organisms produce three broods of young during a seven-day period which provides for the comparative background necessary when testing for a chronic response.

A Ceriodaphnia culture was received from the US Fish and Wildlife Service, Columbia National Fishery Research Laboratory Columbia, Missouri. A test culture was started by isolating one gravid adult in a one gallon jar containing dilution water. After a brood was produced, the adult was removed and a slide mount was prepared for positive identification. The young produced were

transferred to a fresh chamber once a week. New colonies were developed each month to maintain a young population of adults.

Fathead minnows (Pimephales promelas) are widely used in toxicity tests and are considered an acceptable USEPA test organism (Peltier & Weber, 1985). The fathead minnow is an ecologically tolerant fish and native throughout most of the United States in a variety of aquatic habitats. This species is known to be tolerant of low dissolved oxygen concentrations, high temperature and high turbidity. The fathead minnow is a primary or secondary consumer in most aquatic systems and is a forage fish for many other fish.

Fathead minnow cultures were obtained from the USEPA's Newton Fish Toxicology Station, Cincinnati, Ohio. Approximately 700 fathead minnow embryos (fully mature eggs) were express mailed to the Kansas Biological Survey, one day prior to the initiation of each test. Eggs hatched in transit and the larvae were then monitored in the lab for 4-20 hours. Their culture water was adjusted to 25 degrees celsius by raising the temperature one degree celsius for every two hours. Larvae were then selected and placed in the appropriate test solutions which were maintained at 25 degrees Celsius.

Fish larvae were fed newly hatched (less than 24-hour old) Artemia salina nauplii three times daily throughout the test period. Artemia were cultured in aerated 1000 ml separator funnels. The shrimp nauplii were triple rinsed with distilled water to remove brine and fed at a rate of 0.1 ml per chamber at each feeding.

Equipment Used in Test Procedures

All toxicity tests of this study were conducted in an Environmental Chamber, (Model 462, Hotpack Corp., Philadelphia, PA) located at the Kansas Biological Survey, Lawrence, KS. The chamber was equipped to provide a constant temperature of 25° C for all three test species. The chamber is capable of providing the specific amount of lighting required for each particular species. The chamber is also equipped with automatic timers which were set, in this case, to provide a photoperiod of 16 hours of light and 8 hours of dark during each test period.

Dissolved oxygen was measured using the YSI Model 58 Dissolved Oxygen meter equipped with a pressure compensating membrane probe. The meter was standardized using the Standard Method saturated air methodology prior to each set of measurements (APHA, 1985).

The Horizon Analog Mini-pH-Meter with an accuracy of ±0.1 pH unit was utilized throughout test procedures. The meter was calibrated against 7.0 pH buffer solution before each set of measurements.

The YSI 33 Conductivity Meter with accuracy ±2.5% for each of three scale ranges of 0-500, 0-5000 and 0-50,000 umhos/cm.

Weights were obtained with the Sartorius Electronic Analytical Balance (Model 1712, MP8, Westbury, NY). The balance was calibrated prior to measurement to evaluate weight to the 0.001 gram.

Fish were weighed after heating in the Drying Oven, Telco Laboratory Precision Oven, Chicago, IL, for two hours at 100° C.

Water Quality

The dilution water initially used for the first series of experiments was obtained from the reservoir at the University of Kansas Nelson Environmental Studies Area (NESA). All Ceriodaphnia tests were conducted using reservoir water. Also, the first Lemna minor and Pimephales promelas single tests with atrazine and alachlor were conducted in reservoir water. All other Lemna and Pimephales tests were performed using water obtained from a well source located at NESA. All water to be utilized in a test was collected one day prior to the initiation of each bioassay. The well lines were allowed to flush for at least two hours prior to collection to ensure consistency of water quality. Water was kept under refrigeration until needed and aerated by vigorously shaking prior to use. A single pesticide analysis performed by the Wilson Laboratory, Salina, Kansas, indicated that no quantifiable concentrations of atrazine and alachlor were present in the well water, Appendix 2. Daily chemical and physical analyses were performed on the water from at least one replicate chamber for each concentration level. Water quality measurements were obtained for temperature, dissolved oxygen, pH, specific conductance, total alkalinity, and total hardness.

Temperature was taken prior to the onset of each toxicity test procedure. Renewal water was adjusted to 25° C before using in all

test procedures. Dissolved oxygen was measured at the onset of all experiments and daily thereafter prior to static renewal procedures. Daily measurments of pH were taken in at least one representative in each concentration. Specific conductivity measurments were conducted in each concentration once at the onset, once during the test and at the termination of the study.

Water analyses for total alkalinity and total hardness were conducted according to Standard Methods (APHA, 1985) following procedures for titrametric-color change end-point. Alkalinity analysis utilized 100 m/L water samples, bromo cresol green methyl red indicator and 0.02N H2SO4 titrant. Total hardness was measured using 50 ml water sample. The titrant and reagent used were 0.02N EDTA (TitraVer) and UniVer 1, respectively. All reagents, indicators and titrants were obtained from HACH Company (Ames, Iowa).

All tests were conducted for a seven day period in an environmental chamber at the Kansas Biological Survey's ecotoxicology laboratory at the University of Kansas (Lawrence, Kansas). The chamber was set for a photoperiod of 16 hours light and eight hours dark with illumination at test chamber level at approximately 6456 Lux for Lemna tests and 3000 Lux for all other test organisms. Water temperature was maintained at 25 ±1° C. All containers and glassware were cleaned with detergent (Alconox^{R}), 20% hydrochloric acid, and acetone, then distilled water rinsed according to USEPA cleaning guidelines (Peltier and Weber, 1985) prior to their use in any test and periodically (24 hours) during the course of the fish tests.

use in any test and periodically (24 hours) during the course of the fish tests.

Toxicity testing time schedules and summaries of all herbicide concentrations utilized in the testing program are presented in Tables 3 and 4. Table 4 also lists the toxic units tested for all the joint toxicity tests.

Herbicide Test Solutions

Reagent grade atrazine at 99% purity was obtained from Chem Service Inc., West Chester, PA. It was necessary to prepare a fresh stock solution of atrazine prior to each bioassay. As the solubility of atrazine in water is only 32 mg/L special procedures were followed in order to effectively dissolve atrazine in the test water. A 20 mg/L atrazine stock solution was utilized to prepare all the solution concentrations. The following procedures were followed in order to effectively dissolve the reagent grade atrazine powder and to ensure that correct nominal concentrations were prepared for each test. The appropriate milligrams of atrazine powder and corresponding liters of dilution water needed for each experiment were calculated and adjustments were made for 99% purity. Atrazine was weighed to the 0.001 gram using the Sartorius Electronic Balance. The powder was added to 500 ml of dilution water and blended for approximately 15 minutes in a standard household blender with glass jar container attachment. Dilution water needed for stock solution was measured using a glass 1000 ml graduated cylinder and poured into a five

added to the carboy and mechanically stirred for a minimum of 12 hours. Stock solutions were kept refrigerated until needed.

Reagent grade alachlor powder, 97% pure, was also obtained from Chem Service Inc., West Chester, PA. Fresh stock solution of alachlor was prepared one day prior to the initiation of each bioassay. The alachlor stock solution was made by dissolving alachlor powder in the appropriate amount of dilution water. The solution was mechanically stirred approximately two hours in an amber one gallon glass jar until totally dissolved. Calculations included adjustments for 97% purity. Stock solutions of 200 mg/L or 100 mg/L were used in all alachlor testing as alachlor is very soluable in water (232 mg/L at 20° C). Stock solution concentrations were varied according to the concentration and quantity of test solutions needed for each specific toxicity test. All stock solution were kept under refrigeration until needed in an experiment.

Toxicity Test Procedures

The seven day static procedures for Lemna were similar to those methods as described by (Bishop and Perry 1981; Walbridge 1977; Wang 1986). The test chambers were 500 ml Pyrex storage dishes (with covers) measuring 80 mm tall and 100 mm in diameter.

Each toxicity test consisted of four replicate chambers per concentration. An experimental design of five to seven concentrations were utilized in each test run. In all tests each dish was filled with 400 ml of the appropriate test solution

concentrations were utilized in each test run. In all tests each dish was filled with 400 ml of the appropriate test solution (dilution water & herbicide) plus 10 ml of Hutner's Solution (Appendix 1) for all tests. The initial single herbicide tests utilized 20 Lemna minor plants (40 fronds) per vessel, but in later testing (test 2 and the joint test) only 10 plants (20 fronds) were used in each test vessel. A set of Control dishes (four) containing dilutant water, Hutner's Solution and Lemna colonies were concurrently tested with each set of concentrations. Thus, 40 to 80 plants were exposed to each concentration or used as controls in the dilution water. At the initiation of a test, the Lemna colonies were carefully transferred one at a time into each dish until each dish contained the specified number of plants (40 or 80). The containers were closed with the dish covers and were placed in the environmental chamber for a seven-day incubation period. The chamber illumination was provided by cool white light at an intensity of approximately 6456 Lux. At the end of the incubation period all plants were examined with the aid of a dissecting microscope and the number of fronds per dish recorded. The net growth of Lemna in the control, (final frond number minus the beginning frond count) was compared to the net growth recorded in the various concentrations. The concentration-response effect relationship was determined from the percent growth inhibitation data and a EC50 value estimated.

Lemna minor was first tested to determine the EC50 values for atrazine and alachlor, singularly, then a joint test (atrazine &

toxicity tests were concurrently conducted with the joint tests to ensure the accuracy of the first estimated EC50 values for each herbicide.

The basic seven-day static renewal procedures used in the Ceriodaphnia tests are described in detail by Horning and Weber (1985) and Peltier and Weber (1985). Each test consisted of ten 30 ml disposable plastic salad dressing cups per concentration; each cup containing 15 ml of test solution and one neonate less than 24 hours old. The test method requires that all the neonates used in a test must be 2 to 24 hours old and within four hours of the same age. During the test the Ceriodaphnia are fed a daily chow-yeast-Cerophyl(R) diet at a rate of 0.1 ml per vessel.

Tests were started by carefully transferring one neonate (less than 24 hours old) into a plastic dish using a 2mm bore pipet until all cups contained one neonate. Thus, 10 neonates were exposed to each concentration and 10 were used as contol organisms. Test vessels were placed into the environmental chamber and removed once every 24 hours for required cleaning and solution renewal. At this time, adult survival and number of young produced were observed using a stereomicroscope and recorded. The adult was then transferred into fresh solution; and the young discarded and the test vessels returned to the environmental chamber.

All Ceriodaphnia tests were terminated with all observations completed within 2 hours of completion of the seven day test. This

All Ceriodaphnia tests were terminated with all observations completed within 2 hours of completion of the seven day test. This procedure was necessary to prevent the counting of additional broods that could result with a test extension of even a few hours.

The seven-day static renewal procedures used in this test parallel those set forth by the U.S. Environmental Protection Agency (Horning and Weber, 1986) and Norberg and Mount (1985) for estimating the acute and chronic toxicity to fathead minnow.

The test chambers were 1000 ml spoutless beakers, 19 cm tall and 10 cm in diameter. Beaker covers were utilized to reduce evaporation and prevent contamination from outside sources. Each test incorporated four replicate 1000 ml glass beakers per concentration. An experimental design of three to eight concentrations were utilized for each test procedure. Each beaker contained 500 ml of test solution and 10 fathead minnow larvae less than 24 hours old. Additionally, a set of control beakers containing dilution water and fatheads were maintained for each test.

At the beginning of each test the fathead minnow larvae were added one at a time to each beaker until all beakers contained 10 fish. A total of 40 fish were used in each test concentration or control. All beakers were maintained in an environmental chamber at 16 hours light/8 hours dark cycle with illumination set at approximately 3000 Lux. Beakers were removed once a day to record mortality and to renew test solutions. A light box was used to aid in counting the number of surviving fish in each beaker. After

that approximately 200 ml of test water remained. The vessel would be carefully cleaned using a large bore pipette (4 mm) fitted with a rubber bulb. Uneaten dead brine shrimp, other organic debris and dead fish larvae were removed from the bottom of each chamber. Immediately after cleaning the test chamber fresh test solution was slowly added to prevent excessive turbulence and minimize stress on the remaining larvae. Test vessels were then re-covered and returned to the environmental chamber.

At the conclusion of the seven-day period the surviving fish were removed from each of the test vessels, preserved in 4 percent formalin and placed in labeled vials until they could be dried and weighed. Dry weight analysis of the preserved fish were performed within one week of the conclusion of the test. Weighing dishes were formed from heavy duty household aluminum foil and impressed with an identification number. The foil dishes were then heated in a drying oven for one half hour at approximately 60° C and allowed to cool in a standard desiccator for one half hour. Next, the foil heating dishes were weighed to the 0.01 mg using a Sartorius Electronic Analytical Balance. All fish from each of the vials containing the preserved surviving fish form each test chamber were rinsed with deionized water; placed onto individule pre-weighed foil dishes and dried at 100° C for two hours in the drying oven. Dry weights of each fish group were obtained after cooling in a desiccator for one hour.

dishes and dried at 100 degrees Celsius for two hours in the drying oven. Dry weights of each fish group were obtained after cooling in a desiccator for one hour.

Establishing Toxic Units

The toxic effects of a 1:1 mixture of atrazine and alachlor were examined by adding up "Toxic Units" of the individule herbicides. This rather simple method of predicting the effects of chemical mixtures has been used sucessfully by many researchers (e.g. Lloyd 1961; Herbert and Shurben 1964; Sprague and Ramsey 1965; Brown and Dalton 1970). Toxic Units (TU) for all joint tests were based on the LC50 or EC50 values derived from the single atrazine and alachlor tests. The joint test series was conducted by combining atrazine and alachlor at concentrations representing increasing TU levels. For example, the 1.0 TU mixture level would equal one half the LC50 concentration of each herbicide. The first joint toxicity test was conducted using TU values established from the LC50 or EC50 concentrations obtained from the results of the first Lemna and fathead minnow tests with atrazine or alachlor. A second joint test was conducted along with concurrent separate single toxicity tests. This concurrent series of tests were conducted to provide a second estimate of individual LC50 and EC50 values and to utilize these new values obtained under similar test conditions as the joint test to adjust the previously determined TU values if LC50 or EC50 values differed from intial established values. Because of the lack of

precision sometimes associated with toxicity testing (Macek, 1985) this later set of concurrent of toxicity tests were run to examine the potential variability in test results.

Results of the single herbicide tests conducted concurrently with the second joint test indicated that the original TU values would have to be adjusted to account for difference in LC50 or EC50 values. This was accomplished by recalculating the TU values based on the actual concentrations representing the new LC50 or EC50 values. Figure 3 illustrates the method used to adjust each TU level. The Trimmed Spearman-Karber Analysis of the LC50 for the joint test was then calculated using the adjusted TU values.

Chemical A

$$\frac{\text{Original EC50 or LC50}}{\text{Concurrent EC50 or LC50}} = \text{Adjusted Toxic Unit}$$

Chemical B

$$\frac{\text{Original EC50 or LC50}}{\text{Concurrent EC50 or LC50}} = \text{Adjusted Toxic Unit}$$

The two adjusted Toxic Units added together represent the actual amount of the 2.0 TU level used in the original joint test.

Figure 3: Toxic Unit Adjustment Procedure used to establish new TU values based on estimated LC50 and EC50 from concurrent single test results.

Statistical Analyses

Acute Toxicity

In an acute toxicity test groups of aquatic animals are exposed to progressively increasing concentration of a toxicant. It is reasonable to expect that the percentage of deaths will increase monotonically with the increased concentration of the toxicant (Gelber, et al 1985). The primary purpose of the test is to estimate the concentration of the test material that is lethal to 50% of the organisms within a specific time period, in this case 96 hours. The LC50 or EC50 value can be derived by observation, interpolation, or calculation. Calculating these values is often the most desirable method, as both a LC50 or EC50 value and its 95% confidence limits can be determined. The Trimmed Spearman-Karber method was utilized in analysis of all acute toxicity data. This non-parametric technique is an especially reliable method (Hamilton et al 1977) which always produces estimates that satisfy the monotonic relationship normally found between concentration and quanile responses. It is also one of the statistical procedures recommended by Peltier & Weber (1985) for use in calculating the estimated LC50 and its 95% confidence limits. The computer program used for all the analyses of this paper was designed by Alex Slater of the Kansas Biological Survey.

In determing the LC50 and EC50 values associated with the joint toxicity tests it was necessary to convert the Toxic Unit value levels used in these tests to the corresponding herbicide

concentration value represented by that level. An example of this conversion procedure is given in Figure 4.

The following is an example of the procedure that was used to find chemical concentration present in adjusted Toxic Unit values:

Adjusted TU values were used in Trimmed Spearman-Karber analyses with the EC50 or LC50 given by computer program as 0.9 TU.

The proportion of this amount to the original 1.0 TU was obtained:

1.0 TU / 0.9 TU = 1.1 TU

The original concentrations of each chemical set to equal 1.0 TU:

Atrazine at 1.0 TU = 7.5 mg/L

Alachlor at 1.0 TU = 2.0 mg/L

The amount of the chemical present in the EC50 or LC50 estimate at the 1.0 TU level divided by the proportional amount will give the concentration of each chemical present in the EC50 or LC50 estimate:

Equivalence concentration at the 0.9 TU level:

Atrazine = 7.5 mg/L / 1.1 = 6.8 mg/L

Alachlor = 2.0 mg/L / 1.1 = 1.8 mg/L

FIGURE 4: Toxic Unit Chemical Equivalance Procedure used to determine the concentration of each chemical represented at each Toxic Unit level.

Chronic Toxicity

The chronic effects of atrazine and alachlor to Ceriodaphnia and fathead minnow larvae were estimated by examining the growth and/or reproduction of test organisms exposed to various levels of herbicide. The short-term methods for estimation of chronic toxictity proposed by Horning and Weber (1985) were used to predict the effect and no-effect concentrations of these herbicides. The reproduction and growth (weight) results of the tests performed were summarized for each concentration and compared to the responses of the control organisms. The Analysis of Variance (ANOVA) was applied to the data in order to determine whether the observed differences between the control and the test concentrations were due to random variation or real differences. When the standard ANOVA F-test determined that there was a significant difference ($P > 0.05$) between the control and the concentrations used, multiple comparison tests were employed to identify critical concentrations.

The Dunnett's Procedure (Dunnett, 1970) is the currently recommended method in aquatic toxicity testing for comparing several experimental samples to a concurrent control (Horning and Weber, 1985). This procedure provides a cutoff value for the difference in response between the test concentration and the control. The lowest concentration for which the difference in observed response exceeds the cutoff value is defined as the lowest observable effect concentration or LOEC. The highest concentration for which differences in response is not greater than the cutoff value is

defined as the no observable effect concentration or NOEC (Gelber et al, 1985). The maximum allowable toxic concentration (MATC) is some value greater than the NOEC and less than the LOEC.

It has been suggested that the Dunnett's procedure is not the most powerful test available for use in identifing toxic concentration as this procedure does not consider the concentration-response ordering of effects. The William's Test (Williams, 1971,72) is preferable because it is designed to specifically detect an increasing concentration response (Gelber et al, 1985). Therefore, the ANOVA, followed by the Dunnett's Procedure and also the William's Test were utilized in the analyses of chronic response data.

Table 3: Atrazine and Alachlor Single Toxicity Testing Schedule

Lemna minor

Pesticide	Date	Concentrations (ug/L)
Atrazine	5 Nov 1986 - 12 Nov 1986	25.0, 50.0, 100.0, 150.0, 200.0
Atrazine	23 Jun 1987 - 30 Jun 1987	25.0, 50.0, 75.0, 85.0, 200.0
Alachlor	14 Jan 1987 - 21 Jan 1987	5.0, 7.5, 10.0, 15.0, 20.0
Alachlor	23 Jun 1987 - 30 Jun 1987	5.0, 1.00, 15.0, 25.0, 30.0 40.0

Ceriodaphnia reticulata

Pesticide	Date	Concentrations (mg/L)
Atrazine	8 Nov 1986 - 15 Nov 1986	0.50, 1.0, 2.0, 4.0, 6.0
Atrazine	12 Feb 1987 - 19 Feb 1987	0.75, 1.5, 3.0, 6.0, 9.0
Alachlor	16 Jan 1987 - 23 Jan 1987	1.0, 2.5, 5.0, 7.5, 10.0
Alachlor	10 Feb 1987 - 17 Feb 1987	0.5, 1.0, 2.0, 3.0, 4.0

Pimephales promelas

Pesticide	Date	Concentrations (mg/L)
Atrazine	6 Nov 1986 - 13 Nov 1986	0.25, 0.50, 1.00, 2.00, 4.00
Atrazine	25 Mar 1987 - 1 Apr 1987	0.50, 0.75, 1.00. 1.25, 1.50, 3.00, 6.00, 10.00
Atrazine	16 Apr 1987 - 23 Apr 1987	12.00, 15.00, 18.00
Atrazine	3 Jul 1987 - 10 Jul 1987	1.50, 3.00, 6.00, 12.00, 20.00
Alachlor	16 Jan 1987 - 23 Jan 1987	1.00, 2.00, 3.00, 4.00, 5.00
Alachlor	25 Mar 1987 - 1 Apr 1987	0.50, 1.00, 1.25, 1.50, 1.75, 2.00, 3.00, 6.00
Alachlor	3 Jul 1987 - 10 Jul 1987	0.50, 1.00, 2.00, 4.00, 8.00

TABLE 4: Joint Toxicity Testing Schedule of concentrations used at each Toxic Unit level for Lemna minor and Pimephales promelas.

Lemna minor Joint Test 1	13 June 1987 - 20 June 1987	
Toxic Unit	Atrazine Concentration (ug/L)	Alachlor Concentration (ug/L)
1.00	86.30	25.00
0.25	10.80	3.10
0.50	21.60	6.30
0.75	32.30	9.40
1.00	43.10	12.50
1.25	53.90	15.60
1.75	75.50	21.90
2.00	86.30	25.00

Pimephales promelas Joint Test - 1	11 June 1987 - 18 June 1987	
Toxic Unit	Atrazine Concentration (mg/L)	Alachlor Concentration (mg/L)
1.00	13.17	4.14
0.25	1.65	0.45
0.50	3.29	0.89
0.75	4.94	1.34
1.00	6.58	1.79
1.25	8.23	2.24
1.75	11.52	3.13
2.00	13.17	3.58

Pimephales promelas Joint Test - 2	3 July 1987 - 10 July 1987	
Toxic Unit	Atrazine Concentration (mg/L)	Alachlor Concentration (mg/L)
1.00	15.00	4.14
0.50	3.75	1.00
1.00	7.50	2.00
2.00	15.00	4.10
4.00	30.00	8.20

RESULTS

Water Analysis

All water quality parameters recorded for the toxicity tests were within the APHA Standard Method requirements for toxicity testing (APHA, 1985) except dissolved oxygen concentrations in some fathead minnow tests. Low dissolved oxygen levels consistently occurred with all control and test concentration replicates in the fathead minnow tests only. Prescribed measures were utilized to gradually raise dissolved oxygen concentration prior to solution renewal if values were 3.0 mg/L and below. This method prevented the fish from being exposed to sudden changes in dissolved oxygen concentrations. When necessary, and as a last resort, all vessels were gently aerated as outlined in the EPA/600/4-85/014, 1985 manual. Fish were carefully observed and it was determined by analysis of the acute and chronic results that neither mortality or weight reduction were found to correspond to with the low dissolved oxygen levels. The means and ranges of water quality parameters for the control and each test concentration were evaluated by a standard ANOVA F-test for all toxicity tests which were performed. The evaluation of water parameters in the control as compared to the test concentrations revealed that the differences were insignificant. It was therefore concluded that any toxic responses were incurred as a result of exposure to the chemicals and not related to the dilution water. The means () and ranges for all single and joint toxicity tests are presented in Appendix 2.

