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The Effects of Dilute Copper Sulphate Concentrations on Selected Aquatic Invertebrates

Michael D. Campbell

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THE EFFECTS OF DILUTE COPPER
SULPHATE CONCENTRATIONS ON
SELECTED AQUATIC INVERTEBRATES

by

Michael D. Campbell

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment
of the
Degree of Master of Arts

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Michael D. Campbell

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INTRODUCTION

Numerous reports demonstrate the toxicity of copper salts to aquatic life and studies describing the effects on algae and fish predominate in the literature. Copper sulphate can enter natural waters from industrial effluents, particularly from plating works, and from its widespread use as an algicide and molluscicide.

Much of the previous work on copper toxicity was based on short-term testing to determine lethality. This and other end points with a wide range of results have been reported for fish and larger aquatic invertebrates. McKee and Wolf (1963) have summarized much of the earlier work. Water characteristics such as temperature, pH, dissolved oxygen, hardness, alkalinity, and presence of other poisons have been shown to alter the toxicity of copper sulphate (CuSO_4) to aquatic animals. This fact may partially account for the wide range of toxicities of copper sulphate to aquatic organisms that has been reported in the literature.

The majority of investigations on copper sulphate toxicity involved the use of various species of fish as bioassay organisms. This was primarily because of the commercial and recreational importance of certain fish species. However, the bioassay value of aquatic macroinvertebrates must not be overlooked. Their importance as fish food organisms in the food web is obvious and their value as agents for natural purification of polluted water is significant. Also, aquatic macroinvertebrates may be more susceptible to lower concentrations of pollutants than are many fishes. These facts

justify their use in toxicity bioassays. This same thought was expressed by Duodoroff et al. (1951) in a bioassay study evaluating acute toxicity of industrial effluents to fish. He noted that fish have been widely used in bioassay tests but the value of other aquatic organisms in acute toxicity tests should also be considered.

Copper is usually found in natural waters and is an important trace element in most animals. However, a slight increase of copper in the diet or in the aquatic medium has a strong toxic effect. Thus the amount of copper in the environment may be biologically important because of either deficiency or excess. In an extensive limnological study of three Connecticut lakes, Riley (1939) measured the copper content, its occurrence, distribution, reactions, removal, and regeneration in the lakes.

In a study done by Declier et al. (1970) copper determinations in different animals show that mean values of copper content in invertebrates are much higher than in vertebrates. The following values are given by Clarke (1949) with the units being mg copper/g dry weight: insects 0.0919, marine invertebrates 0.1736, and vertebrates 0.0119.

A small increase in copper content of natural waters, originating either from natural or artificial sources, may have deleterious effects upon the aquatic biota. In this respect, aquatic macro-invertebrates are very useful as indices of water quality and Wilham (1970) states that benthic organisms are particularly suited for pollution studies because their habitat preference and low motility causes them to be directly affected by substances that enter the

environment. Associations or populations of benthic macroinvertebrates provide a more reliable measure of water quality than does the occurrence of any one particular species.

Numerous excellent articles have been published describing the use of aquatic invertebrates as indicators of water quality; among these, Patrick (1950), Surber (1952), and Sprague (1973) are noteworthy. A comprehensive study will include physical and chemical data together with qualitative and quantitative studies of the benthic, marginal, and surface fauna. This information aids prediction of aquatic populations which can be expected to develop under certain ecological conditions, and conversely, estimation of variations in environmental conditions which are indicated by various compositions and densities of aquatic organisms (Gauvin and Tarzwell, 1952).

Various authors classify aquatic organisms according to their tolerance of organic wastes. This classification is comprised of three forms: tolerant, facultative, and intolerant. According to this classification (Weber, 1973) three of the organisms chosen for this study (the amphipod Hyaella azteca, the mayfly Ameletus sparsatus, and the clam Sphaerium simile) are facultative forms and one (the worm Tubifex tubifex) is a tolerant form.

The aquatic invertebrates used for this study were chosen because of their importance as fish food organisms, local availability, and ease of maintenance and testing in the laboratory.

It is hoped that this study will give improved understanding and usefulness to distributional studies of these organisms in

relation to water quality. This type of approach can be an important supplement to other biotic approaches.

LITERATURE REVIEW

Copper Toxicity to Fish

A great deal of research involving fish bioassays for copper sulphate toxicity has been published. A wide variety of fish species have been utilized ranging from those which have a high degree of tolerance to pollution to those which have a low level of tolerance to pollution. An equally diverse range of toxic concentrations of copper sulphate has also been reported.

Many experimenters have indicated that the toxicity of heavy metal salts is directly lessened by increases in hardness and alkalinity of the water. Lloyd (1965) states that many heavy metals have been shown to be more toxic in soft water than in hard water and the extent of the differences in toxicity appears to vary for different metals. Lloyd (1960) and Ellis (1937) found that copper sulphate solutions were less toxic to fish in hard water. The chronic toxicity of copper sulphate to fathead minnows (Pimephales promelas) has been studied in hard water (Mount, 1968) and also in soft water (Mount and Stephan, 1969). The authors investigated the percentage of the median tolerance limit* that does not effect growth and reproduction under prolonged, continuous exposure. In every test the toxicity of copper was greater in the soft water.

* The median tolerance limit (TL_m) or the median lethal concentration value (LC_{50}) is the concentration of toxicant which is lethal to 50% of the test organisms.

Wilson (1972) utilized Atlantic salmon parr as test organisms and tried to predict copper toxicity in receiving waters. He was unsuccessful, however, due to the presence of a variety of organic compounds which were known to chelate copper. "Safe" levels of heavy metal pollution, including copper, were investigated by Saunders and Sprague (1967) and the effects upon the spawning migration of Atlantic salmon were also studied. Sprague (1964) also determined the incipient lethal level of copper to young salmon to be 0.048 mg/l.

Numerous investigators have observed the increased toxicity of copper at low dissolved oxygen concentrations. Hynes (1965) observed that many substances become more toxic as the oxygen content of the water decreases. He also noted that the rate of oxygen consumption is altered by the presence of toxicants. Lloyd (1960, 1961a, and 1965) discussed the increased toxicity of copper salts under conditions of low dissolved oxygen to fish. He noted (1961a) that a given reduction in the dissolved oxygen concentration of the water from the air-saturation value to a lower level increases the toxicity of copper salts to rainbow trout. A hypothesis is presented to account for the effect of low oxygen concentrations on the toxicity of poisons to fish. It assumes that a given toxic effect is produced by a specific concentration of poison at the gill surface and that this concentration is governed not only by the concentration of the poison in the water, but also by the velocity of respiratory flow. Thus, an increase in respiratory flow would increase the rate at which a poison reaches the gill surface. He added that this hypothesis is of some importance since it implies that any environmental

factor which increases the respiratory flow will also increase the rate at which the poison reaches the gill surface and this may increase the susceptibility of the fish to the poison.

Oxygen consumption rates were determined for juvenile bluegills which were exposed to various concentrations of copper (O'Hara, 1971). The author noted that respiratory response was strongly correlated with copper concentrations up to the 96-hour TL_m value of 2.4mg/l of copper, and that higher concentrations produced a greater response.

Much experimental work testing the acute toxicity of heavy metals, including copper, to rainbow trout, has been reported by Brown (1968), Brown and Dalton (1970), and Brown et al. (1969). Brown reports the 48- LC_{50} for copper toxicity to rainbow trout as 0.75 mg/l. Lloyd (1961b) and Lloyd and Jordan (1963) also report on the toxicity of copper to rainbow trout. Included in their reports are toxicities of mixtures of heavy metals. The 96-hour TL_m values of copper ion in hard water is reported to be 0.59 mg/l and in soft water it is 0.11 mg/l. McKim et al. (1970) studied the changes in the blood of brook trout after short and long-term exposure to copper, and noted that the measurement of specific physiological and biochemical changes in the blood of fish exposed for short periods of time to sublethal environmental stresses may provide a sensitive method for predicting the effects of chronic exposure.

McKim and Benoit (1971) investigated the effects of long-term exposure to copper on the survival, growth, and reproduction of brook trout and determined that the effects on yearling trout varied with the concentration of the toxicant. The highest concentration

of copper used (32.5 ug/l) decreased survival and reduced growth slightly. The number of viable eggs produced per female at this concentration was considerably less than the control group. Survival, reproduction, and growth of adults in the lower test concentrations did not differ from the control group. The authors determined the maximum acceptable toxicant concentration (MATC) for brook trout under experimental conditions.

Drummond et al. (1973) investigated short-term indicators of sublethal effects of copper on brook trout, Salvelinus fontinalis. Their report was a follow-up to the work of McKim and Benoit (1971) who investigated effects of long-term exposure of copper to brook trout. The work by Drummond et al. evaluated the changes in cough frequency, locomotor activity, and feeding behavior as possible short-term indicators of the long-term effects of copper within 2 to 24 hours at copper concentrations as low as 6-15 mg/l. These changes appear to be useful for predicting the concentration range likely to have no long-term effects.

The acute toxicity of heavy metals on various species of warm-water fish was reported by Pickering and Henderson (1966). In order to evaluate and compare acute toxicity of copper sulphate the 24, 48, and 96-hour TL_m 's were determined. The 48-hour TL_m 's in hard water varied from 1.14 mg/l for fathead minnows to 10.2 mg/l for bluegills. The authors stated that the 48-hour TL_m 's in soft water varied from 0.023 mg/l for fathead minnows to 0.74 mg/l for bluegills. They concluded that the TL_m values of the heavy metals are a direct measure of the acute toxicity under the experimental

conditions and such concentrations would be expected to cause fish mortality under most environmental conditions and would not be safe for fish even under conditions of short exposure. Tarzwell (1966) noted similar effects of pH on copper sulphate toxicity to fish.

Herbert et al. (1965) also ran into considerable difficulties in obtaining a valid threshold concentration for copper. Even under carefully controlled laboratory conditions the values obtained in hard water ranged from 0.27 ppm Cu to 1.1 ppm Cu and factors causing this variation could not be determined.

Fish that have a known tolerance to pollution have also been studied in their response to concentrations of copper. Ozaki et al. (1970) studied the survival and growth of goldfish and carp in dilute solutions of copper sulphate and Brungs et al. (1973) investigated the acute and long-term accumulation of copper in the brown bullhead.

The mode of copper toxicity to fish was first described by Carpenter (1927) and later reviewed by Lloyd (1965). They report that the lethal action of certain soluble salts of the heavy metals on freshwater fishes results in the formation of an insoluble compound of metallic ion with some constituent of the mucus which coats over the skin and gills and finally causes death by suffocation. Lloyd also reported that other tests suggest that the toxic action is not internal and that at least for some heavy metals it is confined to the epithelial cells of the gill lamellae.

Moore and Kellerman (1904) gave the limiting safe dosage of copper sulphate for representative freshwater fish. The information

is given in Table 1. The authors failed to state the experimental conditions under which these values were obtained. They are close to values reported in recent literature and with some risk may be used for estimating "safe" dosages for these fish, under otherwise favorable conditions.

Copper Toxicity to Algae

A review of the literature describing the use of copper sulphate as an algicide is relevant because concentrations added to the aquatic environment to kill algae may also have toxic effects to desirable aquatic organisms.

Numerous reports can be found in the literature describing the ubiquitous use of copper sulphate as an algicide. A wide range of toxic concentrations are recorded for different types of algae in a variety of natural water conditions. As one would expect, there is a range of values of sensitivity among the different orders of algae as well as among the species. In addition, variations in water quality such as the physical and chemical characteristics tend to alter the organisms sensitivity. The physiological state of the algae is an important factor governing sensitivity.

In a general discussion of the characteristics of algae, Bartsch (1954) reviews control by growth limitation and control by algicides. The traditional chemical used for the control of algae is copper sulphate. Copper sulphate is a convenient and inexpensive chemical and can be applied in many ways including bag dragging, power blowers, aircraft, dry feeders, solution boxes, drippers, sprayers, and by placing crystals on ice.

Table 1. Limiting Safe Dosage of Copper Sulphate for Fish
(after Moore and Kellerman 1904)

<u>Fish</u>	<u>Copper Sulphate ppm</u>
Trout	0.14
Carp	0.33
Suckers	0.33
Catfish	0.40
Pickereel	0.40
Goldfish	0.50
Perch	0.67
Sunfish	1.35
Blackbass	2.00

Bartsch notes that copper sulphate has properties that make it much less than an ideal algicide. Some of these properties are:

(1) similar to other heavy metals, copper in sufficient concentrations has the ability to poison fish and other aquatic life; (2) when precipitated it can accumulate in lakes and (3) it is corrosive to equipment and paint.