Lemna minor

Atrazine Test 1

The total frond count for all four replicate chambers were summed at the conclusion of the seven day test period and are presented in Table 5. The net frond growth was found to decrease with increasing concentration of atrazine, Figure 5. The Trimmed Spearman-Karber analysis estimated the EC50 value to be 86.2 ug/L, Table 6. The Dunnett's Procedure, Table 7, and the Williams' Test, Table 7a, were used to calculate the NOEC-LOEC values for reduction in growth. Both statistical procedures indicated the levels of 50 ug/L and 100 ug/L to be the NOEC-LOEC values, respectively.

Table 5: Results of Atrazine Test 1 with Lemna minor conducted November 5 - 11, 1986.

	(ug/L)					
	Control	25.0	50.0	100.0	150.0	200.0
Frond Growth (net)	414	350	308	173	142	153
Mean Growth/chamber	104	88	77	43	36	38
Standard Deviation	24	35	24	12	1	1
Reduction of Growth	0	64	106	241	272	261
Percent Inhibition	0	15	25	58	65	63

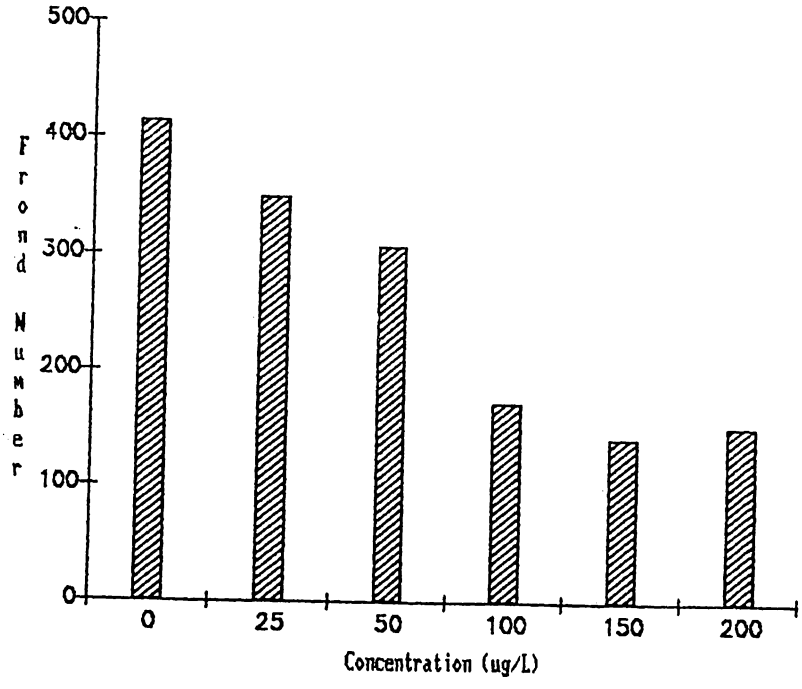


FIGURE 5: Net frond growth of Lemna minor for Atrazine Test 1 conducted from November 5 - 11, 1986.

TABLE 6: Trimmed Spearman-Karber EC50 estimate for Lemna minor frond inhibition based on result from Atrazine Test 1 November 5 - 11, 1986.

CONCENTRATION		ORGANISMS	DEAD	RAW	ADJUSTED
Raw	ln	EXPOSED	ORGANISMS	MORTALITY	MORTALITY
25.00	3.22	414	64	0.16	0.16
50.00	3.91	414	106	0.26	0.26
100.00	4.61	414	241	0.58	0.58
150.00	5.01	414	272	0.66	0.64
200.00	5.30	414	261	0.63	0.64

Spearman Karber Trim = 36.0%

	ln	ug/L
Estimated EC50	4.5	86.3
Lower 95% Confidence Limit	4.4	77.4
Higher 95% Confidence Limit	4.6	96.1

Table 7: Dunnett's procedure for differences from control for Lemna Atrazine Test 1

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	16668.50	5	3333.7	8.10	6.81***
Within	7412.40	18	411.8		

***P<0.001

Observed Differences From Control

ug/L	Difference	ti		Dunnett's Critical t
control	0.0	0.00		
25	16.0	1.10		
50	26.7	1.86	<NOEC	2.41*
100	60.2	4.19	<LOEC	
150	68.0	4.74		
200	65.0	4.53		

*P<0.05

Table 7a: Williams' t test for differences from control for Lemna Atrazine Test 1

Error Mean Square from ANOVA: 411.8

Mean response is expected to decrease as treatment concentration increases.

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML Estimator	$\bar{t}$
0.00	4	104.00					
25.00	4	88.00	88.00	1.12		88.00	1.12
50.00	4	77.00	77.00	1.88	<NOEC	77.00	1.88
100.00	4	43.00	43.00	4.25	<LOEC	43.00	4.25
150.00	4	36.00	37.00	4.67		36.00	4.74
200.00	4	38.00	37.00	4.67		37.00	4.70

k = 5
v = 15 Williams' Critical t = 1.89*

*P<.05

Alachlor Test 1

The results of the seven day Lemna minor test with alachlor conducted on November 5, 1986 are presented in Table 8. Net frond growth declined only slightly over the range of concentrations of alachlor used in this test, Figure 6. The Trimmed Spearman-Karber EC50 value could not be calculated because the highest concentration used in the test, 20 ug/L, did not produce at least 50% decrease in frond development. The EC50 value was expected to occur above the highest concentration used in this test. The graphical analysis of the percent inhibition verses concentration is illustrated in Figure 6a. The NOEC was 5.0 ug/L and the LOEC was 7.5 ug/L as determined by the Dunnett's Procedure, Table 9, and the Williams' Test, Table 9a.

Table 8: Lemna Alachlor Test 1 - 14 January 1987 - Frond Growth Summary

	(ug/L)					
	Control	5.0	7.5	10.0	15.0	20.0
Frond Growth (net)	178	161	123	126	111	105
Mean Growth/chamber	45	40	31	32	28	26
Standard Deviation	10.3	9.1	6.3	6.8	5.8	3.8
Reduction of Growth	0	17	55	52	67	73
Percent Inhibition	0	10	31	30	38	42

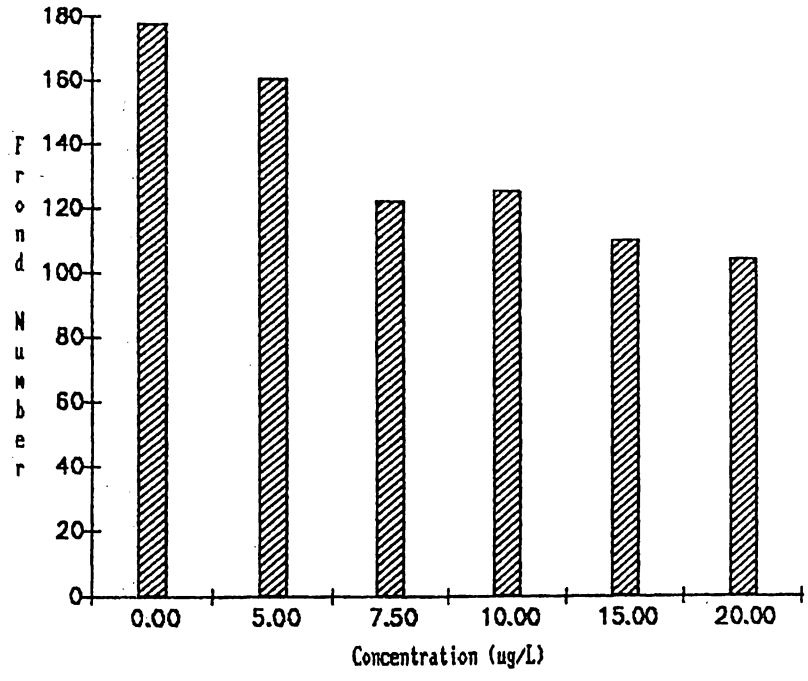


FIGURE 6: Net frond growth of Lemna minor for Alachlor Test 1 conducted from January 14 - 21, 1987.

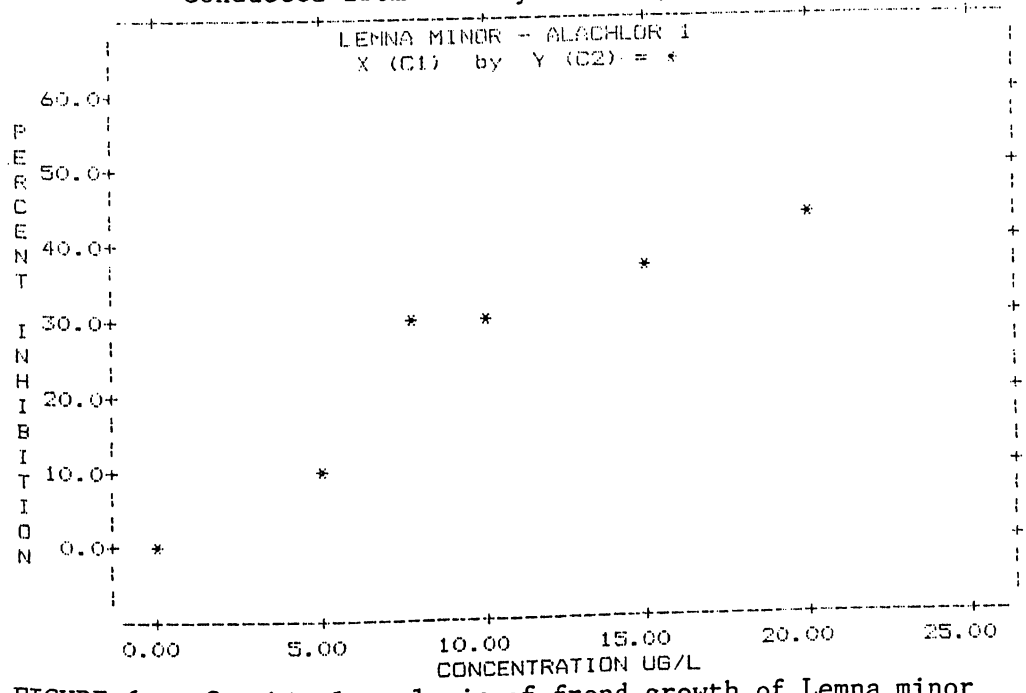


FIGURE 6a: Graphical analysis of frond growth of Lemna minor for Alachlor Test 1 - 14 January 1987.

Table 9: Dunnett's Procedure for Data Set: Lemna - Alachlor Test 1

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	5	211.0	3.88	2.77*	
Within	18	54.3			

*P<0.05

Observed Difference From Control

ug/L	Difference	ti		Dunnett's Critical t
control	0.00	0.00		
5.0	4.25	0.82	<NOEC	2.41*
7.5	18.00	3.45	<LOEC	
10.0	17.25	3.31		
15.0	21.00	4.03		
20.0	22.50	4.32		

*P<0.05

Table 9a: Williams' t Test for Data Set: Lemna - Alachlor Test 1

Error Mean Square from ANOVA: 54.27

Mean response is expected to decrease as treatment concentration increases.

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$	ML' Estimator	$\bar{t}'$
0.00	4	45.00				
5.00	4	40.00	40.00	0.96<NOEC	40.00	0.96
7.50	4	31.00	31.50	2.59<LOEC	31.00	2.68
10.00	4	32.00	31.50	2.59	31.50	2.59
15.00	4	28.00	28.00	3.26	28.00	3.26
20.00	4	26.00	26.00	3.65	26.00	3.65

k = 5
v = 15

Williams' Critical t = 1.89*

*P<.05

Atrazine Test 2

Net frond growth indicated that a significant decrease occurred near the highest concentration of 200 ug/L of atrazine, Figure 7. The results of the Trimmed Spearman-Karber test showed a significantly higher EC50 value for atrazine. The value of 197.4 ug/L was determined as the EC50 at this time, Table 11. The original value estimate tested under slightly different conditions, was 86.2 ug/L. An adjustment of the values used in the joint test was necessary in order to accurately determine the toxic levels, Table 18.

The NOEC-LOEC values were calculated and indicated the levels as the control and 25 ug/L, respectively (Table 12 and 12a). These values indicated that a lower concentration effected the growth of Lemna than was previously estimated in Atrazine Test 1.

Table 10: Lemna Atrazine Test 2 - 23 June 1987 - Frond Growth Summary

	(ug/L)					
	Control	25.0	50.0	75.0	85.0	200.0
Frond Growth (net)	139	100	105	103	102	69
Mean Growth	35	25	21	26	26	16
Standard Deviation	3.4	2.1	8.4	5.1	3.3	2.8
Reduction of Growth	0	39	34	36	37	66
Percent Inhibition	0	28	39	26	27	55

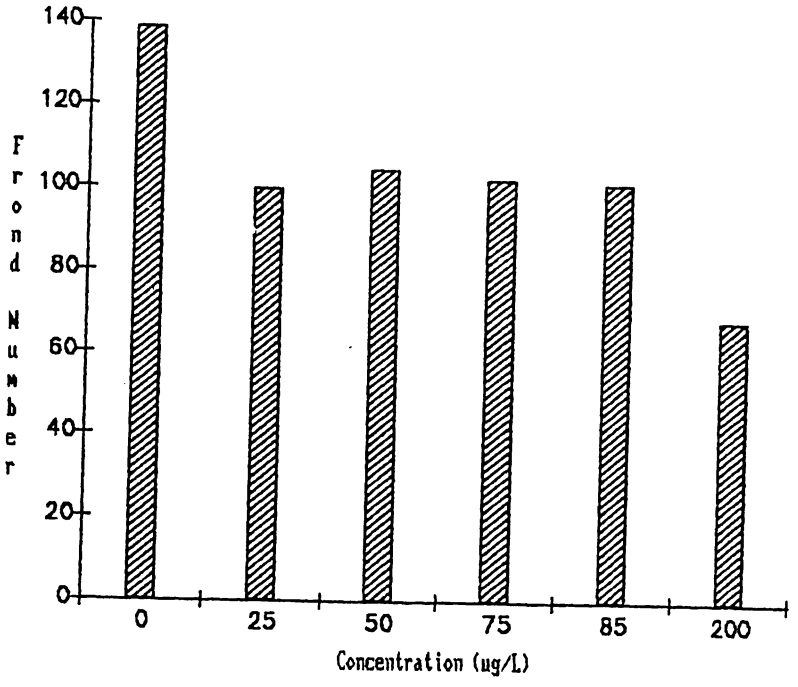


FIGURE 7: Net frond growth of Lemna minor for Atrazine Test 2 conducted from June 23 - 30, 1987.

TABLE 11: Trimmed Spearman-Karber EC50 Estimate for Lemna minor Atrazine Test 2 conducted 23 June 1987.

CONCENTRATION Raw	CONCENTRATION ln	ORGANISMS EXPOSED	DEAD ORGANISMS	RAW MORTALITY	ADJUSTED MORTALITY
25.00	3.22	139	39	0.28	0.26
50.00	3.91	139	34	0.25	0.26
75.00	4.32	139	36	0.26	0.26
85.00	4.44	139	37	0.27	0.27
200.00	5.30	139	70	0.50	0.50

Spearman Karber Trim = 49.7%

	ln	ug/L
Estimated EC50	5.29	197.42
Lower 95% Confidence Limit	4.98	146.10
Higher 95% Confidence Limit	5.59	266.79

Table 12: Dunnett's Procedure for Data Set: Lemna - Atrazine Test 2

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	679.85	5	135.97	6.1*	2.77
Within	399.60	18	22.20		

*P<0.05

Observed differences from Control

ug/L	difference	ti			Dunnett's Critical t
control	0.00	0.00	<	NOEC	
25	9.75	2.52	<	LOEC	2.41*
50	13.50	3.49			
75	9.00	2.32			
85	9.25	2.39			
200	17.50	5.30			

*P<0.05

Table 12a: Williams' t Test for Data Set: Lemna - Atrazine Test 2

Error Mean Square from ANOVA: 29.98

Mean response is expected to decrease as treatment concentration increases.

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML Estimator	$\bar{t}$
0.00	4	35.00			<NOEC		
25.00	4	25.00	25.00	2.58	<LOEC	25.00	2.58
50.00	4	21.00	24.33	2.76		21.00	3.62
75.00	4	26.00	24.33	2.76		23.50	2.97
85.00	4	26.00	24.33	2.76		24.33	2.76
200.00	4	16.00	16.00	4.91		16.00	4.91

k = 5
v = 15

Williams' Critical t = 1.89*

*P<.05

Alachlor Test 2

The second Lemna test with alachlor indicated that the degree of toxicity to Lemna, as determined by the reduction of growth, was somewhat similar to the estimated concentration derived from graphical interpreted value from the first test. The calculated EC50 value from this test was 35.4 ug/L, Figure 8 and Table 14. The results of the multiple comparison tests used to identify the chronic effect, no effect levels for growth inhibition, revealed that the control and the 5.0 ug/L were the NOEC and LOEC values, Table 15 and 15a. These values were only slightly lower than the NOEC of 5.0 ug/L and LOEC of 7.5 ug/L reported for the first alachlor test.

Table 13: Lemna Alachlor Test 2 - 23 June 1987 - Frond Growth Summary

	(ug/L)					
	Control	5.0	15.0	25.0	30.0	40.0
Frond Growth (net)	139	108	85	88	79	62
Mean Growth	35	27	21	22	20	16
Standard Deviation	3.4	2.1	5.9	0.8	3.3	3.9
Reduction of Growth	0	31	54	51	60	77
Percent Inhibition	0	22	39	37	43	55

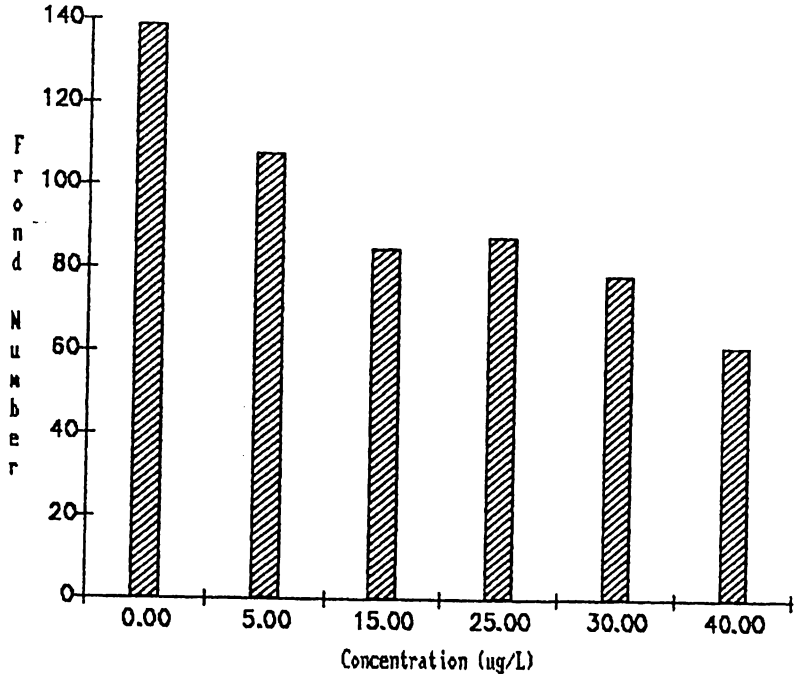


FIGURE 8: Net frond growth of Lemna minor for Alachlor Test 2 conducted from June 23 - 30, 1987.

TABLE 14: Trimmed Spearman-Karber EC50 Estimate for Lemna minor Alachlor Test 2 conducted 23 June 1987.

CONCENTRATION Raw	CONCENTRATION ln	ORGANISMS EXPOSED	DEAD ORGANISMS	RAW MORTALITY	ADJUSTED MORTALITY
0.00	-23.03	139	0	0.00	0.00
5.00	1.61	139	31	0.22	0.23
15.00	2.71	139	54	0.39	0.38
25.00	3.22	139	51	0.37	0.38
30.00	3.40	139	60	0.43	0.43
40.00	3.69	139	77	0.55	0.55

Spearman Karber Trim = 45%

	ln	ug/L
Estimated EC50	3.56	35.23
Lower 95% Confidence Limit	3.42	30.60
Higher 95% Confidence Limit	3.70	40.57

Table 15: Dunnett's Procedure for Data Set: Lemna - Alachlor Test 2

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	896.35	5	179.27	13.85	6.81***
Within	232.92	18	12.94		

***P<0.001

Observed Difference from Control

ug/L	Difference	ti			Dunnett's Critical t
control	0.00	0.00	<	NOEC	2.41*
5.0	7.75	3.05	<	LOEC	
15.0	13.50	5.31			
25.0	12.75	5.01			
30.0	15.00	5.90			
40.0	19.25	7.57			

*P<.05

Table 15a: Williams' t Test for Data Set: Lemna - Alachlor Test 2

Error Mean Square from ANOVA: 12.94

Mean response is expected to decrease as treatment concentration increases.

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML' Estimator	$\bar{t}'$
0.00	4	35.00			<NOEC		
5.00	4	27.00	27.00	3.15	<LOEC	27.00	3.15
15.00	4	21.50	21.50	5.31		21.00	5.50
25.00	4	22.00	21.50	5.31		21.50	5.31
30.00	4	20.00	20.00	5.90		20.00	5.90
40.00	4	16.00	16.00	7.47		16.00	7.47

k = 5
v = 15

Williams' Critical t = 1.89*

*P<.05

The lowest observable effect concentration for atrazine and alachlor are illustrated in Figure 10. The effect concentration caused by atrazine on Lemna minor was statistically determined to occur between 25 and 100 ug/L. The alachlor tests indicated an observable effect in the growth of Lemna was determined to occur between the 5.0 and 7.5 ug/L concentration.