In a study describing the factors involved in the testing and application of algicides, Fitzgerald (1964) discusses relationships between the amount of algae and amount of chemical and also discusses the volume of diluent versus the amount of chemical. Regarding the former, the author stated that an increase in the amount of algae resulted in a corresponding increase in the amount of chemical needed to produce toxicity and regarding the latter, an increase in volume does not necessitate a corresponding increase in chemical. To predict the effectiveness of a chemical for controlling algae growth it is essential to know whether the chemical effect is algicidal or algistatic. In addition, sensitivity of different algae to various chemicals may vary, development of resistance to chemicals may occur and effect of algae growth habit may alter sensitivity.

Steeman-Nielsen and Wium-Anderson (1970) determined that copper in ionic form retards photosynthesis and inhibits growth of unicellular algae at concentrations of copper usually not found in natural waters. This indicates that copper is ordinarily not present in ionic form but is complexed by organic matter. They noted that in Chlorella pyrenoidosa copper influences the algae by blocking mechanisms in the cell membrane in such a way that division does not take place,

however, the copper does not penetrate the cell at first. The decline in the rate of photosynthesis found after some hours seems due primarily to the accumulation of photosynthetic products which secondarily block photosynthesis. They summarized their study by stating that concentrations of ionic copper as low as 1 to 2 mg/l are shown to be poisonous for photosynthesis and growth of Chlorella pyrenoidosa. The effect of toxic doses of copper upon photosynthesis, reproduction, and growth of Chlorella vulgaris was also investigated by McBrien and Hassall (1967).

Gibson (1972) described the sensitivity of Anabaena flos-aquae and Scenedesmus quadricuda to a range of copper sulphate concentrations and the ecological implications of the addition of this algicide. He observed that the sensitivity to copper varied with the stage of growth, with the algae becoming less sensitive as the culture aged.

Fitzgerald and Faust (1963) investigated factors affecting the algicidal and algistatic properties of copper and determined that the minimum level of inhibition of Chlorella to copper sulphate was 1.0 to 8.0 ppm depending on the medium. The minimum level of inhibition of Microcystis was given as 0.05 to 0.03 ppm.

Maloney and Palmer (1965) studied the toxicity of six chemical compounds to a variety of algae. Copper sulphate was used in four different concentrations and the cultures of algae included seven blue-green algae, seventeen green algae, and six diatoms. Copper sulphate showed greater selective toxicity toward the algae than did most other compounds. Four algae required more than 4 ppm of copper sulphate for control, these being the green algae Akistrodesmus

falcatus and Scenedesmus obliquus and the blue-green algae Calothrix braunii and Symploca erecta. At 1.8 ppm copper sulphate controlled the growth of Caccomyxa simplex, a species which was one of the most resistant to the other chemicals. Copper sulphate also displayed selective toxicity to the diatoms at a concentration of 2 ppm. At this concentration it controlled 100 percent of the diatoms, while it was toxic to only 35 percent of the green algae and 57 percent of the blue-green algae.

A screening technique for estimating copper toxicity to estuarine phytoplankton was reported by Erickson et al. (1970). The technique was devised to establish a working range in metal toxicity bioassays which considers the factors affecting copper toxicity. The investigators tested six species of phytoplankton in copper concentrations ranging from 50 to 450 mg/l. Without exception, copper showed greater toxicity in unchelated rather than chelated form.

Whipple and Fair (1927) present a list of the concentrations of copper sulphate required to kill various known algae. The information is presented in Table 2.


Copper Toxicity to Invertebrates

A general account of the biological assessment of the effects of discharges on aquatic benthic organisms is presented by Cairns and Dickson (1970). The authors point out that the type of aquatic organisms destroyed and the extent of destruction are a reflection of the character and quantity of wastes entering the water. They recognize the value of benthic invertebrates for several reasons:

Table 2. Copper Sulphate Required for Eradication of Different Algae
(after Whipple and Fair 1927)

Organism	Copper Sulphate ppm	Organism	Copper Sulphate ppm
CYANOPHYCEAE			
<u>Anabaena</u>	0.12	<u>Nitella</u>	0.10
<u>Aphanizomonon</u>	0.12-0.25	<u>Pandorina</u>	10.00
<u>Clathrocystis</u>	0.12-0.25	<u>Palmella</u>	2.00
<u>Cylindrospermum</u>	0.12	<u>Raphidium</u>	1.00
<u>Coelosphaerium</u>	0.20-0.33	<u>Scenedesmus</u>	1.00
<u>Microcystis</u>	0.20	<u>Spirogyra</u>	0.12
<u>Oscillatoria</u>	0.20-0.50	<u>Staurastrum</u>	1.50
CHLOROPHYCEAE			
<u>Akistrodesmus</u>	1.00	<u>Tribonema</u>	0.25
<u>Chara</u>	0.10-0.50	<u>Ulothrix</u>	0.20
<u>Chlamydomonas</u>	0.50	<u>Volvox</u>	0.25
<u>Cladophora</u>	0.50	<u>Zygnema</u>	0.50
<u>Closterium</u>	0.17	DIATOMACEAE	
<u>Coelastrum</u>	0.05	<u>Asterionella</u>	0.12-0.20
<u>Desmidium</u>	2.00	<u>Fragilaria</u>	0.25
<u>Draparnaldia</u>	0.33	<u>Melosira</u>	0.33
<u>Eudorina</u>	10.00	<u>Navicula</u>	0.07
<u>Enteromorpha</u>	0.50	<u>Nitzschia</u>	0.50
<u>Hydrodictyon</u>	0.10	<u>Synedra</u>	0.50
<u>Microspora</u>	0.40	<u>Stephanodiscus</u>	0.33
		<u>Tabellaria</u>	0.12-0.50

First, many species are extremely sensitive to pollution and respond quickly to it; second, bottom fauna usually have a complex life cycle of a year or more and if at any time during their life cycle, environmental conditions become intolerable, the fauna die; and third, since they have a generally attached or sessile mode of life and are not subject to rapid migrations, they serve as natural monitors of water quality.