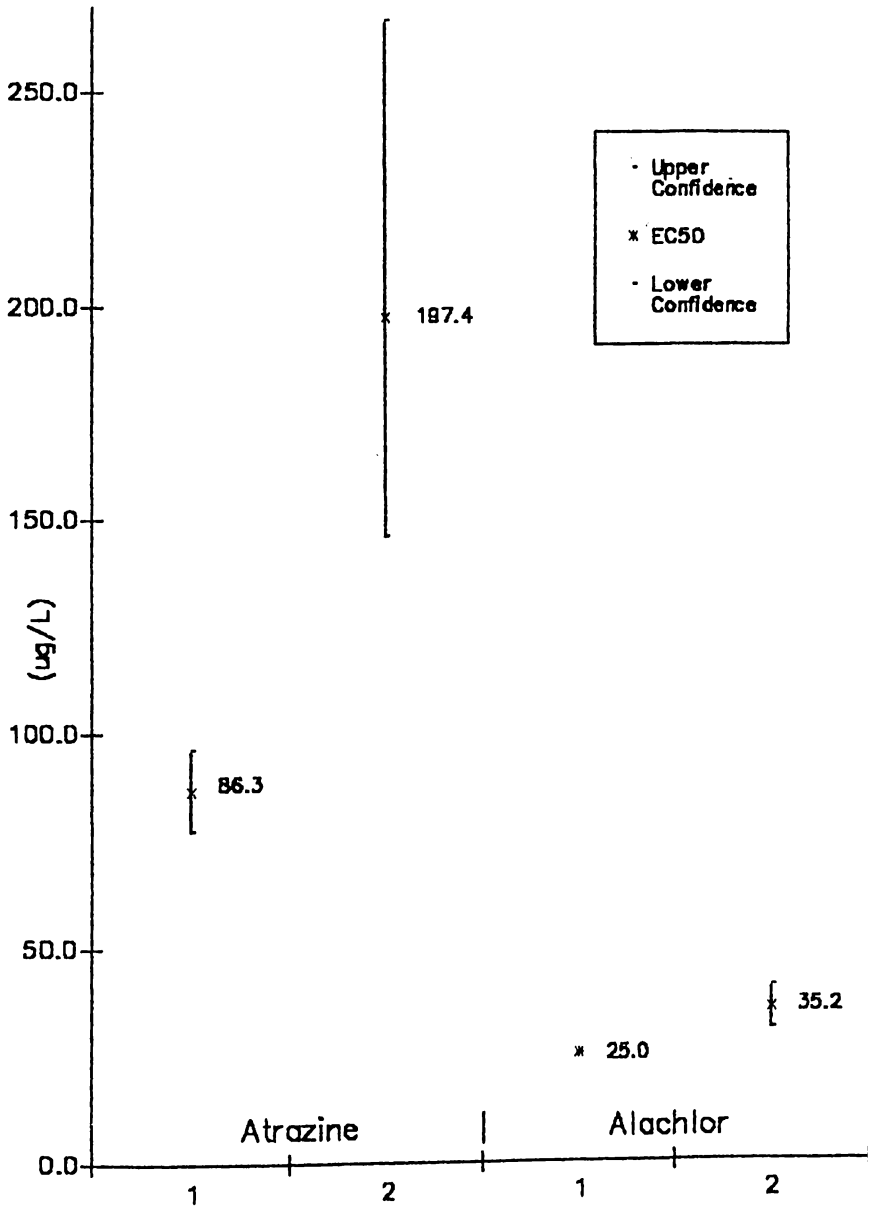


FIGURE 9: Estimated EC50 and Confidence Limits for Lemna minor Atrazine and Alachlor Single Tests

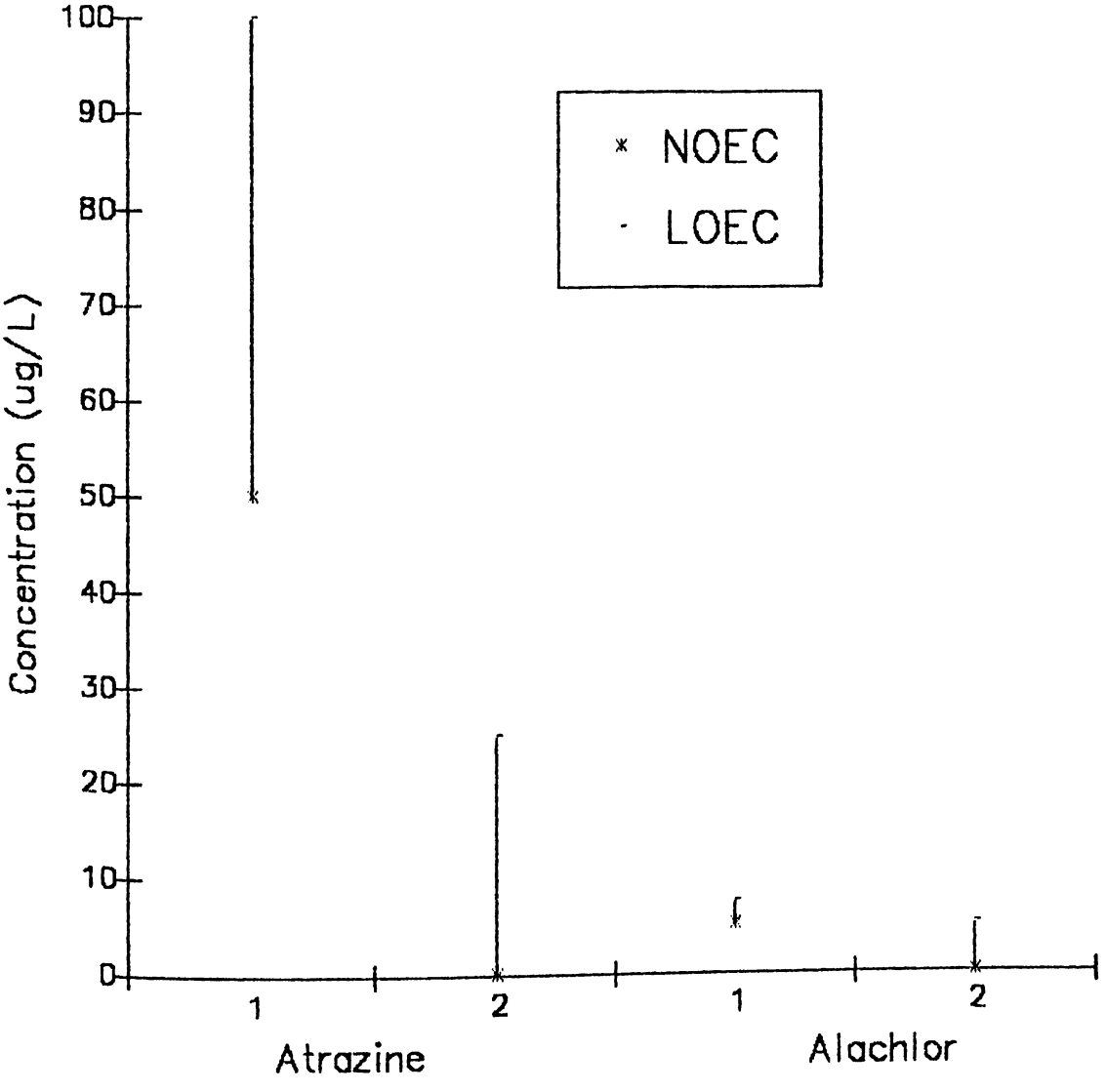


FIGURE 10: Chronic Analysis of Lemna minor for Atrazine and Alachlor Single Tests.

Joint Test

The combined toxicity of atrazine and alachlor was determined to have a greater than additive effect on Lemna. The Trimmed Spearman-Karber Test estimated the EC50 to be 0.52 adjusted Toxic Units. The Toxic Unit represented one half or 0.26 TU of atrazine and 0.26 TU of alachlor, Figure 11 and Table 17. The 0.26 TU represented 22.2 ug/L of atrazine and 6.4 ug/L of alachlor were needed to produce the EC50 effect, Table 18. This indicated that it took significantly less of each chemical, when combined, to produce the same effect the chemical had when applied singularly, Figure 12.

Table 16: Lemna Joint Test (Atrazine/Alachlor) - 20 June 1987
Frond Growth Summary

	(Toxic Units)							
	Control	0.25	0.50	0.75	1.00	1.25	1.75	2.00
Frond Growth (net)	134	112	87	74	78	51	30	43
Mean Growth	34	28	22	19	20	13	8	11
Standard Deviation	2.65	1.8	3.86	4.8	4.6	8.5	3.1	5.8
Reduction of Growth	0	22	47	60	56	83	104	91
Percent Inhibition	0	16	35	45	42	62	78	68

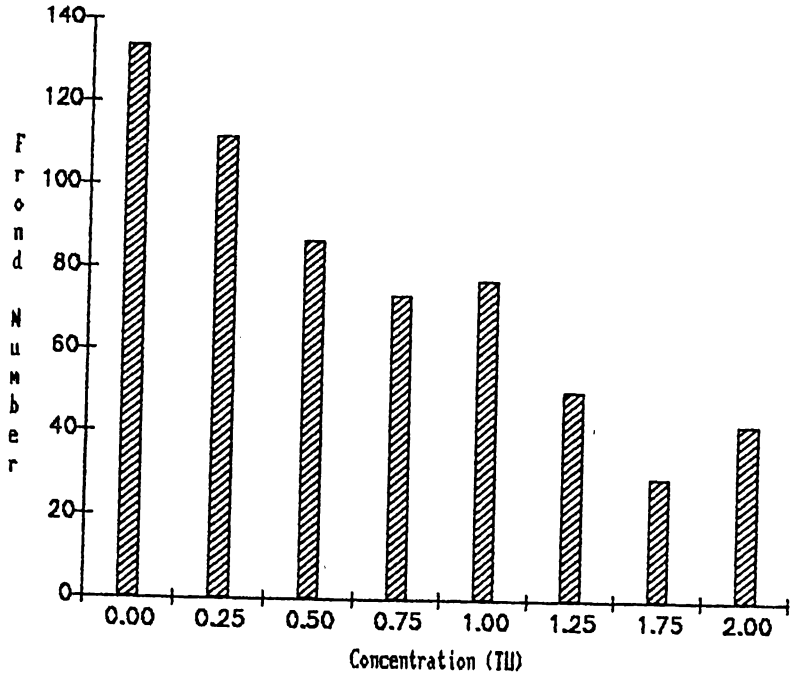


FIGURE 11: Net frond growth of Lemna minor for Joint Test conducted from June 23 - 30, 1987.

TABLE 17: Trimmed Spearman-Karber EC50 Estimate for Lemna minor Joint Test conducted 23 June 1987.

CONCENTRATION Raw	CONCENTRATION ln	ORGANISMS EXPOSED	DEAD ORGANISMS	RAW MORTALITY	ADJUSTED MORTALITY
0.14	-1.97	134	22	0.16	0.16
0.29	-1.24	134	47	0.35	0.35
0.43	-0.84	134	62	0.46	0.44
0.58	-0.55	134	56	0.42	0.44
0.72	-0.33	134	83	0.62	0.62
1.00	0.00	134	104	0.78	0.73
1.15	0.14	134	91	0.68	0.73

Spearman Karber Trim = 27%

	ln	ug/L
Estimated EC50	-0.66	0.52
Lower 95% Confidence Limit	-0.78	0.46
Higher 95% Confidence Limit	-0.53	0.59

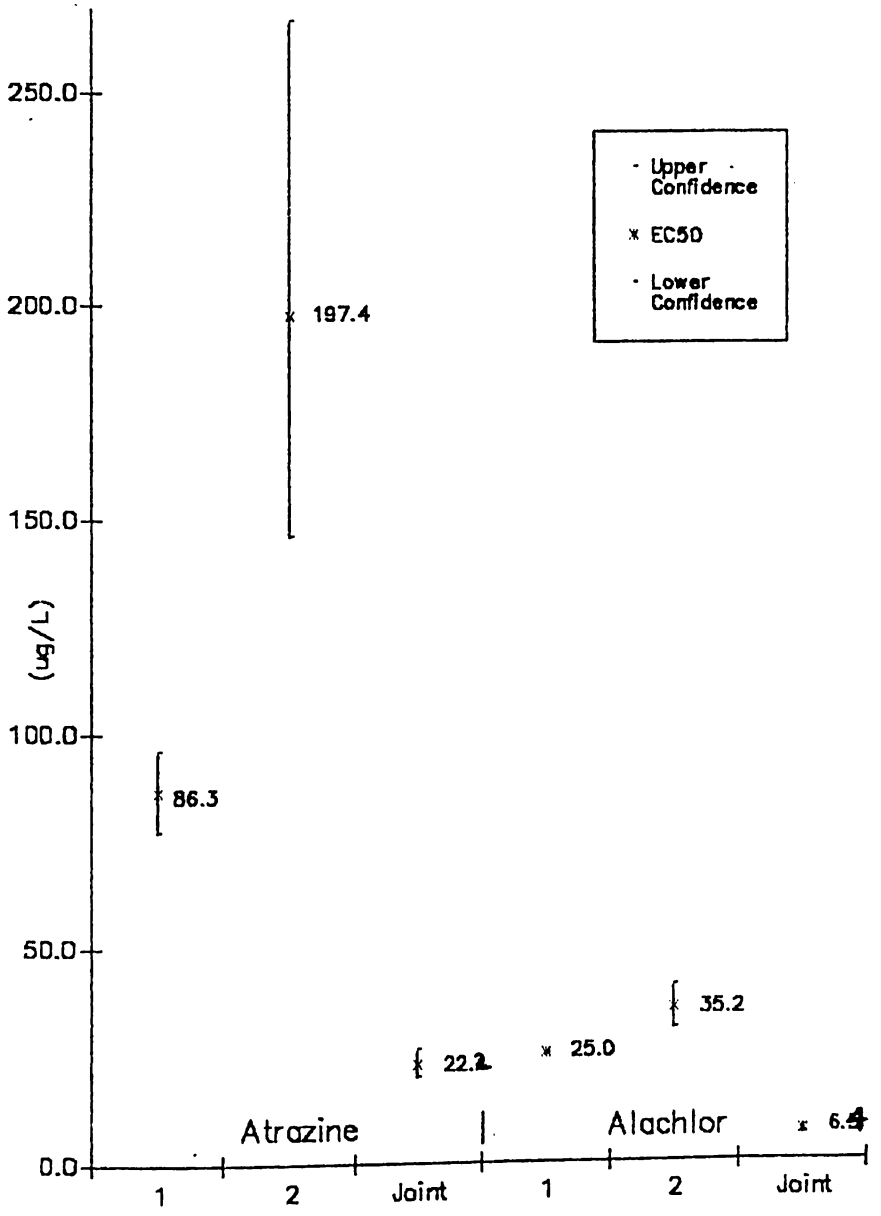


FIGURE 12: Estimated EC50 and Confidence Limits for Lemna minor of All Single Tests and Joint Test.

Table 18: Toxic Unit Adjustments and Chemical Equivalence Calculations for Lemna minor Joint Test

The Atrazine and Alachlor Test 2 results were:

Estimated EC50 = 197.4 atrazine
35.2 alachlor

Therefore, the TU were adjusted to:

(estimated) (actual)
atrazine - 86.2 ug/L divided by 197.4 ug/L = 44% of the EC50 value

(estimated) (actual)
alachlor - 25.0 ug/L divided by 35.2 ug/L = 71% of the EC50 value

Thus, the 2 TU level was actually representative of 1.15 TU.

Toxic Unit	Atrazine (ug/L)	Alachlor	Adjusted Toxic Unit
0.25	10.8	3.1	0.14
0.50	21.6	6.3	0.29
0.75	32.3	9.4	0.43
1.00	43.1	12.5	0.58
1.25	53.9	15.6	0.72
1.75	75.5	21.9	1.00
2.00	86.3	25.0	1.15

Chemical Equivalence represented at the TU EC50

Trimmed Spearman-Karber Estimated EC50 = 0.52 TU
Lower 95% Confidence Limit = 0.46 TU
Higher 95% Confidence Limit = 0.59 TU

The proportion of this amount to the original 1.0 TU was obtained:

EC50 = 1.0 Tu / 0.52 TU = 1.9*
Lower Confidence Limit = 1.0 TU / 0.46 TU = 2.2-
Upper Confidence Limit = 1.0 TU / 0.59 TU = 1.7+

Atrazine

Chemical Equivalence at:
EC50 43.1 / 1.9 = 22.7 ug/L*
Lower Confidence 43.1 / 2.2 = 19.6 ug/L-
Upper Confidence 43.1 / 1.7 = 25.4 ug/L+

Alachlor

Chemical Equivalence at:
EC50 12.5 / 1.9 = 6.6 ug/L*
Lower Confidence 12.5 / 2.2 = 5.7 ug/L-
Upper Confidence 12.5 / 1.7 = 7.4 ug/l+

Ceriodaphnia reticulata

Atrazine Tests

The range of concentrations used in Test 1 did not cause 50 percent mortality in any one level and the Trimmed Spearman-Karber was not performed, Table 19. The ANOVA performed on the reproduction data for Atrazine Test 1, Table 20, indicated that an insignificant decrease in numbers produced by the Control as compared to the numbers produced in any of the concentration levels, Table 21. Thus, the Dunnett's Procedure could not be used to analyze for the observable effect concentrations. The more sensitive Williams' Test was used which determined NOEC/LOEC levels to be 4.0 mg/L and 6.0 mg/L, respectively (Table 21a).

The Trimmed Spearman-Karber analysis, Table 23, of the mortality data in the Atrazine Test 2, Table 22, determined that the LC50 was 7.9 mg/L. Concentrations examined in the Atrazine Test 2 caused a significant decrease in Ceriodaphnia reproduction, Table 24, as determined by the ANOVA. The Dunnett's Procedure was performed, Table 25, with the NOEC reported as 3.0 mg/L and the LOEC as 6.0 mg/L. The same NOEC/LOEC values were found when the Williams' Test was applied to the Atrazine Test 2 reproduction data, Table 25a.

Table 19: Ceriodaphnia Adult Mortality for Atrazine Test 1 - 8 November 1986

	Initial #	End #	Total Dead
Control	10	10	0
0.5 mg/L	10	9	1
1.0 mg/L	10	9	1
2.0 mg/L	10	9	1
4.0 mg/L	10	8	2
6.0 mg/L	10	7	3

Table 20: Total Number of Neonates produced per Ceriodaphnia for Atrazine Test 1 - 8 November 1986

				(mg/L)		
Replicate	Control	0.5	1.0	2.0	4.0	6.0
1	2	6	5	2	4	1
2	7	2	4	6	7	0
3	1	4	1	0	1	8
4	12	9	6	12	2	2
5	5	4	3	3	7	1
6	10	0	4	9	0	4
7	5	0	3	8	4	4
8	2	4	3	0	3	2
9	6	2	3	3	0	3
10	14	4	3	8	0	3
Total	64	28	38	45	44	28
$\bar{X}$	5.4	2.8	3.8	4.5	4.5	2.8
s	4.2	1.9	2.0	3.3	4.5	2.2
s^2	16.9	3.7	4.4	11.1	20.7	5.0

Table 21: ANOVA Table for Atrazine Test 1 Ceriodaphnia Reproduction

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	10.74	5	2.148	1.04	2.38*
Within	554.04	54	10.260		

*P<.05

Standard F Test indicated the reproduction was insignificantly effected.

Table 21a: Williams' t Test for Data Set: Ceriodaphnia - Atrazine Test 1

Error Mean Square from ANOVA: 10.30

Mean response is expected to decrease as treatment concentration increases.

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML' Estimator	$\bar{t}'$
0.00	10	5.40					
0.50	10	2.80	3.90	1.05		2.80	1.81
1.00	10	3.80	3.90	1.05		3.30	1.46
2.00	10	4.50	3.90	1.05		3.70	1.18
4.00	10	4.50	3.90	1.05	<NOEC	3.90	1.05
6.00	10	2.80	2.80	1.81	<LOEC	2.80	1.81

k = 5 Williams' Critical t = 1.79
v = 54

Table 22: Ceriodaphnia Adult Mortality for Atrazine Test 2 - 12 February 1987

	Initial #	End #	Total Dead
Control	10	10	0
0.75	10	10	0
1.50	10	10	0
3.00	10	8	2
6.00	10	7	3
9.00	10	4	6

TABLE 23: Trimmed Spearman-Karber LC50 Estimate for Ceriodaphnia Atrazine Test 2 - 12 February 1987

CONCENTRATION Raw	CONCENTRATION ln	ORGANISMS EXPOSED	DEAD ORGANISMS	RAW MORTALITY	ADJUSTED MORTALITY
0.75	-0.29	10	0	0.00	0.00
1.50	0.41	10	0	0.00	0.00
3.00	1.01	10	2	0.20	0.20
6.00	1.79	10	3	0.30	0.30
9.00	2.20	10	6	0.60	0.60

Spearman Karber Trim = 40%

	ln	mg/L
Estimated LC50	2.06	7.86
Lower 95% Confidence Limit	1.75	5.78
Higher 95% Confidence Limit	2.37	10.70

TABLE 24: Total Number of Neonates produced per Ceriodaphnia for Atrazine Test 2 - 12 February 1987

Replicate	Control	0.75	1.50	(mg/L) 3.00	6.00	9.00
1	36	29	41	29	19	13
2	22	27	26	25	16	9
3	20	36	18	24	11	15
4	30	23	16	23	20	8
5	28	11	21	19	14	6
6	16	20	0	2	17	2
7	25	15	19	17	2	4
8	23	9	17	10	18	12
9	18	6	1	14	15	6
10	17	28	18	12	0	0
Total	235	204	215	255	217	98
$\overline{X}$	23.5	20.4	17.7	17.5	13.2	7.5
s	6.4	9.9	11.6	8.2	6.9	4.8
s^2	40.5	97.8	135.6	66.9	48.2	23.6

TABLE 25: Dunnett's Procedure for Data Set: Ceriodaphnia - Atrazine Test 2

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	1584.30	5	316.86	4.60	3.37**
Within	3712.50	54	68.75		

**P<.01

Observed Difference from Control

mg/L	Difference	ti		Dunnett's Critical t
control	0.00	0.00		
0.75	3.10	0.84		
1.50	5.80	1.57		
3.00	6.00	1.62	<NOEC	2.28*
6.00	10.30	2.78	<LOEC	
9.00	16.00	4.32		

*P<.05

TABLE 25a: Williams' t Test for Data Set: Ceriodaphnia - Atrazine Test 2

Error Mean Square from ANOVA: 68.75

Mean response is expected to decrease as treatment concentration increases.

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML' Estimator	$\bar{t}'$
0.00	10	23.50					
0.75	10	20.40	20.40	0.84		20.40	0.84
1.50	10	17.70	17.70	1.56		17.70	1.56
3.00	10	17.50	17.50	1.62	<NOEC	17.50	1.62
6.00	10	13.20	13.20	2.78	<LOEC	13.20	2.78
9.00	10	7.50	7.50	4.32		7.50	4.32

k = 5 Williams' Critical t = 1.79
v = 54

It was noted that the mean reproduction of control adults in the Atrazine Test 1, 5.4, was dramatically lower than the reproduction of control adults in the Atrazine Test 2, 23.5. Variation in number of young produced between these two sets of adults may have been due to slight changes that occurred in diet, water chemistry, or in culture maintenance factors. Another notable variable was that the Atrazine Test 1 was conducted in NESA Reservoir water while the Atrazine Test 2 utilized NESA Well water. Changes in adult populations could have occurred as the zooplankton were adapting to new water conditions.

Alachlor Tests

The higher concentrations used in the Alachlor Test 1 resulted in immediate acute responses. Significant mortality among adults occurred at the 5.0 mg/L level and above, Table 26. The Trimmed Spearman-Karber analysis estimated the LC50 value to be 4.7 mg/L, Table 27. The Dunnett's and the Williams' Test were used to calculate NOEC-LOEC reproduction response, Table 28, of the Alachlor Test 1, Table 29 and 29a. Both procedures reported the LOEC of the reproduction of Ceriodaphnia occurred at the 1.0 mg/L level and the control was the NOEC. The method for analyzing reproduction responses specified that levels at which significant mortality occurred be excluded. This significance was determined by contingency testing, Appendix 3, which indicated which concentration levels must be omitted from the chronic reproduction analysis (i.e.

Dunnett's Procedure). The two highest concentrations were excluded for this particular analysis.

In order to further quantify the chronic response, the Alachlor Test 2 was conducted using concentration levels below the estimated LC50 concentration. Corresponding mortality results of the test, Table 30, revealed that significant mortality did not occur in any of the concentrations tested. Contingency tests, Appendix 3, indicated that all levels could be included in evaluation of reproduction. The Dunnett's Procedure, Table 32, indicated that the NOEC was 1.0 mg/L and LOEC was 2.0 mg/L as calculated for the reproduction data in Table 31. These values were verified by the results of the Williams' Test, Table 32a.

Mortality of adults was not achieved by the highest concentration of 4.0 mg/L of alachlor. This could indicate that an acute threshold existed between the 4.0 mg/L and 5.0 mg/L levels, near the reported LC50, 4.7 mg/L, estimate. However, it was noted that most adults at the 4.0 mg/L level were included as survivors only because there was visual movement of the gills, a qualifier outlined in the EPA methods. Most "live" adults exhibited very little movement when gently prodded and were not able to swim about the test vessel. Figure 13 illustrates the LC50's and Confidence Limits of the Atrazine Test 1 and the Alachlor Test 2 as estimated by the Trimmed Spearman-Karber analysis.

TABLE 26: Ceriodaphnia Adult Mortality for Alachlor Test 1 - 16 January 1987

	Initial #	End #	Total Dead
Control	10	10	0
1.0	10	9	1
2.5	10	7	3
5.0	10	6	4
7.5	10	2	8
10.0	10	2	8

TABLE 27: Trimmed Spearman-Karber LC50 Estimate for Ceriodaphnia Alachlor Test 1 - 16 January 1987

CONCENTRATION Raw	CONCENTRATION ln	ORGANISMS EXPOSED	DEAD ORGANISMS	RAW MORTALITY	ADJUSTED MORTALITY
1.00	0.00	10	1	0.10	0.10
2.500	0.92	10	3	0.30	0.30
5.000	1.61	10	4	0.40	0.40
7.500	2.02	10	8	0.80	0.80
10.000	2.30	10	8	0.80	0.80

Spearman Karber Trim = 21%

	ln	ug/L
Estimated LC50	1.54	4.68
Lower 95% Confidence Limit	1.10	2.98
Higher 95% Confidence Limit	2.00	7.36

TABLE 28: Total Number of Neonates produced per Ceriodaphnia for Alachlor Test 1 - 16 January 1987

Replicate	Control	(mg/L) 1.0	2.5	5.0	7.5	10.0
1	20	2	0	7	0	0
2	4	15	1	1	0	0
3	9	9	3	0	0	0
4	19	0	14	8	3	3
5	20	1	15	0	0	4
6	9	3	6	0	1	0
7	10	0	10	7	4	0
8	20	3	0	15	0	0
9	33	1	15	0	17	0
10	13	28	0	8	0	0
Total	157	62	64	46	25	7
$\overline{X}$	15.7	6.2	6.4	4.6	2.5	0.7
s	8.3	9.0	6.5	5.2	5.3	1.5
s^2	70.2	81.1	42.5	26.7	28.1	2.2

TABLE 29: Dunnett's Procedure for Data Set: Ceriodaphnia Alachlor Test 1

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	1270	5	254	4.62	3.37**
Within	2970	54	55		

**P<.01

Observed Difference from Control

mg/L	Difference	ti		Dunnett's Critical t
control	0.00	0.00	<NOEC	2.3*
1.0	9.50	2.80	<LOEC	
2.5	9.3	2.80		
5.0	11.1	3.30		

*P<0.5

TABLE 29a: Williams' t Test for Data Set: Ceriodaphnia - Alachlor Test 1

Error Mean Square from ANOVA: 55.00

Mean response is expected to decrease as treatment concentration increases.

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML' Estimator	$\bar{t}'$
0.00	10	15.70			<NOEC		
1.00	10	6.20	6.30	2.83	<LOEC	6.20	2.86
2.50	10	6.40	6.30	2.83		6.30	2.83
5.00	10	4.60	4.60	3.35		4.60	3.35
7.50	10	2.20	2.20	4.07		2.20	4.07
10.00	10	0.70	0.70	4.52		0.70	4.52

k = 5 Williams' Critical t = 1.79
v = 54

TABLE 30: Ceriodaphnia Adult Mortality for Alachlor Test 2 - 10 February 1987

	Initial #	End #	Total Dead
Control	10	10	0
0.5	10	8	2
1.0	10	8	2
2.0	10	7	3
3,0	10	8	2
4.0	10	10	0

TABLE 31: Total Number of Neonates produced per Ceriodaphnia for Alachlor Test 2 - 10 February 1987

				(mg/L)		
Replicate	Control	0.5	1.0	2.0	3.0	4.0
1	26	27	14	3	0	0
2	8	26	1	25	2	6
3	24	0	24	11	0	17
4	26	11	14	11	10	1
5	30	25	21	7	6	0
6	28	20	10	7	25	9
7	15	14	47	22	27	0
8	21	11	21	17	22	22
9	30	27	13	11	18	0
10	40	27	14	12	13	31
Total	248	171	204	101	146	106
$\bar{X}$	24.8	17.0	20.4	10.2	14.6	10.6
s	8.7	9.5	10.7	6.2	10.3	12.3
s^2	76.8	91.6	115.4	38.6	106.7	152.0

TABLE 32: Dunnett's Procedure for Data Set: Ceriodaphnia - Alachlor Test 2

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	1621.30	5	324.26	3.35	2.38*
Within	5229.90	54	96.85		

*P<.05

Observed Difference from Control

mg/L	Difference	ti		Dunnett's Critical t
control	0.00	0.00		
0.5	7.3	1.65		
1.0	3.9	0.88	< NOEC	2.28*
2.0	14.1	3.20	< LOEC	
3.0	9.7	2.20		
4.0	13.7	3.11		

*P<.05

TABLE 32a: Williams' t Test for Data Set: Ceriodaphnia - Alachlor Test 2

Error Mean Square from ANOVA: 96.85

Mean response is expected to decrease as treatment concentration increases

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML' Estimator	$\bar{t}'$
0.00	10	24.80					
0.50	10	17.00	18.70	1.39		17.00	1.77
1.00	10	20.40	18.70	1.39	<NOEC	18.70	1.39
2.00	10	10.20	12.40	2.82	<LOEC	10.20	3.32
3.00	10	14.60	12.40	2.82		12.40	2.82
4.00	10	10.60	10.60	3.23		10.60	3.23

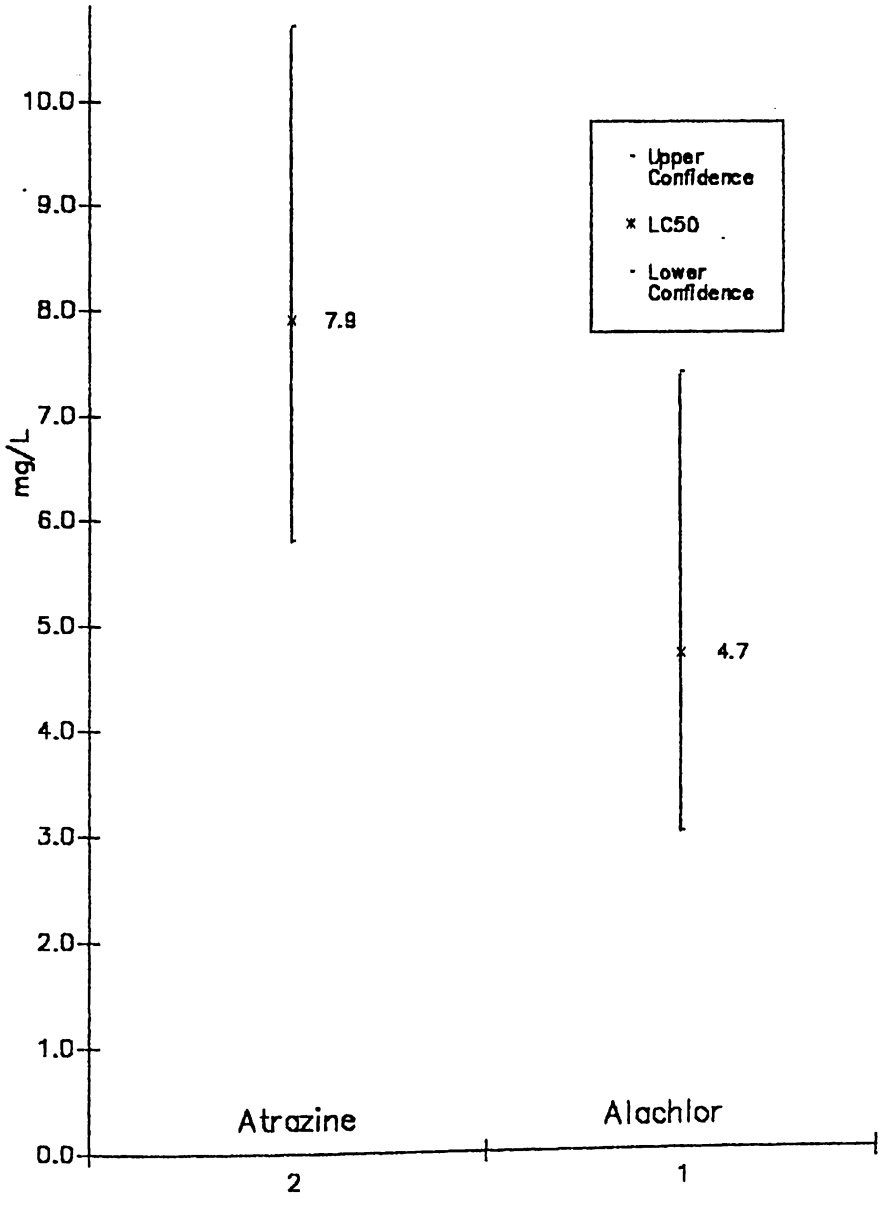


FIGURE 13: Estimated LC50 and Confidence Limits for Ceriodaphnia Atrazine and Alachlor

Stock cultures of Ceriodaphnia were carefully monitored between test periods to determine "normal" reproduction habits. Expected reproduction could then be predicted and maintained. When changes in these numbers began occurring during Atrazine Test 1 and after Atrazine and Alachlor Test 2, efforts were made to determine and correct possible physical or chemical problems. Analyses of many of the different variables that could possibly effect the fertility of the population such as diet, water, etc, were investigated. The population remained stabilized after the Atrazine Test 1, but the problem re-appeared sometime after the conclusion of the other tests. Further testing with Ceriodaphnia was therefore abandoned due to variability in control reproduction of this specific stock population.

Pimephales promelas

Daily survival records for all single and joint Fathead Toxicity testing are presented by individual chamber in Appendix 4.

Atrazine Tests

Acute Analysis (Mortality)

Concentrations tested in Atrazine Test NS (0.25 mg/L - 4.0 mg/L) and Test 1 (0.50 mg/L - 10.00 mg/L) did not produce at least 50% mortality at any level thus the LC50 could not be determined, Figure

14 and 14a. Concentrations evaluated in Atrazine Test 2 produced significant mortality and the Trimmed Spearman-Karber analysis estimated the LC50 to be 13.17 mg/L, Figure 15 and Table 33.

Chronic Analysis (Weight)

A dry weight analysis was performed on each test to determine if there was a chronic response and at which level it occurred. Table 34 summarizes fish weights of Atrazine Test NS. The Analysis of Variance (ANOVA) indicated there was no significant difference of dry weights in all concentrations as compared to the control weights, Table 34a. The No Observable Effect Concentration (NOEC) and the Lowest Observable Effect Concentration (LOEC) could not be calculated by the Dunnett's Procedure as indicated by the ANOVA. Weights could not be tested using the Williams' Test due to the fact that all treatment means were larger than control mean. The response should decrease with increasing concentration.

Final dry weights of the fish in Atrazine Test 1 are presented in Figure 16. The ANOVA indicated that there was a significant difference of weights between at least one of the concentrations. The Dunnett's Procedure determined the NOEC and LOEC to be 1.5 mg/L and 3.0 mg/L, respectively, Table 35. The Williams' Test computer analysis also determined the same NOEC-LOEC values for Atrazine, Table 35a.

A weight analysis was not performed on Atrazine Test 2 due to the fact that the range of test concentrations (10 mg/L - 18 mg/L) were significantly higher than needed to produce the reported lowest observable effect concentration of 3.0 mg/L.

Alachlor Tests

Acute Analysis (Mortality)

Survivorship of Fathead minnows in Test 1, 1.0 mg/L - 5.0 mg/L alachlor, revealed that 50% mortality did not occur and the LC50 could not be defined, Figure 17. Concentrations evaluated in Test 2 0.5 mg/L - 6.0 mg/L, achieved greater than 50% mortality and the LC50 was estimated to be 3.58 mg/L, Figure 18 and Table 38.

Chronic Analysis (Weight)

A summary of dry weights for the Alachlor Test 1, Figure 19, and Test 2, Figure 20, revealed that there was a noticeable reduction in weights in both tests. Test 1 and Test 2 ANOVAs indicated that there were significant differences. The Dunnett's Procedure and the Williams' Test were also performed. Both statistical procedures produced the same NOEC value of 1.0 mg/L and LOEC value of 2.0 mg/L for the Alachlor Test 1, Table 39 and 39a. In Test 2 the NOEC and LOEC were determined to be 0.5 mg/L and 1.0 mg/L, respectively, by both the Dunnett's Procedure and the Williams' Test, Table 40 and 40a.

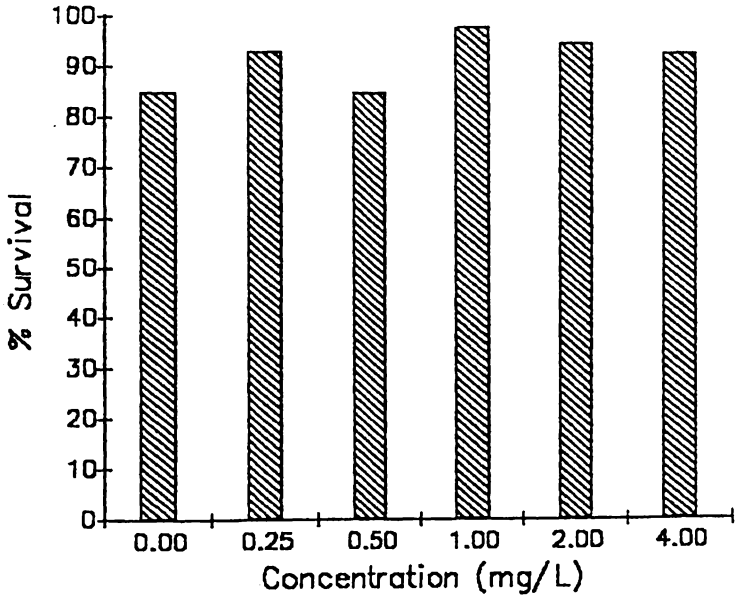


FIGURE 14: Pimephales Mean Survival per Concentration for Atrazine Test NS. 6 November - 12 November 198(

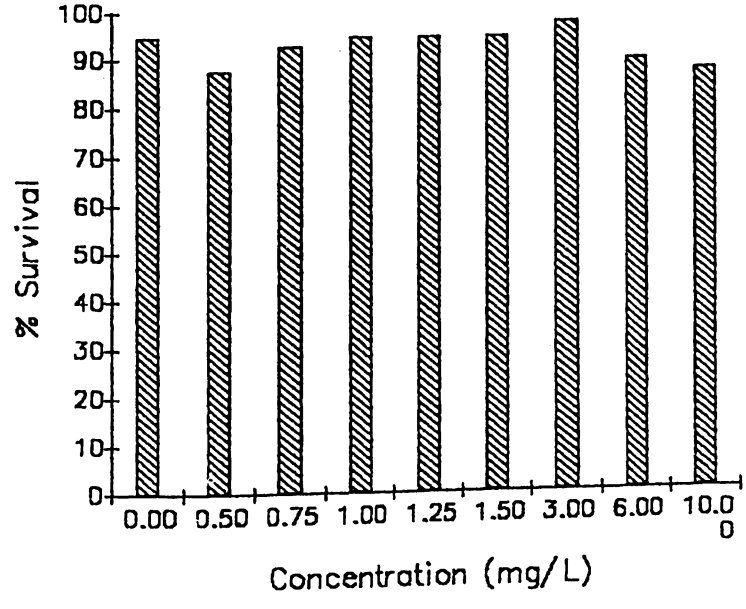


FIGURE 14a: Pimephales Mean Survival per Concentration for Atrazine Test 1. 25 March - 1 April 1987

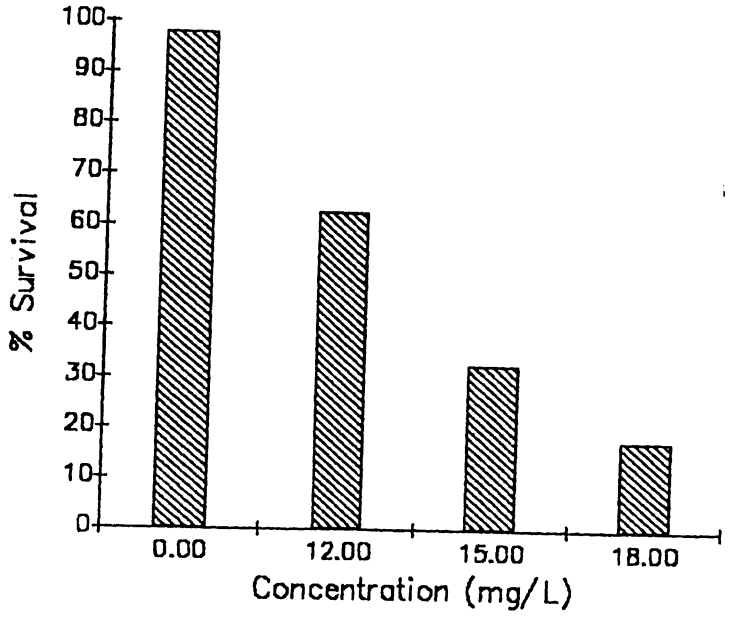


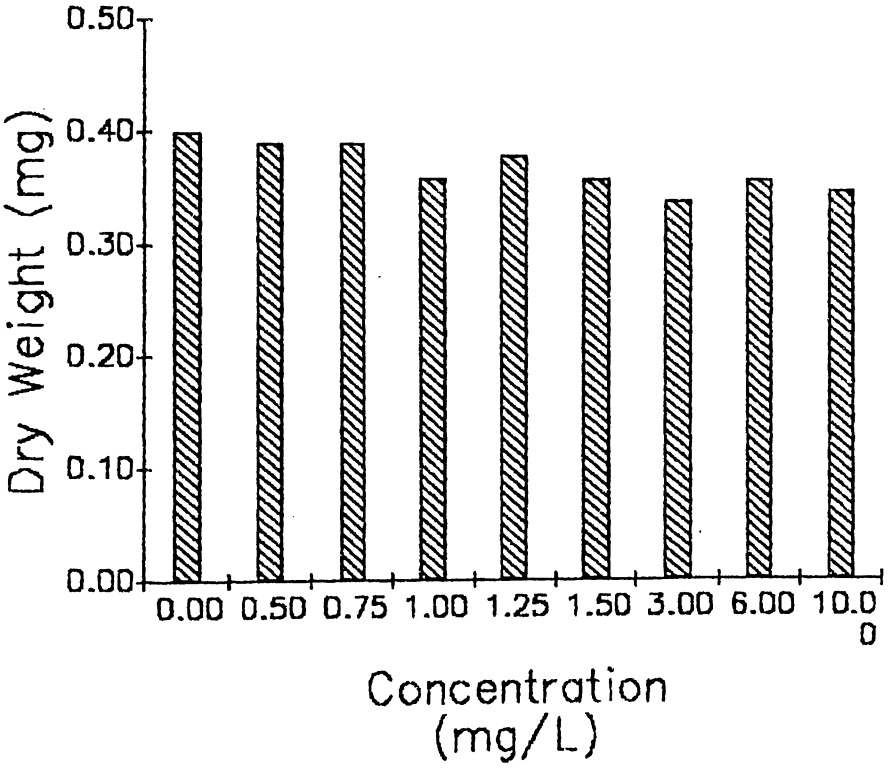
FIGURE 15: Pimephales Mean Survival per Concentration for Atrazine Test 2. 16 April - 23 April 1987

TABLE 33: Trimmed Spearman-Karber Analysis of Atrazine Test 2

CONCENTRATION mg/L	ln	ORGANISMS EXPOSED	DEAD ORGANISMS	RAW MORTALITY	ADJUSTED MORTALITY
12.00	2.49	40	15	0.38	0.38
15.00	2.71	40	27	0.68	0.68
18.00	2.89	40	33	0.83	0.83

Spearman Karber Trim = 38%

	ln	mg/L
Estimated EC50	2.58	13.17
Lower 95% Confidence Limit	2.50	12.15
Upper 95% Confidence Limit	2.66	14.28



	Chamber #	(Atrazine mg/L) Cont	0.50	0.75	1.00	1.25	1.50	3.00	6.00	10.0(
Fish Weights (mg)	1	0.41	0.41	0.39	0.37	0.43	0.37	0.34	0.33	0.32
	2	0.41	0.39	0.42	0.33	0.35	0.37	0.34	0.35	0.35
	3	0.39	0.39	0.38	0.34	0.37	0.33	0.36	0.38	0.34
	4	0.39	0.36	0.37	0.39	0.40	0.35	0.32	0.35	0.38
Mean		0.40	0.39	0.39	0.36	0.38	0.36	0.34	0.36	0.35

FIGURE 16: Atrazine Test 1 - Mean fish weights (mg) from four replicate test chambers for each treatment concentratic of surviving fish. Mean fish weights for each test chamber at each concentration level are presented in the lower panel.

TABLE 34: Atrazine Test NS - Mean fish weights for each test chamber at each concentration level of surviving fish.

	Chamber #	Control	(Atrazine mg/L) 0.25	0.50	1.00	2.00	4.00
Fish Weights (mg)	1	0.16	0.40	0.43	0.37	0.36	0.34
	2	0.38	0.46	0.33	0.30	0.32	0.31
	3	0.40	0.31	0.43	0.41	0.33	0.36
	4	0.32	0.32	0.44	0.36	0.30	0.29
Mean		0.32	0.37	0.41	0.36	0.33	0.32

TABLE 34a: ANOVA Table of Atrazine Test NS Dry Weight Data

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	0.0252	5	0.00504	1.33*	2.77
Within	0.0576	15	0.00384		

*P<0.05

The mean weights in the concentrations did not significantly vary from the mean weights of the Control. The Dunnett's Procedure is not continued.