These same authors also note that immature or larval stages of mayflies, stoneflies, caddisflies, riffle beetles, and hellgramites are quite sensitive and environmental changes often eliminate them from the bottom fauna. Tolerant organisms such as sludgeworms, certain midge larvae, leeches, and certain snails may increase in numbers under polluted conditions. Most snails, sowbugs, scuds, black fly larvae, crane fly larvae, fingernail clams, dragonfly nymphs, and some midge larvae are intermediate in tolerance to environmental changes. 

One of the early studies of copper toxicity to aquatic invertebrates was performed by Riley (1939) in three Connecticut lakes. He chose for his experiment three protozoans, a coelenterate, a rotifer, copepods, cladocerans, and insect larvae. He measured in hours the survival time of animals exposed to different concentrations of copper, ranging from 0.01 to 200 mg/l. He noted that it appears generally true that copper has a toxic effect only when it enters the tissue of an animal, although there are exceptions, particularly with fish, as noted by Carpenter (1927). Riley stated that if the general rule holds true, susceptibility to copper

poisoning is a function of the permeability of the integument. The tolerance level of ten representative freshwater invertebrates was found to range from 0.03 to more than 0.50 mg/l copper.

Jones (1936) investigated the toxicity of dissolved metal salts to Polycelis nigra and Gammarus pulex and observed high toxicity to the animals at low concentrations of copper salts. The author states that aquatic arthropods that breathe atmospheric oxygen are extremely resistant to the toxic action of dissolved salts and their survival times, even at high concentrations are long and variable. Those that make use of oxygen dissolved in the water are probably more sensitive because the integument is more permeable.

The effect of ionic copper on the oxygen consumption of Gammarus pulex and Polycelis nigra was also studied by Jones (1942). Upon exposure to low concentrations of copper both animals exhibited an initial increase in respiration rate followed by a rapid decline. The author observed that if the animals were removed from the dilute solution ten minutes after immersion, washed and placed in freshwater, normal ciliary locomotion of Polycelis was almost immediately resumed. But removal after 25 to 30 minutes resulted in failure to resume gliding and eventually death occurred. The author suggested that depression of respiration rate is merely a symptom of the toxic process.

The effect of copper on Gammarus pseudolimnaeus, Physa integra, and Campeloma decisum was investigated by Arthur and Leonard (1970). The animals were subjected to acute copper exposure followed by long-term copper exposure under continuous-flow conditions. Survival, growth, reproduction, and feeding were the responses used in measuring

toxicity. The 96-hour median tolerance limit (TL_m) values for Campeloma decisum, Physa integra, and Gammarus pseudolimnaeus were 1.7, 0.039, and 0.020 mg/l total copper, respectively. After six weeks exposure for all species the total copper concentration having no effect was between 8.0 and 14.8 ug/l.

Biesinger and Christensen (1972) studied the effects of various metals on survival, growth, reproduction, and metabolism of Daphnia magna. Toxicities of various metals were evaluated on the basis of acute toxicity in terms of a 48-hour LC_{50} . For all metals tested on an acute basis, median lethal concentrations were higher with than without food added. In acute toxicity tests the 48-hour LC_{50} 's for Daphnia magna with and without food were 60.0 and 9.8 ug/l copper, respectively.

The work was considered justifiable because the sensitivity of Daphnia magna to toxic substances was in general representative of other abundant zooplankton and also it has been shown that Daphnia magna is more sensitive to lower concentrations of toxicants than are many desirable fish species.

Warnick and Bell (1969) studied the toxicity of heavy metal salts to different species of aquatic insects. The authors suggested that the results of acute toxicity tests can be used as a basis for long-term tests to establish requirements necessary for the survival of aquatic life. They reported a 48-hour TL_m of 0.32 mg/l copper for the mayfly Ephemerella subvaria.

Fowler and Goodnight (1965) studied the effects of environmental factors on the respiration of Tubifex tubifex. They immersed Tubifex

tubifex in 1 ppm copper sulphate solution and reported that this concentration was not lethal to the animals. The duration of exposure time was not reported by the authors.

Scott and Major (1972) studied the effect of copper on survival, respiration, and heart rate of the blue mussel Mytilus edulis. Upon exposure to copper, the blue mussel showed decreased survival, decreased respiration, and decreased heart rate. The threshold for all of these effects was 0.2 mg/l. Respiratory recovery occurred in time with detoxification of the medium. Similar detoxification occurred if the original medium was treated with heat killed Mytilus edulis homogenate. The probable mode of detoxification was reported to be by organic binding. After seven days, 55% of the animals kept in 0.2 mg/l were dead and only 5% of the 0.1 mg/l group were dead.

An in-depth study of the effect of copper on the crayfish Orconectes rusticus was done by Hubschman (1967a). Experiments were performed to determine the possible delayed effects of short or periodic exposure as well as continuous exposure during different stages of the life cycle. In the continuous-flow experiments, 50% of the animals survived 96 hour exposure to 3.0 mg/l and all animals survived exposure to 6.0 mg/l for 24 hours. The experiments were repeated to observe possible delayed effects of exposure. Fifty organisms were exposed to 6.0 mg/l for 24 hours and then maintained in flowing freshwater and all were dead at 10.5 days following exposure to copper. Adults exposed for 24 hours at 2.5 mg/l, then removed from the copper solution and placed in freshwater were all dead in 15 days.

Hubschman also studied the effect of continuous exposure to low concentrations of copper to Orconectes rusticus. The results showed that copper toxicity was greatly influenced by the age of the animals. Adults exposed to 1.0 mg/l copper were all dead at the end of 16 days, while most survived in concentrations of 0.5 mg/l. Juveniles were exposed to 1.0 mg/l and 50% were dead in less than 24 hours and all were dead at the end of six days. Young organisms were also exposed to 1.0 mg/l and 50% of these were dead in six hours.

The purpose of the study was to determine basic environmental requirements for aquatic invertebrates. In terms of water quality criteria it is of little satisfaction to state that an organism survived for 24 or 48 hours in a given concentration if delayed effects are expected. Hubschman's studies have shown that mortality may in fact result long after initial exposure, thus making it difficult to define exact levels of acute toxicity.

These experiments showed that the toxic effect of copper to crayfish is dependent not only upon the concentrations and duration of exposure but also on the age of the animal.