TABLE 35: Dunnett's Procedure for Data Set: Fathead minnow - Atrazine Test 1.

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	0.016	8	0.00220	4.23**	3.26
Within	0.014	27	0.00052		

**P<0.01

Observed Difference from Control

mg/L	Difference	ti		Dunnett's Critical t
control	0.00	0.00		
0.50	0.01	0.62		
0.75	0.01	0.62		
1.00	0.04	2.48		
1.25	0.01	0.62		
1.50	0.04	2.48	<NOEC	2.53*
3.00	0.06	3.72	<LOEC	
6.00	0.05	3.10		
10.00	0.06	3.72		

*P<0.0

Table 35a: Williams' t Test for Data Set: Fathead Minnow - Atrazine Test 1

Error Mean Square from ANOVA: 0.001
Mean response is expected to decrease as treatment concentration

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML' Estimator	$\bar{t}'$
0.00	4	0.40					
0.50	4	0.39	0.39	0.62		0.39	0.62
0.75	4	0.39	0.39	0.62		0.39	0.62
1.00	4	0.36	0.38	1.55		0.36	2.48
1.25	4	0.39	0.38	1.55	<NOEC	0.38	1.55
1.50	4	0.36	0.36	2.48	<LOEC	0.36	2.48
3.00	4	0.34	0.35	3.41		0.34	3.72
6.00	4	0.35	0.35	3.41		0.35	3.41
10.00	4	0.34	0.34	3.72		0.34	3.72

k = 8 v = 24 Williams' Critical t = 1.85

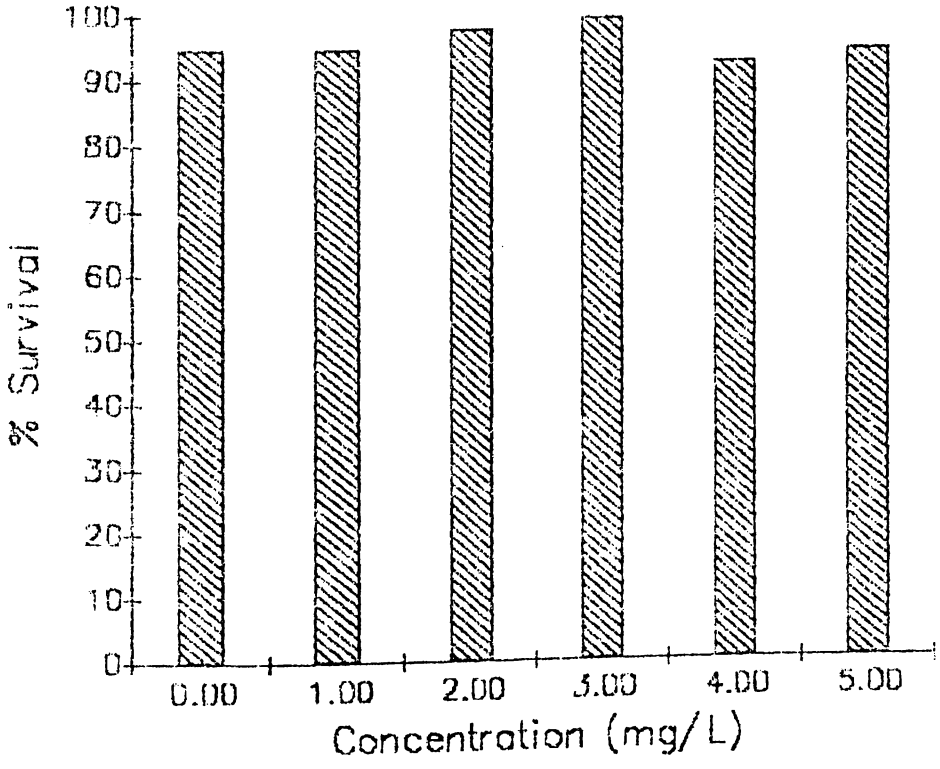


FIGURE 17: Pimephales Mean Survival per Concentration for Alachlor Test 1. 16 Jan - 23 Jan 1987

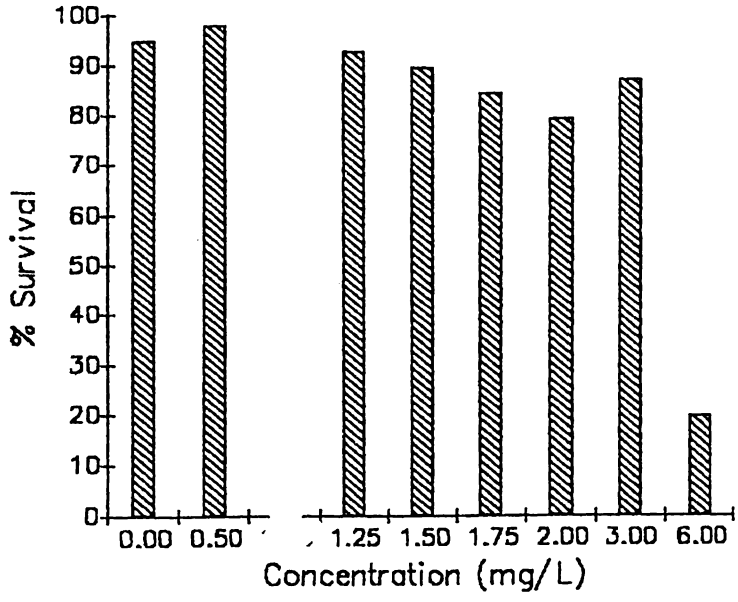


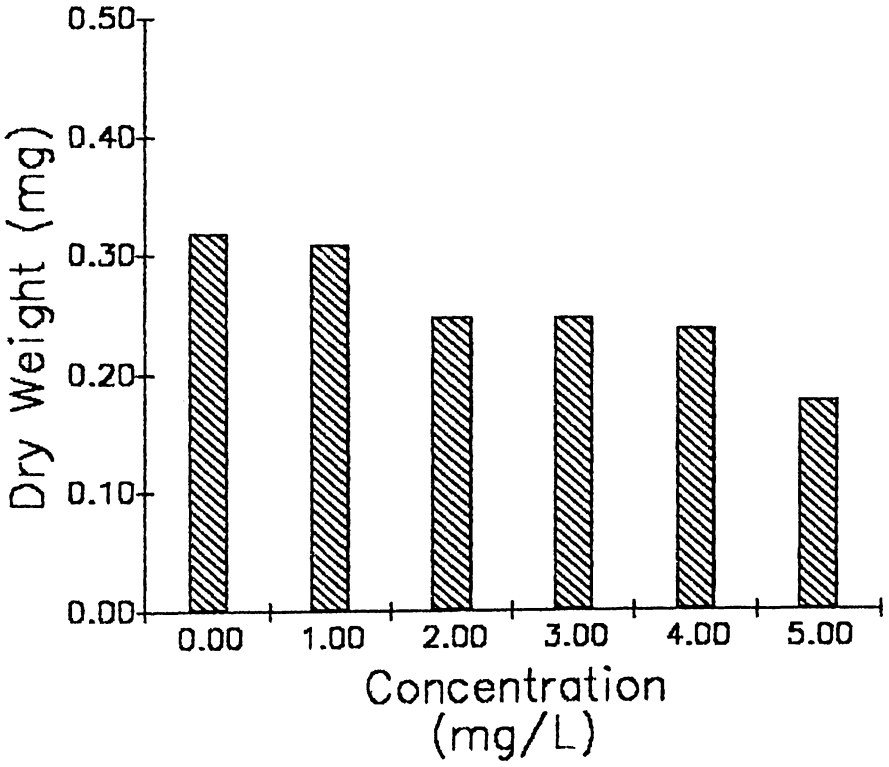
FIGURE 18: Pimephales Mean Survival per Concentration for Alachlor Test 2. 25 March - 1 April 1987

TABLE 36: Trimmed Spearman-Karber Analysis of Alachlor Test 2

CONCENTRATION mg/L	CONCENTRATION ln	ORGANISMS EXPOSED	DEAD ORGANISMS	RAW MORTALITY	ADJUSTED MORTALITY
0.50	-0.69	40	1	0.03	0.03
1.25	0.00	40	3	0.03	0.08
1.50	0.22	40	4	0.10	0.10
1.75	0.41	40	6	0.15	0.15
2.00	0.56	40	8	0.20	0.16
3.00	0.69	40	5	0.13	0.16
6.00	1.79	40	32	0.80	0.80

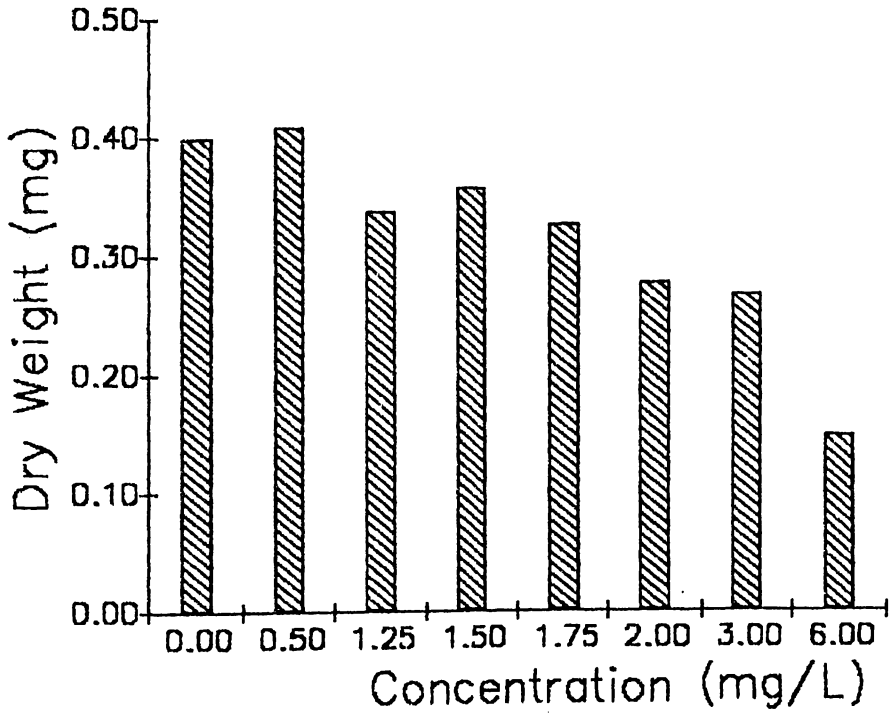
Spearman Karber Trim = 21%

	ln	mg/L
Estimated LC50	1.28	3.58
Lower 95% Confidence Limit	1.13	3.08
Upper 95% Confidence Limit	1.42	4.15



	Chamber #	(Alachlor mg/L) Control	1.00	2.00	3.00	4.00	5.00
Fish Weights (mg/L)	1	0.31	0.31	0.27	0.24	0.22	0.22
	2	0.32	0.34	0.26	0.25	0.24	0.16
	3	0.30	0.32	0.28	0.29	0.27	0.18
	4	0.34	0.28	0.20	0.24	0.22	0.20
Mean		0.32	0.31	0.25	0.26	0.24	0.19

FIGURE 19: Alachlor Test 1 - Mean fish weights (mg) from four replicate test chambers for each treatment concentration of surviving fish. Mean fish weights for each test chamber at each concentration level are presented in lower panel.



	Chamber #	(Alachlor mg/L) Control	0.50	1.25	1.50	1.75	2.00	3.00	6.00
Fish Weights (mg/L)	1	0.41	0.41	0.37	0.34	0.35	0.28	0.26	0.21
	2	0.41	0.41	0.31	0.35	0.28	0.24	0.31	0.09
	3	0.39	0.45	0.38	0.42	0.37	0.28	0.26	0.16
	4	0.39	0.40	0.34	0.32	0.33	0.32	0.26	0.15
Mean		0.40	0.42	0.35	0.36	0.33	0.28	0.34	0.15

FIGURE 20: Alachlor Test 2 - Mean fish weights (mg) from four replicate test chambers for each treatment concentration of surviving fish. Mean fish weights for each test chamber at each concentration level are presented in the lower panel.

TABLE 37: Dunnett's Procedure for Data Set: Fathead minnow - Alachlor Test 1.

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	0.0480	5	0.0096	15.30***	6.81
Within	0.0108	18	0.0006		

***P<0.0001

Observed Difference from Control

mg/L	Difference	ti		Dunnett's Critical t
control	0.00	0.00		
1.00	0.00	0.11	<NOEC	2.41*
2.00	0.06	3.44	<LOEC	
3.00	0.06	3.28		
4.00	0.08	4.28		
5.00	0.13	7.22		

Table 37a: Williams' t Test for Data Set: Fathead Minnow - Alachlor Test 1

Error Mean Square from ANOVA: 0.001

Mean response is expected to decrease as treatment concentration increases.

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML' Estimator	$\bar{t}'$
0.00	4	0.32					
1.00	4	0.31	0.31	0.57	<NOEC	0.31	0.57
2.00	4	0.25	0.26	3.72	<LOEC	0.25	4.01
3.00	4	0.26	0.26	3.72		0.26	3.72
4.00	4	0.24	0.24	4.58		0.24	4.58
5.00	4	0.19	0.19	7.44		0.19	7.44

k = 5 Williams' Critical t = 1.89

v = 15

TABLE 38: Dunnett's Procedure for Data Set: Fathead minnow - Alachlor Test 2.

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	0.22	8	0.0278	6.3**	3.26
Within	0.03	27	0.0010		

Observed Difference from Control

mg/L	Difference	ti		Dunnett's Critical t
control	0.00	0.00		
0.50	0.01	0.45	<NOEC	2.53*
1.25	0.06	2.69	<LOEC	
1.50	0.05	2.24		
1.75	0.07	3.14		
2.00	0.12	5.38		
3.00	0.13	5.83		
6.00	0.25	11.21		

*P<0.05

Table 38a: Williams' t Test for Data Set: Fathead Minnow - Alachlor Test 2

Error Mean Square from ANOVA: 0.001

Mean response is expected to decrease as treatment concentration increases.

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML' Estimator	$\bar{t}'$
0.00	4	0.40					
0.50	4	0.41	0.41	-0.22	<NOEC	0.41	0.224
1.00	4	0.32	0.34	2.68	<LOEC	0.32	3.578
1.50	4	0.36	0.34	2.68		0.34	2.683
1.75	4	0.33	0.33	3.13		0.33	3.130
2.00	4	0.28	0.28	5.37		0.28	5.367
3.00	4	0.27	0.27	5.81		0.27	5.814
6.00	4	0.15	0.15	11.18		0.15	11.180

k = 8 Williams' Critical t = 1.85

v = 24

Joint Toxicity (Atrazine/Alachlor)

Acute Analysis

Joint Test 1

LC50 values established from single Atrazine and Alachlor Test 2 were utilized to determine Toxic Unit levels for this test. The 2.0 TU level was derived by combining 13.17 mg/L of atrazine and 3.58 mg/L of alachlor. A strictly additive effect would have been achieved at the 1.0 TU level which represented one half the LC50 for atrazine (6.59 mg/L) and one half the LC50 for alachlor (1.79 mg/L) which when combined would produce 50% mortality.

Analysis of survivorship results indicated that concentrations tested did not produce at least 50% mortality at any of the Toxic Unit levels (Figure 21). The Trimmed Spearman-Karber analysis was terminated due to lack of significant mortality at any one concentration.

The statistical results of this test indicated that a highly antagonistic response was occurring. The joint test was performed again and the accuracy of estimated LC50 concentrations of the chemicals applied singularly were tested.

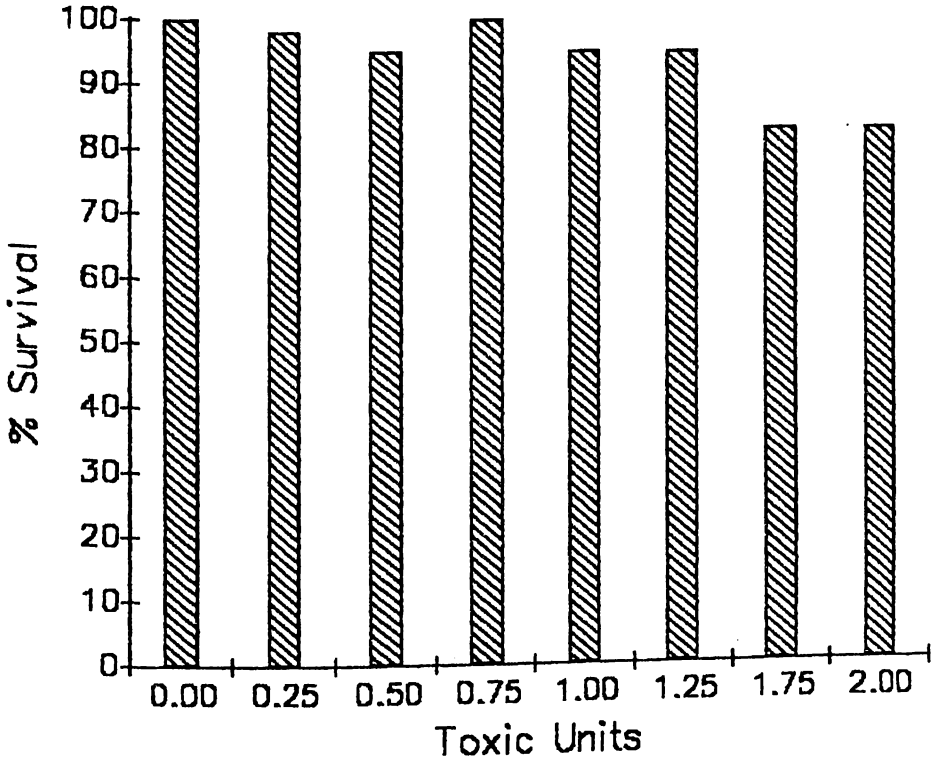


FIGURE 21: Pimephales Mean Survival per Concentration for Joint Test 1. 11 June - 18 June 1987

Joint Test 2

A separate set of single toxicity tests (Atrazine and Alachlor Test 3) were performed concurrently with Joint Test 2. These tests were utilized to verify the accuracy of the estimated LC50 values which were used to determine Toxic Unit values. The Toxic Unit adjustments were then made according to LC50 results obtained from Atrazine and Alachlor Test 3.

Atrazine Test 3

Acute Analysis

The Trimmed Spearman-Karber analysis, Table 39, of the mortality produced in Atrazine Test 3 estimated the LC50 to be 14.70 mg/L, Figure 22. The original value estimate of 15.00 mg/L, obtained from Test 2, was used to set the Toxic Units in Joint Test 2. The new value was 0.3 mg/L above the actual value achieved in the concurrently run Atrazine Test 3; thus, the Toxic Units were adjusted accordingly, Table 44.

Chronic Analysis

NOEC and LOEC values were reported as 1.50 mg/L and 3.00 mg/L, respectively, by the Dunnett's Procedure, Table 40, and the Williams' Test, Table 40a. Mean dry weights are presented in Figure 23.

FIGURE 22: Pimephales Mean Survival per Concentration for Atrazine Test 3. 3 July - 10 July 1987

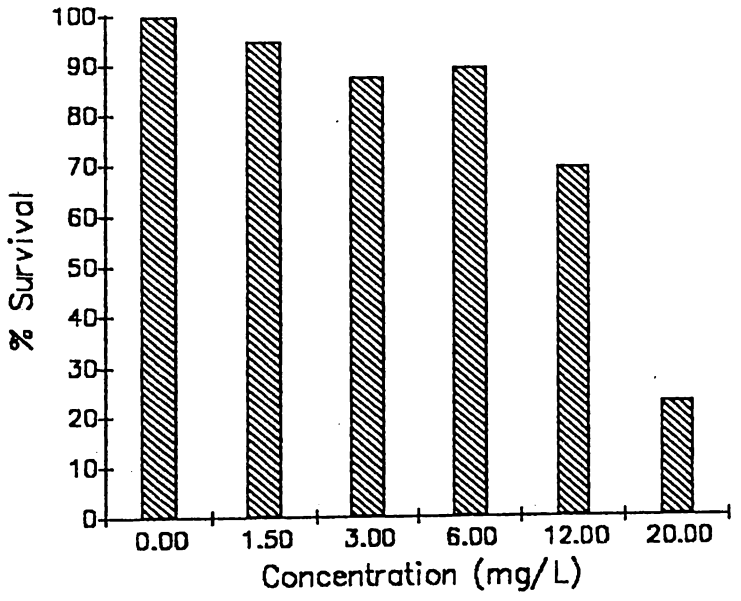
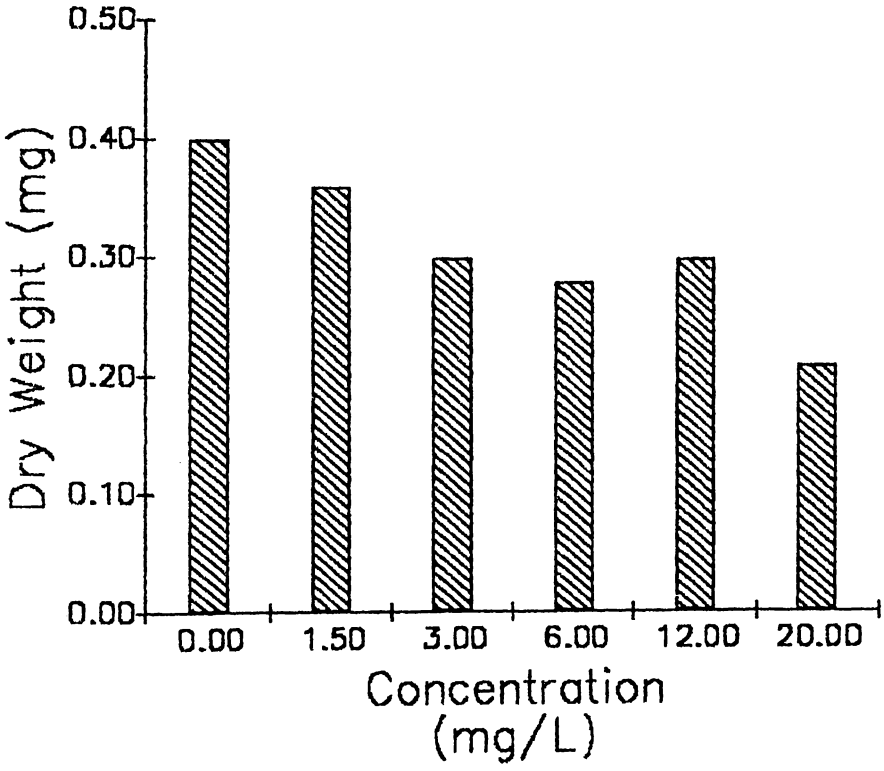


TABLE 39: Trimmed Spearman-Karber Analysis of Atrazine Test 3

CONCENTRATION mg/L	CONCENTRATION ln	ORGANISMS EXPOSED	DEAD ORGANISMS	RAW MORTALITY	ADJUSTED MORTALITY
1.50	0.41	40	2	0.05	0.05
3.00	1.01	40	5	0.13	0.11
6.00	1.79	40	4	0.10	0.11
12.00	2.49	40	12	0.30	0.30
20.00	3.00	40	31	0.78	0.78

Spearman Karber Trim = 24%

	ln	mg/L
Estimated LC50	2.69	14.75
Lower 95% Confidence Limit	2.47	11.82
Upper 95% Confidence Limit	2.91	18.39



	Chamber #	Atrazine (mg/L) Control	1.50	3.00	6.00	12.00	20.00
Fish Weights (mg)	1	0.34	0.35	0.24	0.26	0.26	0.20
	2	0.36	0.36	0.34	0.30	0.34	0.20
	3	0.42	0.37	0.32	0.26	0.30	0.00
	4	0.49	0.37	0.32	0.28	0.31	0.25
Mean		0.40	0.36	0.32	0.28	0.30	0.21

FIGURE 23: Atrazine Test 3 - Mean fish weights (mg) from four replicate test chambers for each treatment concentration of surviving fish. Mean fish weights for each test chamber at each concentration level are presented in the lower panel.