Hubschman (1967b) reviewed the mode of toxic action of copper to invertebrates. He reports that Jones (1942) studied the effect of copper on the amphipod Gammarus pulex and to the flatworm Polycelis nigra and showed that exposure resulted in reduced respiration rate. Hunter (1949) studied the effect of copper on the marine amphipod Marinogammarus marinus and indicated that the toxic action of copper results from interference with either the respiratory system or osmoregulatory system or both. Corner and Sparrow (1956) worked

with the brine shrimp Artemia salina, the copepod Acartia clausi, and the barnacle Eliminius modestis and concluded that the mode of action of copper is specific in its effect on respiratory mechanisms.

Kerkut and Munday (1962) found that in the crab Carcinus maenas not all tissues are equally effected by copper and that the heart and gills are most sensitive. Hubschman suggests that at copper concentrations in the part per million range, at least two modes of toxic action are operative. In concentrations above 1 mg/l respiratory enzymes are inhibited quite rapidly as the mechanism of detoxification is apparently overwhelmed. At concentrations below 1 mg/l an entirely different toxic action is observed which is the degenerative effect on cells and tissues.

Hunter (1949) put forth a hypothesis about the toxic action of heavy metals on complex animals: (1) at very low concentrations the only effect may be stimulatory; (2) at a slightly higher concentration an enzyme system might be inactivated; (3) at a higher concentration the metal might interfere with respiration by decreasing the transfer efficiency of the blood pigments or interfere with excretion or with nervous coordination; and (4) at an even higher concentration, the metal might coagulate mucus or other essential secretions while at very high concentrations coagulation of the protoplasm itself or fixation will occur.

Copper Toxicity Tests

A wide range of copper toxicity for aquatic organisms is reported in the literature. Certain reports are difficult to

interpret because the experimenters fail to include a statement of the physical and chemical characteristics of the water being used or fail to state the form of copper being added to the water as a toxicant. All of these factors are extremely important and no conclusive results can be stated without knowledge of these factors.

The wide range of lethal or harmful values of copper reported may also reflect the use of different test organisms, dilution water, exposure time, and other experimental conditions. The toxicity of the same or similar wastes may also vary widely in different receiving waters. The Aquatic Life Advisory Committee states that permissible concentrations of toxicants in waters receiving industrial wastes are those which can be tolerated independently by all individuals, instead of the standard 50% which is the normal criteria. This includes all significant species of aquatic organisms and takes into account those which serve in food chains as well as fish and others of direct economic and recreational importance. This criteria is applicable to all stages of the life cycles for these organisms. Therefore, the concentration of toxic industrial wastes should never be more than a small fraction which under experimental conditions is demonstrably fatal within a limited period of time to 50% of the test organisms used in the bioassay. The authors conclude that the result of the 48-hour TL_m multiplied by an application factor of 0.1 represents a concentration of waste which usually will not produce adverse effects on the total population of aquatic organisms.

Certain reports on copper toxicity to aquatic organisms are equally hard to interpret or even accept because of the bioassay methods which are employed. The use of the static type bioassay was previously preferred (Duodoroff et al., 1951), however, this type of bioassay can yield ambiguous results. Recent experimenters have preferred a continuous-flow bioassay test (Zillich, 1972; Sprague, 1973; Burke and Ferguson, 1968; and Freeman, 1971).

Burke and Ferguson (1968) noted that the objectional features of static tests include a decline in concentration of the toxicant during the exposure period caused by uptake by the experimental animals and adsorption of the toxicant onto the container or other surfaces, and its chemical alteration. Furthermore, accumulation of waste products, reduction of dissolved oxygen supply, and the growth of microbial populations may produce an undesirable test environment. Burke and Ferguson favor the continuous-flow apparatus for several reasons and note that there was no more than 1% difference in expected concentration in samples from the dilution as compared with samples of known concentration.

The purposes of the flow rate in continuous-flow tests are as follows: (1) to provide exposure to a constant concentration of toxicant, because it may be depleted by the test organisms if the flow rate is too slow; (2) to remove all waste products adequately, since waste products may foul the water if they are not removed; and (3) to provide an adequate supply of dissolved oxygen for the test organisms, because if the flow is too slow the organisms may lower the dissolved oxygen to a point where stress may be increased.

Occurrence of Copper in the Aquatic Environment

The natural occurrence and regeneration of copper in aquatic environments is extremely important and an excellent discussion of this topic was prepared by Riley (1939). The author determined that the copper content varied tremendously with the season and from one year to the next and the total range in all samples varied from 0.005 to 0.383 mg/l. The distribution of total copper was highly irregular and was especially marked during the overturns. His results showed that the annual variation was extreme, with the amount of copper greatest in the autumn and least in the late winter and spring.

Riley also investigated the factors influencing the distribution of copper in the lakes and noted that the copper in natural waters is derived ultimately from the soil of the drainage basin. All soils contain copper, ranging from a trace to several percent and groundwater dissolves traces of copper from the relatively insoluble salts in the soil and eventually brings it into streams and lakes. The amount coming into a body of water is dependent on several factors: the quantity in the soil, its availability, and the amount of precipitation. The author stated that if copper were inert biologically and chemically, the amount in a given lake would remain fairly constant. Riley's experiments showed that major variations in copper cannot solely be accounted for on the basis of precipitation effects, but are at least partially due to reactions in the lake basin.

The chief means of removal of copper from the lake water is by adsorption on organic particles. It was also shown that certain variations of copper are too great to be explained on the basis of precipitation effects. Therefore, there must be sources of copper within the basin which are available at certain times to the lake water and periodically increase the copper content. The two possible sources are decaying littoral plants and lake mud.

Riley concluded that there are five factors which affect the copper content of lake water: (1) precipitation which lowers the copper content by dilution; (2) sedimentation which entails the removal of copper from solution by adsorption on organic matter; (3) regeneration from the mud; (4) liberation of copper from littoral plants in the autumn when they die and decompose and removal of copper from the water during the growing season; and (5) liberation of copper in the autumn by the decomposition of vegetation surrounding the lake.

The physical state in which copper is present in the aquatic environment may determine its toxicity by affecting its availability. Stiff (1971) tried to establish the forms in which copper could exist in the natural waters and to relate this knowledge to the results of fish toxicity tests in order to determine the relative toxicities of the different forms of copper. He stated that in the aquatic environment three physical states of copper are possible, and these are particulate, colloidal, and soluble. Particulate forms could include oxide, sulphide, and malachite precipitates as well as insoluble organic complexes, copper absorbed on clays, and

metallic hydroxide precipitates. Soluble matter includes copper both as free cupric ions and as soluble complexes. Colloidal matter includes polypeptide material, some clays, and metallic hydroxide precipitates. Experimental evidence from his work suggests that only the soluble forms of copper are available to the aquatic organisms.

The most significant artificial means of copper addition to natural waters is the application of copper sulphate and other copper compounds for control of unwanted vegetation and for control of swimmers itch disease. The State of Michigan regulates the distribution and addition of copper compounds to the public waters of this state for both of these uses.

The algicidal use of copper sulphate is employed to rid water of unwanted vegetation including submerged, emergent, and floating plants, as well as algal populations. Incomplete records of weed control are available and it has only been since 1970 that the Weed Control Commission of the Michigan Department of Natural Resources has kept a very close record of weed control with copper compounds. This commission recommends an application of a concentration of 0.21 ppm copper sulphate for algal control and 0.40 ppm for control of Chara (Wandel, 1974).

In the years 1970-1973 the following amounts of copper compounds were added to Michigan public waters to control unwanted vegetation: 20,009 pounds of copper sulphate, 2,926 pounds of chelated copper, and 2,660 pounds of sequestrene copper (Wandel, 1974).

Much more complete records of copper sulphate addition for control of swimmers itch disease exist in the State of Michigan. Freshwater snails are intermediate hosts for the cercariae which cause the schistosome dermatitis and the snails in Michigan which are primarily responsible are Stagnicola emarginata, Lymnaea stagnalis juglaris, Physa parkeri and a number of other species of Physa. The Department of Natural Resources attempts to control the swimmers itch disease by eliminating these freshwater snails and suggests an application of a concentration of 32.0 ppm copper sulphate to eradicate them (Wandel, 1974).

Records are available since 1947 for the application of copper sulphate for the control of swimmers itch. In the years 1947-1970, 1,371,655 pounds of copper sulphate have been applied to Michigan public waters to control this disease (Wandel, 1974).

Investigations on the effect of copper sulphate treatment on lake ecology and accumulation in lake muds in the Madison, Wisconsin area were performed by Mackenthun and Cooley (1952) and Nichols et al. (1946). Over a 26 year period beginning in 1925, 1,697,639 pounds of copper sulphate have been applied to Lake Manona. Nichols et al. reported that although the total amount of copper deposited in the lake muds were unknown, it appeared that by far the greatest amount of copper applied remains as a deposit in the mud. The latter investigators reported that another lake, Lake Monona, contains up to 480 milligrams of copper per kilogram of mud and stated that this concentration is lower than the amounts experimentally determined to have a deleterious effect on the bottom-dwelling organisms studied.

They also reported that the toxic limit of copper sulphate, precipitated and accumulated in the bottom muds, could not be accurately determined for certain bottom-dwelling organisms. Nichols et al. reported that the solubility of precipitated copper in lake muds is determined to a large extent by the total alkalinity and the pH of the overlying water.

Such high concentrations of copper sulphate in natural waters may affect aquatic organisms other than the target organisms. If the concentration of copper sulphate added is above the tolerance limit for non-target organisms then undoubtedly they will be adversely affected. Aquatic macroinvertebrates and perhaps certain fish species may be particularly affected. If the copper precipitates out of the water, combines with organic matter or otherwise becomes unavailable to the aquatic organisms then the effect may be reduced.

The possibility of deleterious effects upon non-target aquatic organisms exists and is an important consideration in evaluating the desirability of copper sulphate addition to freshwater environments.

MATERIALS AND METHODS

The continuous-flow bioassay test system utilized in this experiment was modeled after the original design developed by the Michigan Water Resources Commission used to test effluent water (Zillich, 1972). However, a modified construction design was implemented (Figure 1) resulting in several noteworthy improvements. In contrast to the original design the test water at each concentration was not reused. After the water passed out through the overflow it was drained out of the test containers into a sink. A uniform volume was maintained in each test container by the use of standpipes fitted into the bottom of each container. The volume in each container was approximately 800 ml. A simplified construction design aided in the observation of experimental organisms, set-up, and maintenance of the test system. Instead of testing effluent from an industrial source where a variety of toxic substances may be present, this test involved only the use of copper sulphate. In addition, the modified design resulted in equal dilution increments in intervals of 20% ranging from zero to 100 percent of the test concentrations. Therefore, the only toxic substance contributing variably to mortality would be copper sulphate.

A wooden frame measuring 199.0 cm X 124.4 cm X 60.9 cm was constructed of 0.9 cm plywood which made up the sides and back and 3.8 cm X 7.6 cm boards were used for the corners. Each shelf was made from 1.9 cm boards and 1.9 cm plywood was used for the top of the frame. This construction resulted in a durable and secure frame

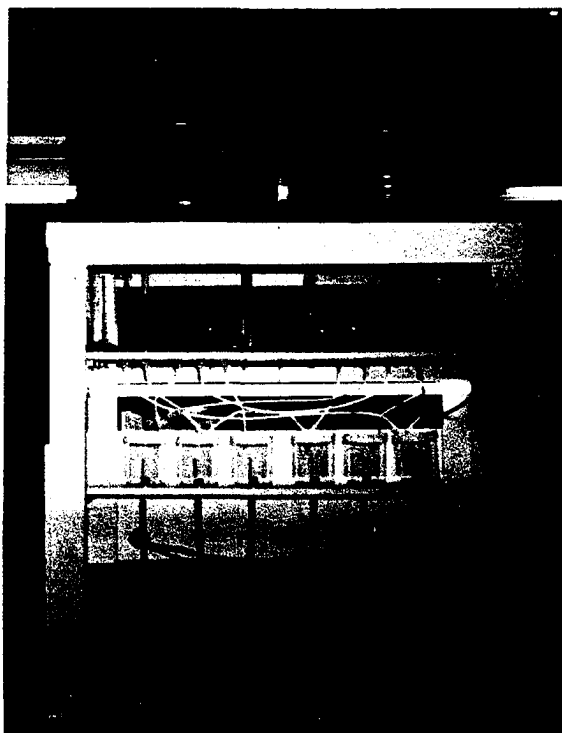


Figure 1. Continuous-Flow Diluter System

which was necessary to support the weight of the water reservoirs that were used.