TABLE 40: Dunnett's Procedure for Data Set: Fathead minnow - Atrazine Test 3.

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	0.08675	5	0.01735	6.45**	4.25
Within	0.04842	18	0.00269		

**P<0.01

Observed Difference from Control

mg/L	Difference	ti		Dunnett's Critical t
control	0.00	0.00		
1.50	0.04	1.09	<NOEC	2.41*
3.00	0.10	2.73	<LOEC	
6.00	0.12	3.27		
12.00	0.10	2.73		
20.00	0.19	5.18		

*P<0.05

Table 40a: Williams' t Test for Data Set: Fathead Minnow - Atrazine Test 3

Error Mean Square from ANOVA: 0.003

Mean response is expected to decrease as treatment concentration increases.

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML' Estimator	$\bar{t}'$
0.00	4	0.40					
1.50	4	0.36	0.36	1.09	<NOEC	0.36	1.09
3.00	4	0.30	0.30	2.73	<LOEC	0.30	2.73
6.00	4	0.28	0.29	3.00		0.28	3.27
12.00	4	0.30	0.29	3.00		0.29	3.00
20.00	4	0.21	0.21	5.18		0.21	5.18

k = 5 Williams' Critical t = 1.89
v = 15

Alachlor Test 3

Acute Analysis

Mortality results of this test are presented in Figure 24. The LC50 was estimated at 4.9 mg/L by the Trimmed Spearman-Karber analysis, Table 41. This new value was slightly higher than the value used in setting the Toxic Units for the joint test. The appropriate adjustments were made to the Toxic Units as shown in Table 44.

Chronic Analysis

Analysis of dry weight results, Figure 25, revealed that the 2.0 mg/L concentration produced larger fish than were produced in control. Weights of the fish in the next higher concentration, 4.0 mg/L, are shown to decrease dramatically. The Dunnett's Procedure, Table 42, and the Williams' Test, Table 42a, determined the 2.0 mg/L level was the NOEC and 4.0 mg/L was the LOEC.

The second joint test was designed to include concentrations ranging from 0.50 TU to 4.0 TU. These concentrations were used to provide for any unexpected antagonistic responses that may have been occurring. The 2.0 TU level was now represented by the higher confidence interval of the LC50 estimate, derived from single Test 2 for Atrazine and Alachlor. Thus, 15.00 mg/L of atrazine and 4.1 mg/L of alachlor were combined to achieve the 2.0 TU level. The 4.0 TU level represented two times the amount of each chemical which would be needed to kill 50% of the fish when used singularly.

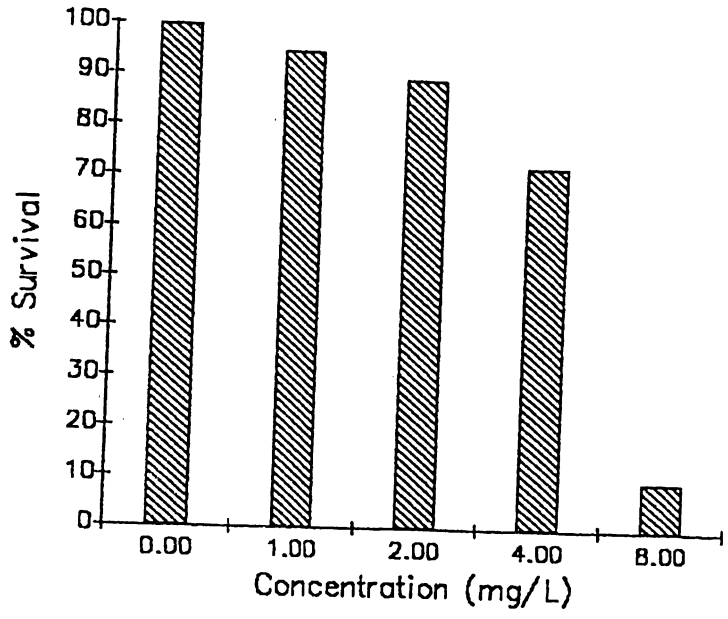


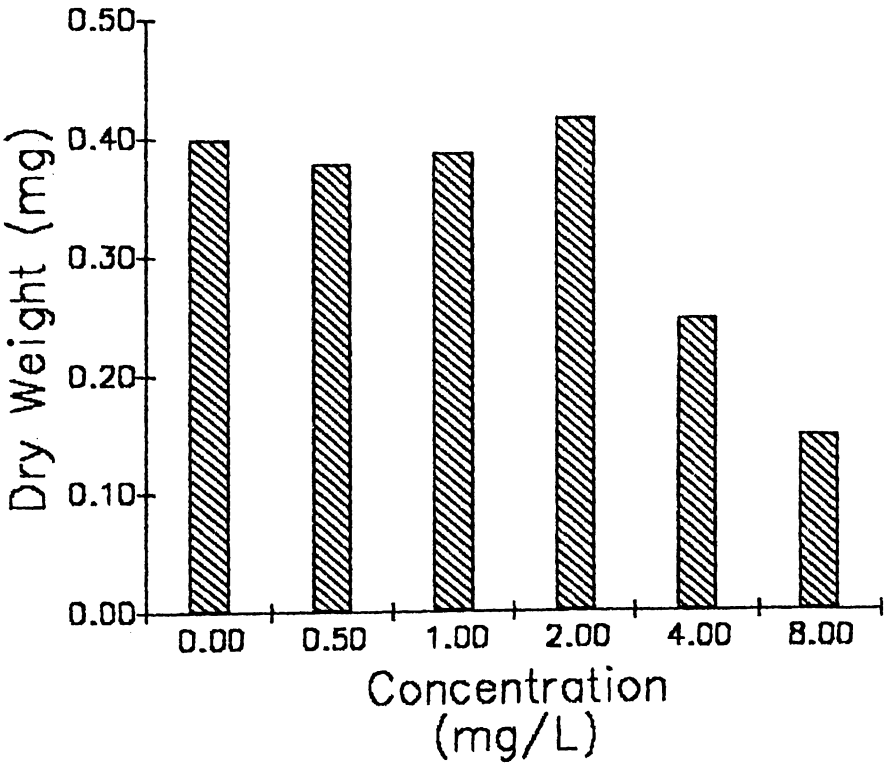
FIGURE 24: Pimephales Mean Survival per Concentration for Alachlor Test 3. 3 July - 10 July 1987

TABLE 41: Trimmed Spearman-Karber Analysis of Alachlor Test 3

CONCENTRATION mg/L	ln	ORGANISMS EXPOSED	DEAD ORGANISMS	RAW MORTALITY	ADJUSTED MORTALITY
0.50	-0.69	40	0	0.00	0.00
1.00	0.00	40	2	0.05	0.05
2.00	0.69	40	4	0.10	0.10
4.00	1.39	40	11	0.28	0.28
8.00	2.08	40	36	0.90	0.90

Spearman Karber Trim = 11%

	ln	mg/L
Estimated LC50	1.59	4.88
Lower 95% Confidence Limit	1.45	4.26
Upper 95% Confidence Limit	1.72	5.60



	Chamber #	Control	Alachlor (mg/L) 0.50	1.00	2.00	4.00	8.00
Fish Weights (mg/L)	1	0.34	0.34	0.36	0.46	0.22	0.20
	2	0.36	0.35	0.42	0.45	0.21	0.00
	3	0.42	0.41	0.40	0.40	0.31	0.15
	4	0.49	0.42	0.40	0.37	0.28	0.10
Mean		0.40	0.38	0.39	0.42	0.25	0.15

FIGURE 25: Alachlor Test 3 - Mean fish weights (mg) from four replicate test chambers for each treatment concentration of surviving fish. Mean fish weights for each test chamber at each concentration level are presented in lower panel.

TABLE 42: Dunnett's Procedure for Data Set: Fathead minnow - Alachlor Test 3.

ANOVA Table

Source	Sum of Squares	D.F.	Mean Square	F Ratio	Significance
Between	0.2315	5	0.04630	20.59**	4.35
Within	0.0405	18	0.00225		

**P<0.01

Observed Difference from Control

mg/L	Difference	ti		Dunnett's Critical t
control	0.00	0.00		
0.50	0.02	0.60		
1.00	0.01	0.30		
2.00	-0.02	-0.60	<NOEC	2.41*
4.00	0.15	4.47	<LOEC	
8.00	0.25	7.45		

Table 42a: Williams' t Test for Data Set: Fathead Minnow - Alachlor Test 3

Error Mean Square from ANOVA: 0.002

Mean response is expected to decrease as treatment concentration increases.

Concentration	Replicates	Mean Response	ML Estimator	$\bar{t}$		ML' Estimator	$\bar{t}'$
0.00	4	0.40					
0.50	4	0.38	0.40	0.10		0.38	0.60
1.00	4	0.39	0.40	0.10		0.39	0.45
2.00	4	0.42	0.40	0.10	<NOEC	0.40	0.10
4.00	4	0.25	0.25	4.47	<LOEC	0.25	4.47
8.00	4	0.15	0.15	7.45		0.15	7.45

k = 5 Williams' Critical t = 1.89
v = 15

Acute Analysis

The fish survival summary is presented in Figure 26. The Trimmed Spearman-Karber analysis estimated that the LC50 occurred at the 1.35 adjusted TU level, Table 43. This toxic unit equivalence value represented 10.2 mg/L of atrazine and 2.7 mg/L of alachlor, Table 44, indicating that less of each chemical was required to cause the LC50 when they were combined. Lower and Higher Confidence Limits were analyzed in order to determine if the joint results were actually causing a greater effect. Figure 27 illustrates that the confidence intervals of the estimated LC50 values do not overlap the estimated LC50 value. The results show that the joint toxicity of the chemicals caused a greater than additive effect.

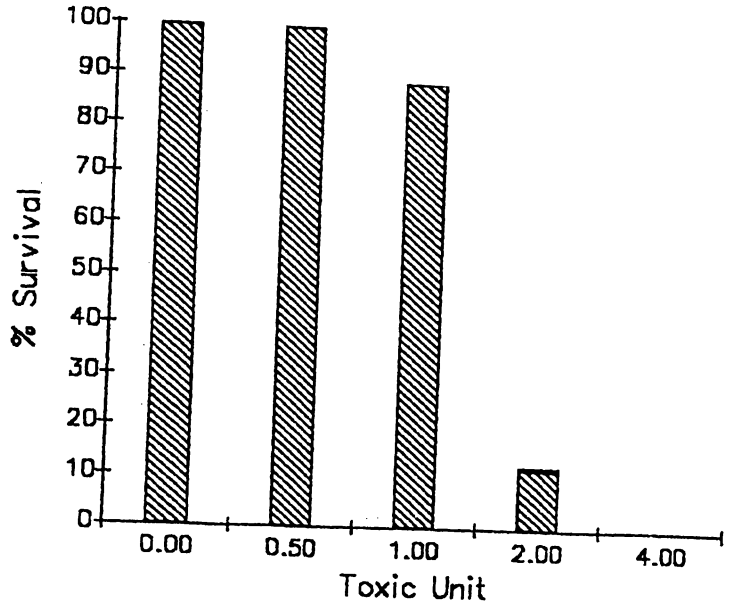


FIGURE 26: Pimephales Mean Survival per Concentration for Joint Test 2. 3 July - 10 July 1987

TABLE 43: Trimmed Spearman-Karber Analysis of Joint Test 2

CONCENTRATION TU	CONCENTRATION 1n	ORGANISMS EXPOSED	DEAD ORGANISMS	RAW MORTALITY	ADJUSTED MORTALITY
0.47	-0.76	40	0	0.00	0.00
0.94	-0.06	40	4	0.10	0.10
1.87	0.63	40	35	0.88	0.88
3.74	1.32	40	40	1.00	1.00

Spearman Karber Trim = 1.0%

	1n	mg/L
Estimated LC50	0.30	1.35
Lower 95% Confidence Limit	0.20	1.22
Upper 95% Confidence Limit	0.40	1.49

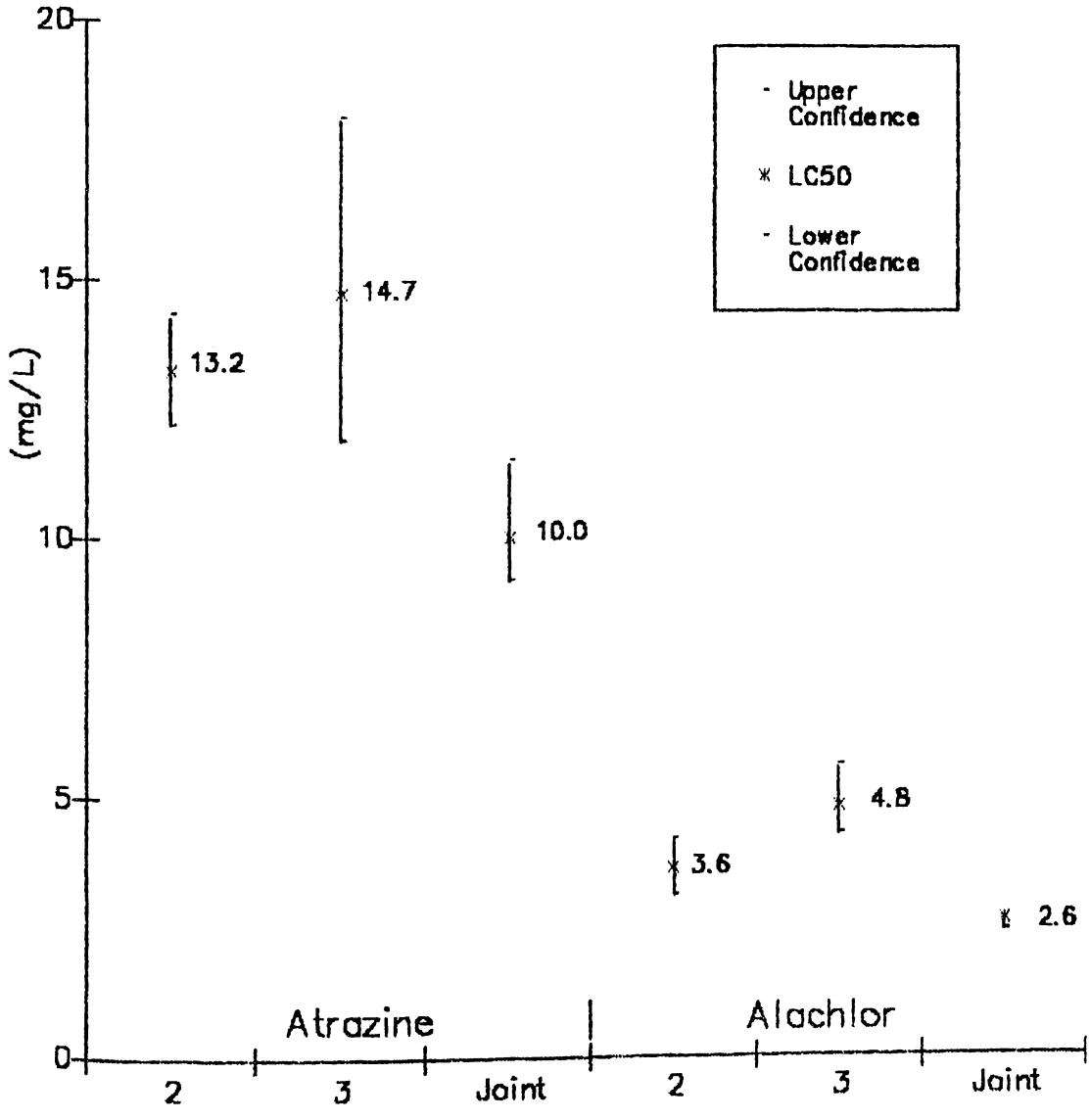


FIGURE 27: LC50 and Confidence Limits of Pimephales promelas for Single Atrazine/Alachlor Tests and Joint Test 2

Table 44: Toxic Unit Adjustments and Chemical Equivalence Calculations for Fathead Joint Test 2.

The Atrazine and Alachlor Test 3 results were:

Estimated LC50 = 14.70 Atrazine
4.88 Alachlor

Therefore, the TU were adjusted to:

(estimated) (actual)
Atrazine - 15.00 mg/L divided by 14.70 mg/L = 102 % of the LC50 value.

(estimated) (actual)
Alachlor - 4.10 mg/L divided by 4.88 mg/L = 85 % of the LC50 value.

Thus, the 2 TU level was actually representative of 1.87 TU.

Toxic Unit		Atrazine (mg/L)		Alachlor	Adjusted Toxic Unit
0.5	=	3.75	+	1.00	0.47
1.0	=	7.50	+	2.00	0.94
2.0	=	15.00	+	4.10	1.87
4.0	=	30.00	+	8.20	3.74

Chemical Equivalence as represented at the TU LC50

Trimmed Spearman-Karber Estimated LC50 = 1.35 TU
Lower 95% Confidence Limit = 1.22 TU
Upper 95% Confidence Limit = 1.49 TU

The proportion of this amount to the original 1.0 TU was obtained:

LC50 = 1.0 TU / 1.35 TU = 0.74*
Lower Confidence Limit = 1.0 TU / 1.22 TU = 0.82-
Upper Confidence Limit = 1.0 TU / 1.49 TU = 0.67+

Atrazine

Chemical Equivalence at:
EC50 7.5 / 0.74 = 10.1 mg/L*
Lower Confidence 7.5 / 0.82 = 9.2 mg/L-
Upper Confidence 7.5 / 0.67 = 11.2 mg/L+

Alachlor

Chemical Equivalence at:
EC50 2.0 / 0.74 = 2.7 mg/L*
Lower Confidence 2.0 / 0.82 = 2.4 mg/L-
Upper Confidence 2.0 / 0.67 = 3.0 mg/l+

DISCUSSION

The separate acute toxic effects caused by atrazine and alachlor were established for Lemna minor (EC50), Ceriodaphnia reticulata and Pimephales promelas (LC50s). The chronic no observable and lowest observable effect concentrations for all organisms to each chemical were also calculated, Table 45.

Effects of Atrazine

Lemna minor and other aquatic macrophytes have been demonstrated to be the most sensitive non-target species to the effects of atrazine. This was expected since the herbicidal properties of the chemical are known to be photosynthetic inhibition. Atrazine was demonstrated to cause a significant acute response to the plants in the presence of less than 200 ug/L. Toxic levels are often dependent on the type of dilution water particular to each test. In the case of Atrazine Test 1 the acute effect was reported at 86.4 ug/L. The chronic effect was detected at levels ranging as low as 0.5 ug/L to 7.5 ug/L. Atrazine has been observed at concentrations up to 42 ug/L in the natural aquatic environment (Richard et al., 1974).

Relatively few similar studies have been done to analyze the effects of herbicides on Lemna and other aquatic plants. Research with Lemna has been directed toward examining the effects atrazine has on the structural changes within the plant (Beaumont & Grenier, 1979). Other research focused on the possibility of utilizing

TABLE 45: EC50, LC50 and NOEC and LOEC values for all organisms tested in single toxicity tests for atrazine and alachlor

ATRAZINE

	EC50/LC50	95% Confidence Limits	NOEC/LOEC	
Lemna minor (ug/L)				
5 Nov 1986	86.3	77.4 - 96.1	50	100
23 June 1987	197.4	146.1 - 266.8	0	25
Ceriodaphnia (mg/L)				
8 Nov 1966	*	*	4.0	6.0
12 Feb 1987	7.9	5.8 - 10.7	3.0	6.0
Pimephales (mg/L)				
6 Nov 1986	*	*	*	*
25 Mar 1987	*	*	1.5	3.0
16 Apr 1987	13.2	12.2 - 14.3	*	*
3 Jul 1987	14.8	11.8 - 18.4	1.5	3.0

ALACHLOR

Lemna minor (ug/L)				
14 Jan 1987	*	*	5.0	7.5
23 Jun 1987	35.6	30.6 - 40.6	0	5.0
Ceriodaphnia (mg/L)				
16 Jan 1987	4.6	3.0 - 7.4	0	1.0
10 Feb 1987	*	*	1.0	2.0
Pimephales (mg/L)				
16 Jan 1987	*	*	1.0	2.0
25 Mar 1987	3.6	3.1 - 4.2	0.5	1.0
3 Jul 1987	4.9	4.3 - 5.6	2.0	4.0

* Values cannot be calculated from data

Lemna species as indicators or purifiers of heavy metal pollution due to its rapid absorption capability (Nasu etal, 1979). Stratton (1984) detected the EC50 of Anabaena spp., a bluegreen algae, to range from 0.1 to 0.5 mg/L atrazine. Acute toxicity to Elodea was caused by 80 ug/L atrazine (Forney & Davis, 1981) and 650 ug/L was found to be toxic to Potomogeton, a macrophyte, (Correll & Wu 1982).

The acute effects on the other non-target organisms caused by atrazine do not occur unless a relatively high concentration of the chemical is present. The known occurrence of atrazine in the environment is considerably lower than is needed to elicit the acute response of the Ceriodaphnia and the fathead minnow. The LC50 to the Ceriodaphnia was estimated at 7.9 mg/L and the rate of reproduction was observed to significantly decrease at the 6.0 mg/L level. Acute testing with Ceriodaphnia provided LC50 values for atrazine, 7.9 mg/L, and alachlor, 4.7 mg/L. Both the values are reported, as calculated by the Trimmed Spearman-Karber method, with 95% probability of occurring within the range of confidence limits. The confidence intervals which have been associated with the Ceriodaphnia tests indicated that the actual LC50 may occur within the range of 5.8 mg/L to 10.7 mg/L for atrazine and between 3.0 mg/L to 7.4 mg/L for alachlor. The atrazine LC50 value obtained from this test was found to be within the LC50 confidence intervals reported by Macek et al, 1976, to another zooplankton species, Daphnia magna. Other zooplankton information was found to be inconsistent with these results. Hartman & Martin, 1985 reported a considerably higher LC50

of 36.5 mg/L to Daphnia pulex. These values were all higher than the previously estimated values of 3.6 mg/L (FWPCA, 1968). Macek, (1976) found the chronic effect of Daphnia magna to occur at 0.25 mg/L which better reflects the lowest observable effect concentration. Further testing would be necessary to determine whether the chronic response attained in this series of tests were accurate.

Tests conducted with the fathead minnow were found to provide consistent LC50 data. Analysis of acute toxicity data indicated that approximately 14.7 mg/L of atrazine and 4.9 mg/L of alachlor caused significant mortality to the fish. These values were in agreement with other research conducted with the fathead minnow. Much of the aquatic toxicity testing have been performed using the fathead minnow and other fish species as the test organisms. Macek et al (1979) tested the effects of atrazine which provided very similar values to the results of this study. His study concluded that 15.0 mg/L caused the LC50 to the fathead minnow.

The chronic analysis of mean dry weight indicated that the NOEC and LOEC values were 1.5 mg/L and 3.0 mg/L, respectively. These values were significantly lower than the acute response. However, other studies reported that the chronic response occurred at much lower concentrations than indicated here. Macek et al. (1979) found that the maximum allowable toxicant concentration was between 0.11 mg/L, NOEC, and 0.23 mg/L, LOEC. These values were probably more indicative of the expected levels due to the extensive parameters examined in the chronic analysis performed in the study.

Effects of Alachlor

Testing with alachlor demonstrated that it produces a much higher degree of toxicity to organisms than atrazine. The acute response of all of the organisms tested was usually demonstrated within three days of the onset of each test. Fifty percent reduction in growth of Lemna was achieved at the 35.2 ug/L concentration. The concentration of Test 1, 0.5 ug/L - 20 ug/L, did not elicit a fifty percent reduction in growth. The graphical procedure utilized in order to estimate the amount of alachlor that would have produced fifty percent reduction in growth was adapted from the chronic procedures described in the EPA/600/4-85/014 document. The method in the manual illustrates the LC50 value graphically and is used when at least fifty percent mortality has occurred. In this analysis the graph, Figure 2a, was plotted even though 50 percent mortality had not occurred. A line was manually fitted to the plotted points and crossed the fifty percent reduction of growth line between the 25 ug/L and 35 ug/L concentrations. The second set of tests statistically determined that the EC50 was 35.2 ug/L. This example illustrated that it may be possible to estimate the EC50 or LC50 without actually achieving the necessary 50 percent mortality.

The concentrations used in the fathead minnow Test 1 included the estimated LC50 derived from Test 2. The LC50 acute response was indicated to occur at 4.9 mg/L. Only one other study was available

which reported the LC50 caused by alachlor to fathead minnow was 5.0 mg/L (Call et al., 1984). The 4.0 mg/L concentration of Test 1 should have produced significantly greater mortality than actually occurred. However, the fish in the highest level of Test 1, 5.0 mg/L, were noted to be immobile and sometimes lying at the bottom of the chamber. These fish were recorded as alive because gill movement, an EPA established survival quantifier, was observed. The apparent discrepancy was therefore resolved upon examination of the confidence intervals of Test 2 and Test 3 which indicated that the LC50 could occur anywhere from 3.1 mg/L to 5.6 mg/L. Analysis of the criteria which determine the LC50 reveals that the death of one fish can cause the significance needed to declare the toxic level. Thus, when fish are killed inadvertently during the necessary renewal process or when one weaker individual occurs in a concentration level and dies sooner than would normally be expected, the estimated LC50 may be reported at slightly higher or lower concentrations than actually exist. The chronic response to alachlor was found to occur between 1.0 and 4.0 mg/L.

Test Variations

Many of the aspects that occurred during this series of experiments illustrate the common variables that are encountered when utilizing toxicity test procedures. The dynamic nature of each individual aquatic ecosystem and its particular inhabitants cause the physical and the chemical properties of each to be unique. The toxicity of a chemical is most often influenced by the properties

occurring in the specific medium or environment. It is difficult and, sometimes impossible to assess all parameters and predict precisely the level of toxic response from one system to another. Although dilution water for toxicity testing in the laboratory can sometimes be obtained from the actual site that is to be evaluated, but within the laboratory it cannot accurately represent the possible interactions that may occur in the natural environment. The conclusion may result in an inaccurate estimate by increasing or decreasing the toxic effect of the chemical.

The dilution water in this experiment was obtained from surface water for some of the test procedures and from a ground water source for other tests. The difference in hardness and alkalinity between sites is known to influence the toxic effect of some types of chemicals on the test organisms. Pesticides are not known to be one of these types of chemicals (Sprague, 1985). However, the change in water may have been responsible for the noted change in the growth of the control Lemna and could not be used as dilution water for the Ceriodaphnia since the adult Ceriodaphnia could not tolerate the well water even with a gradual acclimation.

Another variable was created when the original stock colony of Lemna clones failed to maintain over the duration of the test period, eight months, making it necessary to develop a new colony of Lemna obtained from a different source for the second series of tests. The Lemna tests specifically demonstrated the variability of results that may occur due to numerous factors known to occur when utilizing

bioassay type experimentation. Atrazine caused a greater than 50% reduction of growth in the presence of 86.4 ug/L in Test 1 while it took 197.2 ug/L to cause a similar response in Test 2. Preliminary examination of the different values reported between the two tests appeared to be significant. A further examination of the results of the acute response of Lemna revealed that the real differences between the two EC50 values actually must be calculated from the Upper Confidence Interval of 96.1 ug/L in Test 1 and the Lower Confidence Interval of 146.1 ug/L attained in Test 2. Thus, the difference, 50 ug/L, was well within the variation range reported to be from 25% to 40% between tests (Macek, 1985).

Problems with the culture and maintainance of Ceriodaphnia have been noted by other researchers and high culture mortality has been associated with some types of dilution water. When it became necessary to switch the dilution water used for all experiments after Lemna Test 1, the Ceriodaphnia did not adjust to the change. Consultation with Columbia National Pesticide Research Labortatory personnal indicated that the organism is quite sensitive to slight changes in hardness and pH (pers.comm., S. Finger, May 1986). The only criteria acceptable for dilution water for Ceriodaphnia testing is that the organism thrives and exhibits normal reproductive behavior. Reservoir water was therefore utilized throughout Ceriodaphnia testing. Tests conducted with the Ceriodaphnia provided inconsistent results especially with regard to the reproduction data. This was indicative of possible environmental stress related to

laboratory maintenance. The chronic results are reported although they seem to be quite high when compared to other reported chronic effects. Normally the chronic effect occurs at a level approximately 10 times lower than the acute of lethal concentration level. The estimated LC50's for atrazine and alachlor to Ceriodaphnia are reported for tests that were conducted in which no observed abnormalities occurred. The estimated LC50 result for atrazine exhibits a large confidence interval. The acute effect to Ceriodaphnia occurred within the range provided by the higher and lower confidence intervals. These Trimmed Spearman-Karber results reflect the high degree of standard deviation in the data but can report the general area of toxicity which can now be further quantified.

The tests conducted with fathead minnows provided the most consistent data. Problems of low dissolved oxygen concentrations were associated with most of the tests. The dissolved oxygen levels were monitored periodically and corrected by the prescribed methods (EPA/600/4-85/013). Fathead minnows are known to occur naturally in areas and are found to be tolerant of low dissolved oxygen concentrations. The low dissolved oxygen condition, ranging from 2.00 mg/L - 4.00 mg/L, consistently appeared in all test concentrations including the control vessels. Examination of the survival records indicated that mortality was never associated with low dissolved oxygen conditions. It was therefore concluded that

this was not a significant determining factor in the results of the toxicity of atrazine and alachlor to the fathead minnow.

A laboratory error occurred in Alachlor Test 2. Eighty percent of the fish in one of the four test chambers of the 1.00 mg/L concentration died within 24 hours of test initiation. It may have been attributed to contamination in that particular beaker or the temperature of test solution may not have been adjusted prior to fish transfer. The next higher concentration level did not exhibit significant mortality; therefore, the 1.00 mg/L concentration series was eliminated from any acute and chronic analysis.

Effects of Joint Exposure

Joint testing with atrazine and alachlor have been studied and the combination was found to prolong weed control at low rates (Akabundu, 1975). This desirable effect has been analyzed for the use in agricultural management for use to control target weeds (Anderson, 1963; Bayer, 1966; Curry & Cole, 1966; Kenyon & Ball, 1967; Liu et al., 1966; Raleigh, 1967; Thomson, 1966; Wilson et al., 1966). The extensive practice of mixing the two chemicals has become common in the midwest region causing the combination to be found in natural aquatic environments. The effects on non-target aquatic organisms which are unavoidably exposed have not previously been evaluated. The combined acute toxic effects of atrazine and alachlor were evaluated for Lemna minor and the fathead minnow. The joint

effects indicated a significantly lower amount of the two combined chemicals was needed to produce the same effect caused by applying the chemicals singularly, Table 46.

A few studies of other types of combined herbicides on aquatic organisms have also provided synergistic effects. Finlayson and Faggella (1986) found additive toxicity when Moliate and Thiobencarb pesticides were combined on four different fish species. The combined effects of other organic chemical mixtures have been analyzed with conclusive evidence of synergism by others (Broderius & Kahl, 1985; Woodward, 1982; Konemann, 1981).

The results of the Fathead Minnow Joint Test 1 were unexpected and contradictory to the results of the preceding single toxicity tests. The TU concentrations used in the test were calculated from the LC50 values of the single tests. The series of Toxic Unit concentrations ranged from 0.5 - 2.0. The 2.0 TU level contained the concentration that produced the estimated LC50 using atrazine and the LC50 of alachlor for the fathead minnow. A purely additive response would have occurred at the 1.0 TU level. However, analysis of the mortality results determined that at least 50% mortality had not occurred at either the 1.0 TU or the 2.0 TU levels. It was noted that the organisms in the 2.0 TU level were barely alive, as indicated by their inactive behavior and did not move away or react to the cleaning pipet during solution renewal transfer. Initially, the results indicated that an unpredicted, highly antagonistic response may have been produced by the combined chemicals.

Joint Test 2 was thus designed to (1) incorporate concurrent single tests for each chemical to verify the accuracy of the first series of single tests and (2) to utilize the upper confidence intervals of the single Atrazine and Alachlor Test 2 to set TU concentrations. Two important interpretations were provided by Joint Test 2 data. The results of the two single tests verified that the LC50 values derived from the first single tests were accurate. Secondly, use of the upper confidence values to set TU concentrations caused the 1.0 TU level to be toxic whereas in Joint Test 1 the mortality was not significant at even the 2.0 TU level. This indicated that a threshold effect may have occurred with the use of the slightly higher concentration to the set the TU concentrations in Joint Test 2.

An important realization was established by the information obtained from the data provided by Joint Test 1 and Test 2. Since the LC50 values are estimates which are calculated by means of regressing a line through the given mortality verses concentration points, it is necessary to narrow the range of the reported confidence intervals and to include the upper confidence limit in the determination of the Toxic Unit concentrations.

The results of this series of tests indicated a greater than additive response may be expected to occur when atrazine and alachlor are found together.

Many of the toxicity studies to organisms has evaluated exposure to individual chemicals. Relatively few studies have focused on the

TABLE 46: Acute single and joint toxicity values (EC50/LC50 with 95% confidence intervals) for Lemna minor and Pimephales promelas as caused by atrazine and alachlor.

	Single Toxicity Values		Joint Toxicity Values	
	Atrazine	Alachlor	Atrazine	Alachlor
Lemna (ug/L)				
Test 1	86.3 77.4-96.1	*	*	*
Test 2	197.7 (146.1-266.8)	35.6 (30.6-40.6)	22.2 (19.6-26.1)	6.4 (2.4-3.1)
Fathead (mg/L)				
Test 2	13.2 (12.2-14.3)	3.6 (3.1-4.2)	*	*
Test 3	14.8 (11.8-18.4)	4.9 (4.3-5.6)	10.0 (9.2-11.5)	2.6 (2.4-3.1)

* Values cannot be calculated from data

joint toxicity to aquatic organisms although it is inherent that the occurrence of mixtures of chemicals is the rule in the real world. A summary of the publications that review the toxicity of combined mixtures of chemicals on aquatic organisms indicated the acute and chronic effects caused from combined chemicals occur at significantly lower concentration than observed with individule chemicals (Sprague, 1970; Anderson & Weber, 1975; Muska & Weber, 1977; Alabaster & Lloyd, 1980; Calamari & Alabaster, 1980; Konemann, 1981a; Hermans & Leewangh, 1982; Finlayson and Faggella, 1986). More and more chemicals have become regarded as essential to daily life creating a greater urgency for evaluating the joint effects of the combined chemicals. The chronic effect is a more sensitive measure to an organism and should be used as a guildline when determining the toxicity of a chemical. The implications that the knowledge of joint toxicity provides must be environmentally applied when determining the biological significance of the simultaneous occurrence of aquatic toxicants. The significantly higher toxic effects that are caused by chemical mixtures should be considered when determining the criteria of the discharge and concentration levels acceptable for maintaining the integrity of the earth's waters.

The knowledge is available to define, at the very least, a conservative estimate of the concentration level that a severe response is likely to occur and that information should be utilized to provide the guildlines needed to avoid acute damage to an ecosystem.

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CONCLUSIONS

The following conclusions were made from the this toxicity testing research:

1. Separate single toxicity testing with atrazine and alachlor revealed that the relative acute toxicity in both cases was plant > invertebrate > vertebrate.

2. Alachlor consistently caused acute toxicity to all test species at significantly lower concentrations than did atrazine.

3. Joint toxicity testing with atrazine and alachlor combined at 1:1 ratios demonstrated that the relative toxicity was plant > vertebrate.

4. Joint results of the combined chemicals caused the acute response at lower concentrations than when the chemicals were tested singularly. The combined chemicals were superadditive to the test species.

5. The chronic response of test species when exposed to atrazine or alachlor were produced at significantly lower concentrations than was needed to elicit the acute response.

RECOMMENDATIONS FOR FUTURE STUDY

Future joint testing with atrazine and alachlor should be conducted utilizing combined ratios, other than 1:1, that are known to occur in the natural environment. This information would determine if superadditivity is maintained at the different concentration ratios.

Joint toxicity testing should necessarily be supplemented with concurrent single toxicity testing to assure the integrity of the originally obtained EC50 or LC50 values.

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APPENDIX 1

Hutner's Stock Solution (from Hutner, 1953)

STOCK SOLUTION I

SOLUTION A:

While stirring about 200 ml distilled water, add:

17.7 gm	$Ca(NO_3)_2.4H_2O$
25.0 gm	ethylenediaminetetraacetic acid (EDTA)
20.0 gm	K_2HPO_4
10.0 gm	NH_4NO_3

To dissolve EDTA, add 12-13 gm KOH (85 pellets).

SOLUTION B:

To another volume of water (about 150 ml), add the following with constant stirring:

3.295 gm	$ZnSO_4.7H_2O$
0.710 gm	H_3BO_3
1.260 gm	$Na_2MoO_4.2H_2O$
0.197 gm	$CuSO_4.5H_2O$

At this point add 1 N HCl (about 13 drops) until the cloudiness disappears. Then add:

0.010 gm	$Co(NO_3)_2.6H_2O$
0.897 gm	$MnCl_2.4H_2O$

SOLUTION C:

To a third volume of water (about 50 ml) add:

1.245 gm	$FeSO_4.7H_O$

Now, with constant stirring, combine Solutions A, B, and C. Take to 1,000 ml with distilled water. This makes Stock Solution I. Keep refrigerated.

STOCK SOLUTION II

To make Stock Solution II, take 25.0 gm $MgSO_4.7H_2O$ to 1,000 ml with distilled water.

For full strength medium, use 20 ml of Stock Solution I plus 20 ml Stock Solution II per liter of medium.

APPENDIX 2

WILSON LABORATORIES

525 NORTH EIGHTH STREET - P.O. BOX 1884 - SALINA, KANSAS 67402-1884 - (913)825-7186

LABORATORY REPORT

CLIENT: KANSAS BIOLOICAL SURVEY - 2
ATTN: DEAN KETTLE
2291 IRVING HILL DRIVE
LAWRENCE, KS 66045

DATE RPTD: 11/20/87
DATE RCVD: 11/04/87
PURCHASE AUTH: AL 59447
FILE NO.: 87-9844
ORDER NO.: 8976

LAB NUMBER: 87120193
SAMPLE DESCRIPTION: NESA WELL WATER

ANALYSIS	CONCENTRATION	UNITS	BOOK-PAGE
ALACHLOR	ND(0.25)	UG/L	1088-15
ATRAZINE	ND(1.0)	UG/L	1088-15

—CONCLUSION—LAB NUMBER: 87120193 NESA WELL WATER

ND(), WHERE NOTED, INDICATES NONE DETECTED WITH THE DETECTION LIMIT IN PARENTHESES.

ANALYSES WERE PERFORMED ON SAMPLES AS RECEIVED IN ACCORDANCE WITH PROCEDURES REFERENCED IN THE FEDERAL REGISTER, VOL. 49, NO. 209, OCT. 26, 1984; PUBLISHED IN EPA PUBLICATION, SW 846, 2ND ED., JULY 1982 AND IN THE PROPOSED ADDITION TO SW 846, 1984; OR IN EPA PUBLICATION, SW846 3RD ED.,SEPTEMBER 1986. WHERE APPROVED METHODS ARE NOT AVAILABLE, EFFORTS ARE MADE TO USE APPROPRIATE STANDARD METHODS.

SAMPLES WILL BE RETAINED FOR 30 DAYS UNLESS OTHERWISE NOTIFIED.

WILSON LABORATORIES

LYNN R. NEWCOMER
CHIEF CHEMIST

Water Analysis Summary for Lemna minor Atrazine Test 1 and Alachlor Test 1. Water Quality Means () and Ranges.

Atrazine Test 1

	Day 0		Day 7	
	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	7.9	(7.5) 7.4-7.5	9.6	(8.2) 7.5-10.1
pH	7.2	(6.9) 6.8-6.9	8.3	(8.0) 7.8-8.2
Total Hardness (mg/L CaCo3)	105	(100) 98-100	122	(121) 105-130
Total Alkalinity (mg/L)	92	(93) 89-100	86	(77) 70-82
Specific Conductance (umhos)	171	(173) 171-181	165	(165) 160-172

Alachlor Test 1

	Day 0		Day 7	
	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	8.8	(7.6) 7.5-7.8	7.8	(7.6) 7.5-7.6
pH	7.4	(7.4) 7.3-7.4	8.3	(8.3) 8.2-8.3
Total Hardness (mg/L CaCo3)	94	(94) 94	129	(142) 137-152
Total Alkalinity (mg/L)	86	(86) 86	104	(98) 82-114
Specific Conductance (umhos)	225	225	350	(322) 300-350

Water Analysis Summary for Lemna minor Atrazine Test 2 and Alachlor Test 2. Water Quality Means () and Ranges.

Atrazine Test 2

	Day 0		Day 7	
	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	8.1	(8.2) 8.1-8.3	7.5	(7.6) 7.2-8.0
pH	7.2	(7.2) 7.2	8.2	(8.3) 8.0-8.8
Total Hardness (mg/L CaCo3)	335	(335) 335	340	328 300-340
Total Alkalinity (mg/L)	330	330 330	310	308 300-310
Specific Conductance (umhos)	600	610 600-630	750	(770) 720-880

Alachlor Test 2

	Day 0		Day 7	
	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	8.0	(8.1) 8.0-8.1	7.1	(7.1) 7.0-7.2
pH	7.2	(7.2) 7.2	8.5	(8.6) 8.4-8.7
Total Hardness (mg/L CaCo3)	335	(330) 330	325	316 290-325
Total Alkalinity (mg/L)	330	335 330-340	322	317 300-320
Specific Conductance (umhos)	600	608 600-610	740	(765) 720-800

Water Analysis Summary for Ceriodaphnia reticulata Atrazine Test 1. Water Quality Means () and Ranges.

	Atrazine Test 1					
	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	8.4	(8.0) 7.9-8.1	7.6	(7.4) 7.4	6.6	(6.9) 6.9-7.3
pH	8.2	(8.2) 8.0-8.3	8.4	(8.4) 8.4	8.4	(8.4) 8.4-8.5
Total Hardness (mg/L CaCO3)	105	(100) 98-100	NA	NA	122	(121) 105-130
Total Alkalinity (mg/L)	92	93 89-100	NA	NA	86	(77) 70-82
Specific Conductance (umhos)	310	(300) 300	410	(350) 330-381	380	(340) 320-360

Water Analysis Summary for Ceriodaphnia reticulata Alachlor Test 1. Water Quality Means () and Ranges.

	Alachlor Test 1					
	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	8.2	(8.2) 8.0-8.2	6.7	(6.5) 6.3-6.7	8.2	(7.9) 7.8-8.2
pH	8.0	(8.0) 8.0	7.9	(8.0) 8.0	8.0	(7.9) 7.9-8.0
Total Hardness (mg/L CaCO3)	158	(163) 140-180	162	(140) 140	170	(186) 186
Total Alkalinity (mg/L)	119	(130) 128-131	NA	(141) 130-153	160	(143) 143
Specific Conductance (umhos)	285	(285) 285	280	272 271-272	252	255 230-272

Water Analysis Summary for Ceriodaphnia reticulata Atrazine Test 2. Water Quality Means () and Ranges.

	Atrazine Test 2					
	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	8.7	(8.7) 8.7	7.3	7.3 7.2-7.4	7.6	7.4 7.4-7.5
pH	8.0	(8.0) 8.0-8.1	8.3	(8.3) 8.3	8.3	(8.3) 8.3
Total Hardness (mg/L CaCO3)	96	(96) 96	110	(120) 120	118	(110) 104-114
Total Alkalinity (mg/L)	90	(90) 90	116	(121) 119-122	114	(125) 124-128
Specific Conductance (umhos)	235	(234) 330-335	262	265 250-280	270	272 265-280

Water Analysis Summary for Ceriodaphnia reticulata Atrazine Test 2. Water Quality Means () and Ranges.

	Atrazine Test 2					
	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	9.2	(9.2) 9.2	7.6	(7.4) 7.3-7.5	7.6	7.5 7.4-7.7
pH	8.1	(8.1) 8.1	8.2	(8.3) 8.2-8.3	8.3	(8.3) 8.3
Total Hardness (mg/L CaCO3)	92	(94) 94-95	120	(115) 112-118	118	(132) 120-154
Total Alkalinity (mg/L)	139	(140) 136-144	NA	(116) 108-124	118	(141) 132-153
Specific Conductance (umhos)	245	(245) 245	280	(303) 285-320	290	(293) 230-330

Water Analysis Summary for Pimephales promelas Atrazine Test 2. Water Quality Means () and Ranges.

Atrazine Test NS

	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	(7.6)	(7.6) 7.5-7.7	(5.7)	(6.1) 5.8-6.4	(5.7)	(6.3) 6.0-6.7
pH	(8.2)	(8.5) 8.4-8.5	8.0	(8.1) 8.0-8.2	(7.8)	(7.8) 7.8
Total Hardness (mg/L CaCO3)	105	(114) 112-117	110	(122) 114-128	122	(111) 100-122
Total Alkalinity (mg/L)	100	(109) 100-118	118	(107) 100-110	100	(103) 100-110
Specific Conductance (umhos)	350	(778) 275-290	269	262 260-270	255	(257) 255-260

Water Analysis Summary for Pimephales promelas Atrazine Test 1. Water Quality Means () and Ranges.