Four 220 liter barrels were used as reservoirs for the water and for delivery barrels to the system. Each barrel was coated thoroughly with a fiberglass resin to protect against direct contact between metal and the water. The two barrels which were placed on top of the frame were fitted with a 20.3 cm X 1.5 cm polyvinylchloride pipe which delivered the water to the constant level dilution tanks. The two remaining barrels were used as reservoirs for preparing the diluting and test water so that when the water in the delivery barrels became low these could be refilled from the reserve barrels. A submersible pump was used to transfer the water from the reserve barrels to the delivery barrels. A length of vinyl hose was connected to the pump and led into the delivery barrel. After the reserve barrels were emptied, each was subsequently refilled for later use.

The delivery barrels supplied water to the diluting tanks which were constructed of 0.6 cm plexiglass and were cemented together with silicone rubber cement (Figure 2). The water level in these tanks was maintained at a near constant level by the use of float valves which were placed in each tank.

Sets of from one through five holes were drilled into the bottom of the diluting tanks and one millimeter bore glass tubing which was approximately 2.5 cm in length was cemented into each of these holes. Each glass tube extended exactly the same distance

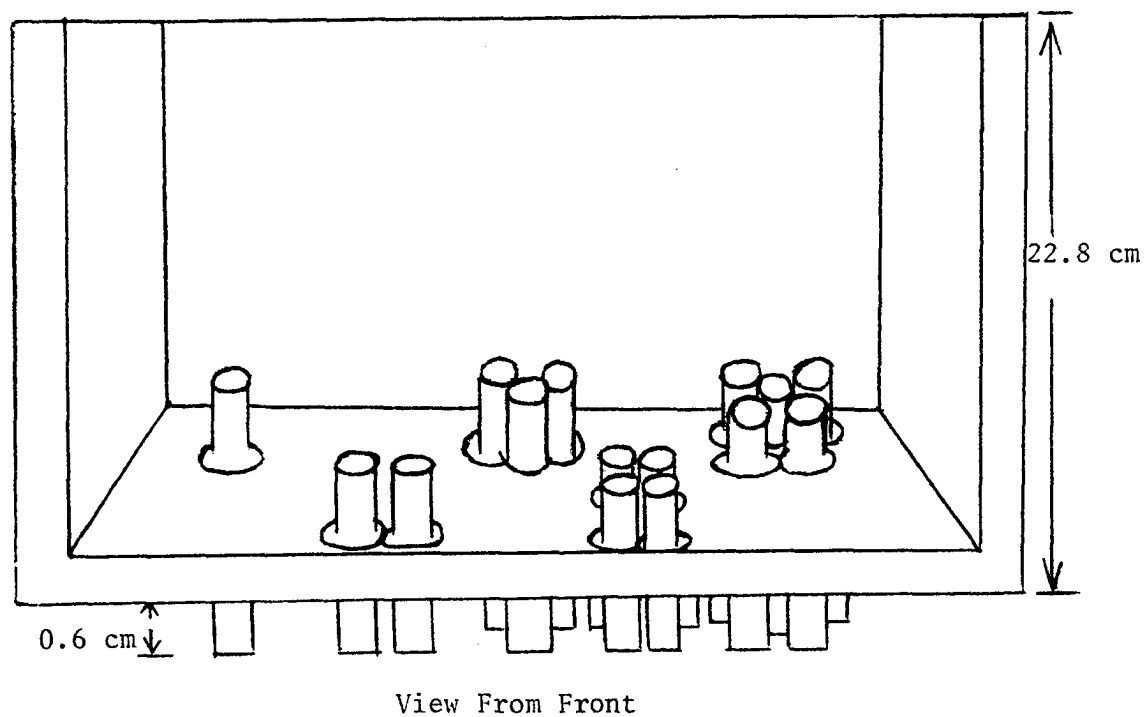
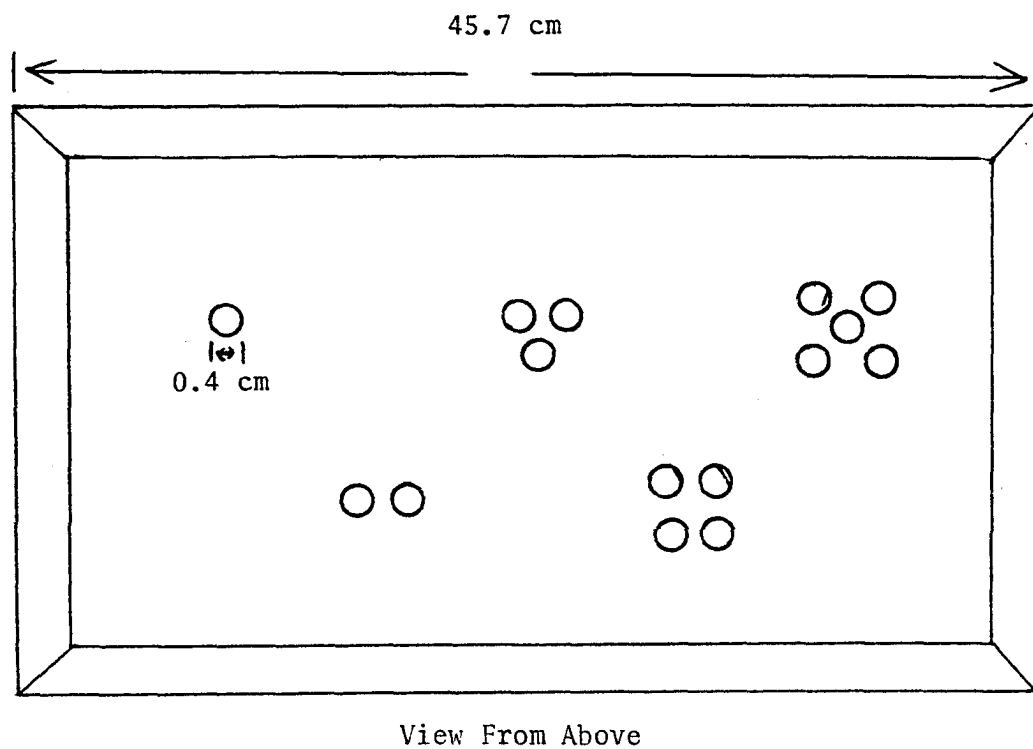


Figure 2. Dilution Tank

below the bottom of the diluting tanks so that the same head of water was effective in producing a uniform rate of flow from each tube in the diluting tank.