	Atrazine Test 1					
	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	9.3	(8.8) 8.0-9.3	2.8	(2.2) 1.6-2.7	6.0	(5.5) 2.0-6.2
pH	7.6	(7.7) 7.6-7.8	8.1	(8.1) 8.0-8.1	7.8	(7.8) 7.8-7.9
Total Hardness (mg/L CaCO3)	328	(325) 320-328	276	(318) 312-324	320	(331) 322-332
Total Alkalinity (mg/L)	320	(321) 314-320	270	(319) 318-319	338	(332) 325-340
Specific Conductance (umhos)	590	(590) 590	520	(625) 620-630	640	(605) 600-610

Water Analysis Summary for Pimephales promelas Atrazine Test 2. Water Quality Means () and Ranges.

	Atrazine Test 2					
	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	7.6	(7.4) 7.3-7.7	3.3	(2.5) 1.7-3.8	6.0	(4.9) 3.2-6.1
pH	7.8	(8.1) 8.0-8.1	7.9	(7.9) 7.9-8.0	8.0	(7.9) 7.8-8.0
Total Hardness (mg/L CaCO3)	272	(325) 274-358	238	(339) 311-389	268	(235) 204-276
Total Alkalinity (mg/L)	321	(321) 337-341	293	(305) 289-316	283	(285) 278-292
Specific Conductance (umhos)	680	(590) 610-650	650	(683) 650-700	600	(597) 590-610

Water Analysis Summary for Pimephales promelas Atrazine Test 3. Water Quality Means () and Ranges.

	Atrazine Test 3					
	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	8.4	(8.5) 8.4-8.7	6.8	(7.0) 6.7-7.4	4.5	(3.9) 3.6-4.3
pH	7.1	(8.1) 7.3-7.5	7.5	(7.9) 7.7-8.0	7.9	(8.0) 7.9-8.0
Total Hardness (mg/L CaCO3)	308	(325) 300-306	320	(314) 314	320	(303) 300-306
Total Alkalinity (mg/L)	326	(321) 320-328	331	(317) 317	325	(314) 311-317
Specific Conductance (umhos)	650	(590) 630-660	600	NA	600	(612) 600-620

Water Analysis Summary for Pimephales promelas Alachlor Test 1. Water Quality Means () and Ranges.

	Alachlor Test 1					
	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	8.2	(8.2) 8.2	6.1	(6.2) 5.8-7.2	6.7	(6.4) 4.3-7.2
pH	8.2	(8.2) 8.2	7.6	(7.6) 7.6	7.5	(7.5) 7.2-7.6
Total Hardness (mg/L CaCO3)	99	(99) 99	104	(91) 90-92	104	(96) 86-100
Total Alkalinity (mg/L)	88	(88) 88-90	81	86 84-88	76	(98) 76-101
Specific Conductance (umhos)	285	(285) 285	242	(232) 231-232	205	(221) 200-225

Water Analysis Summary for Pimephales promelas Alachlor Test 2. Water Quality Means () and Ranges.

	Alachlor Test 2					
	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	9.3	(8.8) 8.0-9.2	2.8	(2.2) 1.7-2.7	6.0	(4.9) 4.2-5.8
pH	7.6	(7.5) 7.3-7.6	8.1	(7.8) 7.8	7.8	(7.8) 7.7-7.8
Total hardness (mg/L CaCO3)	320	(317) 308-328	270	(294) 288-300	338	(323) 306-330
Total Alkalinity (mg/L)	590	(313) 300-312	520	(303) 300-305	640	(333) 320-340
Specific Conductance (umhos)	328	(596) 88-90	276	600 600	320	(608) 600-615

Water Analysis Summary for Pimephales promelas Alachlor Test 3. Water Quality Means () and Ranges.

	Alachlor Test 3					
	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	8.4	(8.4) 8.3-8.5	6.8	(7.3) 6.9-7.5	4.5	(3.7) 3.4-3.9
pH	7.1	(7.2) 7.1-7.5	7.5	(7.8) 7.7-8.0	7.9	(7.8) 7.7-7.9
Total hardness (mg/L CaCO3)	308	(308) 308	320	NA	320	(314) 310-318
Total Alkalinity (mg/L)	326	(323) 320-328	331	(323) 318-328	325	(310) 308-312
Specific Conductance (umhos)	600	(614) 610-620	600	NA	600	(600) 600

Water Analysis Summary for Pimephales promelas Joint Test 1. Water Quality Means () and Ranges.

	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	8.0	(8.0) 7.8-8.2	6.6	(6.3) 5.8-6.6	5.5	(5.5) 4.8-6.3
pH	7.2	(7.6) 7.5-7.7	7.6	(7.7) 7.6-7.9	7.8	(7.9) 7.8-8.0
Total hardness (mg/L CaCO3)	336	(332) 320-335	218	(293) 244-338	234	(279) 262-294
Total Alkalinity (mg/L)	335	(323) 320-328	318	(330) 318-328	330	(310) 308-312
Specific Conductance (umhos)	685	(614) 610-620	660		690	(600) 600

Water Analysis Summary for Pimephales promelas Joint Test 2. Water Quality Means () and Ranges.

	Day 0		Day 3		Day 7	
	Control	Treatments	Control	Treatments	Control	Treatments
Dissolved Oxygen (mg/L)	8.3	(8.4) 8.3-8.4	6.8	(7.4) 7.1-7.6	4.5	(3.5) 3.0-3.8
pH	7.1	(7.3) 7.3	7.5	(7.8) 7.8-8.0	7.9	(8.0) 8.0-8.1
Total hardness (mg/L CaCO3)	308	(328) 328	320	NA	320	(332) 320-346
Total Alkalinity (mg/L)	326	(330) 330	331	(328) 328	325	(334) 334
Specific Conductance (umhos)	600	(615) 610-620	600	NA	600	(605) 600-610

APPENDIX 3

FISHER'S EXACT TEST CONTINGENCY TABLES

Ceriodaphnia Survival = Atrazine Test 1

	Alive	Dead	# Observations		Calculated b	Critical b
Level 1	10	0	10	A=10	b=10	≥ 6
Control	10	0	10	B=10		
				a=10		
Total	20	0	20		Not different	
Level 2	9	1	10	A=10	b=9	≥ 6
Control	10	0	10	B=10		
				a=10		
Total	19	1	20		Not different	
Level 3	9	1	10	A=10	b=9	≥ 6
Control	10	0	10	B=10		
				a=10		
Total	19	1	20		Not different	
Level 4	8	2	10	A=10	b=8	≥ 6
Control	10	0	10	B=10		
				a=10		
Total	18	2	20		Not different	
Level 5	7	3	10	A=10	b=7	≥ 6
Control	10	0	10	B=10		
				a=10		
Total	17	3	20		Not different	

All levels may be used in reproduction analysis.

FISHER'S EXACT TEST CONTINGENCY TABLES

Ceriodaphnia Survival = Atrazine Test 2

	Alive	Dead	# Observations		Calculated b		Critical b
Level 1	10	0	10	A=10	b=10	≥	6
Control	10	0	10	B=10			
				a=10			
Total	20	0	20		Not different		
Level 2	10	0	10	A=10	b=10	≥	6
Control	10	0	10	B=10			
				a=10			
Total	10	0	20		Not different		
Level 3	9	1	10	A=10	b=9	≥	6
Control	10	0	10	B=10			
				a=10			
Total	19	1	20		Not different		
Level 4	9	1	10	A=10	b=9	≥	6
Control	10	0	10	B=10			
				a=10			
Total	19	1	20		Not different		
Level 5	7	3	10	A=10	b=7	≥	6
Control	10	0	10	B=10			
				a=10			
Total	17	3	20		Not different		

All levels may be used in reproduction analysis.

FISHER'S EXACT TEST CONTINGENCY TABLES

Ceriodaphnia Survival = Alachlor Test 1

	Alive	Dead	# Observations		Calculated b		Critical b
Level 1	10	0	10	A=10	b=9	≥	6
Control	10	0	10	B=10			
				a=10			
Total	20	0	20		Not different		
Level 2	7	3	10	A=10	b=7	≥	6
Control	10	0	10	B=10			
				a=10			
Total	17	3	20		Not different		
Level 3	7	3	10	A=10	b=7	≥	6
Control	10	0	10	B=10			
				a=10			
Total	17	3	20		Not different		
Level 4	2	8	10	A=10	b=2	≥	6
Control	10	0	10	B=10			
				a=10			
Total	12	8	20		Different		
Level 5	2	8	10	A=10	b=2	≥	6
Control	10	0	10	B=10			
				a=10			
Total	12	8	20		Different		

Levels 4 and 5 may not be included in reproduction analysis.

FISHER'S EXACT TEST CONTINGENCY TABLES

Ceriodaphnia Survival = Alachlor Test 2

	Alive	Dead	# Observations		Calculated b		Critical b
Level 1	8	2	10	A=10	b=8	≥	6
Control	10	0	10	B=10			
				a=10			
Total	18	2	20		Not different		
Level 2	8	2	10	A=10	b=8	≥	6
Control	10	0	10	B=10			
				a=10			
Total	18	2	20		Not different		
Level 3	7	3	10	A=10	b=7	≥	6
Control	10	0	10	B=10			
				a=10			
Total	17	3	20		Not different		
Level 4	8	2	10	A=10	b=8	≥	6
Control	10	0	10	B=10			
				a=10			
Total	18	2	20		Not different		
Level 5	10	0	10	A=10	b=10	≥	6
Control	10	0	10	B=10			
				a=10			
Total	20	0	20		Not different		

All levels may be included in reproduction analysis.

APPENDIX 4

Live Pimephales larvae per test chamber for Atrazine Test NS.
6 November - 13 November 1986.

Day	Chamber	Control	0.25	0.50	1.00	2.00	4.00
0	1	10	10	10	10	10	10
	2	10	10	10	10	10	10
	3	10	10	10	10	10	10
	4	10	10	10	10	10	10
1	1	10	10	10	10	10	10
	2	10	10	10	10	10	10
	3	10	10	10	10	10	10
	4	10	10	10	10	10	10
2	1	10	10	10	10	10	10
	2	10	10	10	10	10	10
	3	9	10	10	9	10	10
	4	8	10	10	10	10	10
3	1	10	10	10	10	10	10
	2	10	10	10	10	10	9
	3	9	10	10	9	10	10
	4	8	10	10	10	10	10
4	1	10	10	10	10	10	10
	2	10	10	10	10	10	9
	3	8	10	10	9	10	10
	4	8	10	10	10	10	10
5	1	9	10	10	10	10	10
	2	10	9	10	10	10	8
	3	8	10	10	9	9	10
	4	8	10	9	10	10	10
6	1	9	10	10	10	10	10
	2	9	8	10	10	10	8
	3	8	10	10	9	9	10
	4	8	10	9	10	10	10
7	1	9	10	9	10	10	10
	2	9	7	9	10	9	8
	3	8	10	7	9	9	10
	4	8	10	9	10	10	9
Total		34	37	34	39	38	37

Live Pimephales larvae per test chamber for Atrazine Test 1.
5 March - 1 April 1987.

Day	Chamber	Control	0.50	0.75	1.00	1.25	1.50	3.00	6.00	10.00
0	1	10	10	10	10	10	10	10	10	10
	2	10	10	10	10	10	10	10	10	10
	3	10	10	10	10	10	10	10	10	10
	4	10	10	10	10	10	10	10	10	10
1	1	10	10	10	10	10	10	10	10	10
	2	10	10	10	10	10	10	10	10	10
	3	10	10	10	10	10	10	10	10	10
	4	10	10	10	10	10	10	10	10	10
2	1	10	10	10	10	10	10	10	10	10
	2	10	10	10	10	10	10	10	10	10
	3	10	10	10	10	10	10	10	10	10
	4	10	10	9	10	9	10	10	10	10
3	1	10	10	10	10	9	10	10	10	10
	2	9	10	10	10	10	10	10	10	8
	3	10	10	10	10	10	9	10	10	10
	4	10	10	7	10	9	10	10	10	10
4	1	10	10	10	8	9	10	10	9	10
	2	9	8	10	10	10	10	9	9	8
	3	10	10	10	10	10	9	10	10	9
	4	9	8	7	10	9	10	10	10	8
5	1	10	10	10	8	9	10	10	9	10
	2	9	8	10	10	10	10	9	9	8
	3	10	10	10	10	10	9	10	8	9
	4	9	7	7	10	9	10	10	10	8
6	1	10	10	10	8	9	10	10	9	10
	2	9	8	10	10	10	10	9	9	8
	3	10	10	10	10	10	9	10	8	9
	4	9	7	7	10	9	10	10	10	8
7	1	10	10	10	8	9	9	10	9	10
	2	9	8	10	10	10	10	9	9	8
	3	10	10	10	10	10	9	10	8	9
	4	9	7	7	10	9	10	10	10	8
Total		38	35	37	38	38	38	39	36	35

Live *Pimephales* larvae per test chamber for Atrazine Test 2
16 April - 23 April 1987.

Day	Chamber	Control	12.00	15.00	18.00
0	1	10	10	10	10
	2	10	10	10	10
	3	10	10	10	10
	4	10	10	10	10
1	1	10	10	7	9
	2	10	10	9	7
	3	10	10	10	6
	4	10	10	8	7
2	1	10	10	7	8
	2	10	9	8	6
	3	10	10	10	6
	4	10	10	8	6
3	1	10	9	6	8
	2	10	9	8	6
	3	10	7	10	6
	4	10	10	7	6
4	1	10	8	5	3
	2	10	9	7	3
	3	9	7	6	2
	4	10	8	3	2
5	1	10	6	4	2
	2	10	7	4	2
	3	9	7	5	1
	4	10	8	1	2
6	1	10	6	4	2
	2	10	5	3	2
	3	9	6	5	1
	4	10	8	1	2
7	1	10	6	4	2
	2	10	5	3	2
	3	9	6	5	1
	4	10	8	1	2
Total		39	25	13	7

Live Pimephales larvae per test chamber for Atrazine Test 3
3 July - 10 July 1987.

Day	Chamber	Control	1.50	3.00	6.00	12.00	20.00
0	1	10	10	10	10	10	10
	2	10	10	10	10	10	10
	3	10	10	10	10	10	10
	4	10	10	10	10	10	10
1	1	10	10	10	10	10	10
	2	10	10	9	10	10	10
	3	10	10	9	10	10	10
	4	10	10	10	10	10	9
2	1	10	10	10	10	10	10
	2	10	10	9	10	10	10
	3	10	10	9	10	10	10
	4	10	10	10	10	10	9
3	1	10	10	10	9	10	4
	2	10	10	9	10	9	7
	3	10	10	9	10	10	1
	4	10	10	10	10	10	5
4	1	10	10	10	9	9	3
	2	10	10	9	10	7	4
	3	10	10	9	10	7	0
	4	10	10	10	10	7	2
5	1	10	9	10	8	9	3
	2	10	10	9	9	5	4
	3	10	10	8	9	7	0
	4	10	9	9	10	7	2
6	1	10	9	9	8	9	3
	2	10	10	9	9	5	4
	3	10	10	8	9	7	0
	4	10	9	9	10	7	2
7	1	10	9	9	8	9	3
	2	10	10	9	9	5	4
	3	10	10	8	9	7	0
	4	10	9	9	10	7	2
	Total	40	38	35	36	28	9

Live Pimephales larvae per test chamber for Alachlor Test 1
16 January - 23 January 1987.

Day	Chamber	Control	1.00	2.00	3.00	4.00	5.00
0	1	10	10	10	10	10	10
	2	10	10	10	10	10	10
	3	10	10	10	10	10	10
	4	10	10	10	10	10	10
1	1	10	9	10	10	10	10
	2	10	10	10	10	10	9
	3	10	10	10	10	10	10
	4	10	10	10	10	10	10
2	1	9	8	10	10	10	10
	2	10	10	9	10	10	9
	3	10	10	10	10	10	10
	4	10	10	10	10	10	10
3	1	9	8	10	10	10	10
	2	10	10	9	10	10	9
	3	10	10	10	10	10	10
	4	10	10	10	10	10	10
4	1	9	8	10	10	10	10
	2	10	10	9	10	9	9
	3	10	10	10	10	10	10
	4	10	10	10	10	10	10
5	1	9	8	10	10	10	10
	2	10	10	9	10	9	9
	3	10	10	10	10	9	9
	4	9	10	10	10	9	10
6	1	9	8	10	10	10	10
	2	10	10	9	10	9	9
	3	10	10	10	10	9	9
	4	9	10	10	10	9	10
7	1	9	8	10	10	10	10
	2	10	10	9	10	9	9
	3	10	10	10	10	9	9
	4	9	10	10	10	9	10
Total		38	38	39	40	37	38

Live *Pimephales* larvae per test chamber for Alachlor Test 2
25 March - 1 April 1987.

Day	Chamber	Control	0.50	1.00	1.25	1.50	1.75	2.00	3.00	6.00
0	1	10	10	10	10	10	10	10	10	10
	2	10	10	10	10	10	10	10	10	10
	3	10	10	10	10	10	10	10	10	10
	4	10	10	10	10	10	10	10	10	10
1	1	10	10	10	10	10	10	10	10	9
	2	10	10	8	10	10	10	10	10	8
	3	10	10	10	10	10	10	10	10	10
	4	10	10	0	10	10	10	10	10	10
2	1	10	10	10	10	10	10	9	10	9
	2	10	10	1	10	10	10	8	10	7
	3	10	10	8	10	10	10	10	10	7
	4	10	10	0	10	10	10	8	10	7
3	1	10	10	10	10	10	10	9	10	5
	2	9	10	1	10	9	10	8	10	6
	3	10	10	7	10	10	9	10	10	7
	4	10	10	0	10	10	10	8	10	5
4	1	10	10	10	9	9	10	9	10	3
	2	9	10	1	10	9	8	6	10	5
	3	10	10	7	9	9	9	10	10	4
	4	9	10	0	10	9	10	8	10	5
5	1	10	10	10	9	9	10	9	10	3
	2	9	10	1	10	9	8	6	8	2
	3	10	10	7	9	9	9	9	10	2
	4	9	10	0	10	9	10	8	10	4
6	1	10	10	10	9	9	10	9	10	1
	2	9	9	1	9	9	8	6	8	2
	3	10	10	7	9	9	8	9	10	2
	4	9	10	0	10	9	10	8	10	4
7	1	10	10	10	9	9	9	9	10	1
	2	9	9	1	9	9	7	6	7	1
	3	10	10	6	9	9	8	9	10	2
	4	9	10	0	10	9	10	8	8	4
Total		38	39	17	37	36	34	32	35	8

Live Pimephales larvae per test chamber for Alachlor Test 3
3 July - 10 July 1987.

Day	Chamber	Control	0.50	1.00	2.00	4.00	8.00
0	1	10	10	10	10	10	10
	2	10	10	10	10	10	10
	3	10	10	10	10	10	10
	4	10	10	10	10	10	10
1	1	10	10	10	10	10	10
	2	10	10	10	10	10	10
	3	10	10	10	10	10	10
	4	10	10	10	10	10	10
2	1	10	10	10	10	10	9
	2	10	10	10	10	10	10
	3	10	10	10	10	10	10
	4	10	10	10	10	10	10
3	1	10	10	10	9	10	9
	2	10	10	10	10	10	10
	3	10	10	10	10	10	9
	4	10	10	10	10	10	10
4	1	10	10	10	8	10	8
	2	10	10	10	9	10	7
	3	10	10	10	10	10	9
	4	10	10	10	10	10	9
5	1	10	10	10	8	10	4
	2	10	10	10	9	10	5
	3	10	10	10	9	9	7
	4	10	10	10	10	9	7
6	1	10	10	9	8	10	4
	2	10	10	10	9	8	5
	3	10	10	10	9	8	3
	4	10	10	10	10	7	7
7	1	10	10	9	8	8	1
	2	10	10	9	9	8	0
	3	10	10	10	9	7	1
	4	10	10	10	10	6	2
	Total	40	40	38	36	29	4

Live *Pimephales* larvae per test chamber for Joint Test 1
11 June - 18 June 1987.

Day	Chamber	Control	0.25	0.50	0.75	1.00	1.25	1.75	2.00
0	1	10	10	10	10	10	10	10	10
	2	10	10	10	10	10	10	10	10
	3	10	10	10	10	10	10	10	10
	4	10	10	10	10	10	10	10	10
1	1	10	10	10	10	10	10	10	10
	2	10	10	10	10	10	10	10	10
	3	10	10	10	10	10	10	10	10
	4	10	10	10	10	10	10	10	10
2	1	10	10	10	10	10	10	10	10
	2	10	10	10	10	10	10	10	10
	3	10	10	10	10	10	10	10	10
	4	10	10	10	10	10	10	10	10
3	1	10	10	10	10	10	10	10	9
	2	10	10	9	10	10	10	10	10
	3	10	10	10	10	10	10	10	10
	4	10	10	10	10	9	10	10	10
4	1	10	10	10	10	10	10	10	8
	2	10	9	9	10	10	9	9	9
	3	10	10	10	10	10	10	10	10
	4	10	10	10	10	9	10	9	8
5	1	10	10	10	10	10	10	10	8
	2	10	9	9	10	10	9	8	9
	3	10	10	10	10	9	10	7	9
	4	10	10	10	10	9	10	8	7
6	1	10	10	10	10	10	10	10	8
	2	10	9	8	10	10	9	8	9
	3	10	10	10	10	9	10	7	9
	4	10	10	10	10	9	10	8	7
7	1	10	10	10	10	10	10	10	8
	2	10	9	8	10	10	9	8	9
	3	10	10	10	10	9	9	7	9
	4	10	10	10	10	9	10	8	7
Total		40	39	38	40	38	38	33	33

Live Pimephales larvae per test chamber for Joint Test 2
3 July - 10 July 1987.

Day	Chamber	Control	0.50	1.00	2.00	4.00
0	1	10	10	10	10	10
	2	10	10	10	10	10
	3	10	10	10	10	10
	4	10	10	10	10	10
1	1	10	10	10	10	5
	2	10	10	10	9	5
	3	10	10	10	10	4
	4	10	10	10	10	6
2	1	10	10	10	10	1
	2	10	10	10	8	2
	3	10	10	10	10	0
	4	10	10	10	10	3
3	1	10	10	10	9	0
	2	10	10	10	6	0
	3	10	10	10	7	0
	4	10	10	9	8	0
4	1	10	10	10	5	0
	2	10	10	10	2	0
	3	10	10	10	3	0
	4	10	10	9	1	0
5	1	10	10	10	4	0
	2	10	10	10	1	0
	3	10	10	9	2	0
	4	10	10	8	0	0
6	1	10	10	9	4	0
	2	10	10	10	1	0
	3	10	10	9	2	0
	4	10	10	8	0	0
7	1	10	10	9	4	0
	2	10	10	10	0	0
	3	10	10	9	1	0
	4	10	10	8	0	0
	Total	40	40	36	5	0