Each container in which the organisms were tested received the flow from five holes. The dilutions of the test water were obtained by determining the number of holes, out of the total of five holes, which delivered the test water to the test containers. Therefore, dilutions of the original concentration of test water ranged from zero to one hundred percent in intervals of twenty percent.

In order to level the tanks, a number of holes were drilled through the shelf along the edges of the tanks and each was fitted with leveling screws topped with a flat piece of cork stopper fitted onto the end of each bolt where it made contact with the bottom of the diluting tank. If any portion of the tank required raising or lowering in order to level the tank, then the appropriate bolt could simply be adjusted to achieve the desired level.

Polyethylene collecting funnels were fixed under each set of holes in the bottom of the diluting tanks and were connected to polyethylene tubing which emptied into each of the test containers. Polyethylene "Y" connectors received water from the diluting water tank and the test water tank which allowed for complete mixing of the various concentrations prior to entry into the test containers.

The test containers were polypropylene, liter capacity jars (Figure 3). A number three rubber stopper was fitted into the bottom of each container and a 0.7 cm I.D. glass standpipe was inserted through the stopper. The glass standpipe drained the

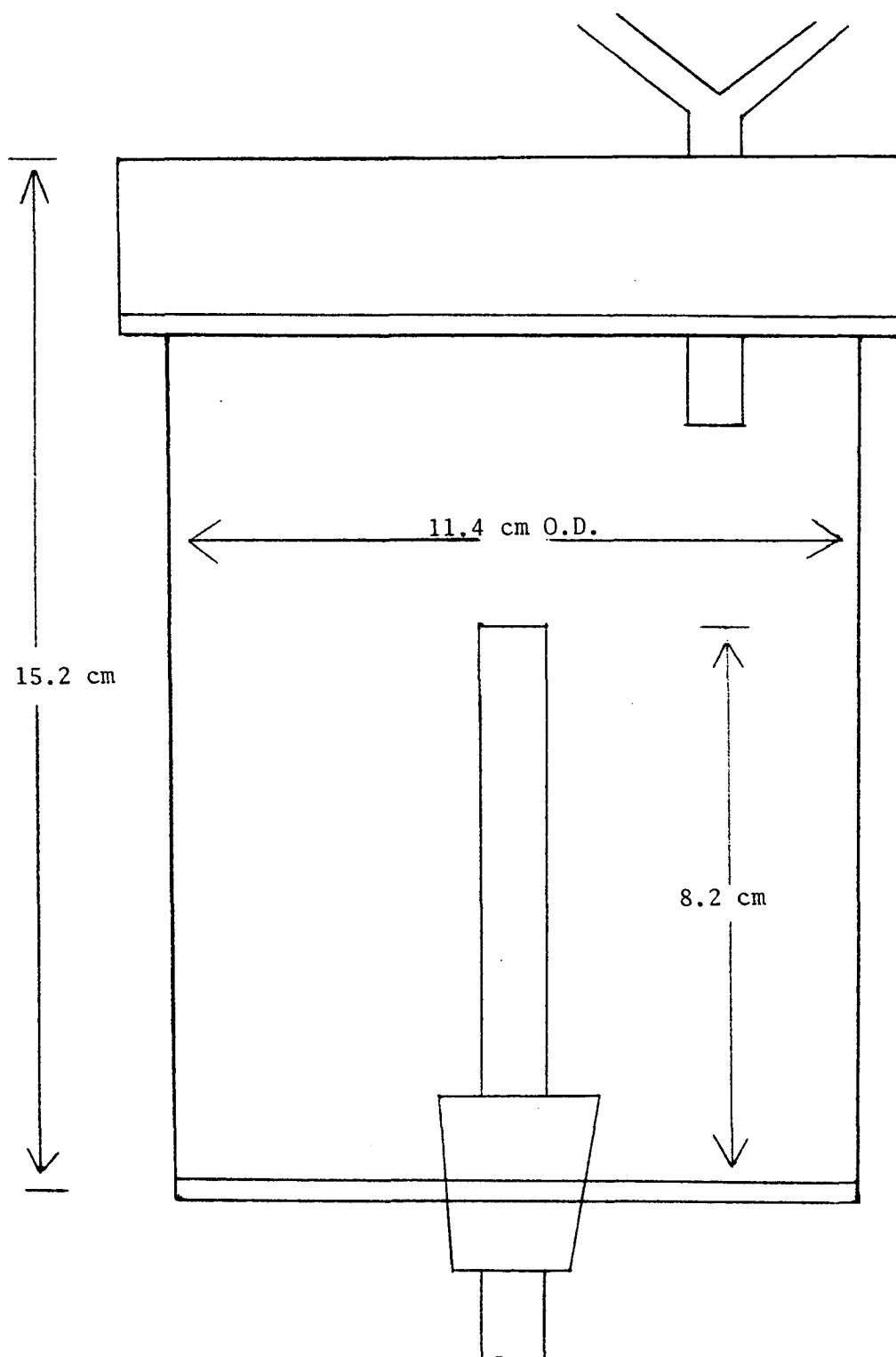


Figure 3. Bioassay Test Container

overflow into a sink via 0.4 cm I.D. plastic tubing. The top of the standpipe measured 8.2 cm from the bottom in each test container so that the volume of water was identical in each of the six test containers.

The method of collecting, holding, and testing the organisms was followed from the procedure set forth by Sprague (1973). The test organisms which were utilized in this experiment were Ameletus sparsatus, Hyalella azteca, Tubifex tubifex, and Sphaerium simile. The test organisms were identified using the following texts: Needham et al. (1969), Pennak (1953), and Burch (1972).

Tubifex tubifex was collected from the Portage Creek near Lake Street in Kalamazoo. Ameletus sparsatus, Hyalella azteca, and Sphaerium simile were collected from the Portage Creek at 12th Street and N Avenue in Portage. The Tubifex were collected only once for this experiment and were maintained in the laboratory until the tests were conducted. Ameletus, Hyalella, and Sphaerium were collected and identified prior to each test. These organisms were acclimated for four days in the laboratory prior to testing and were deprived of food for one day prior to testing.

During and after the experiments, these organisms were held and tested in glass petri dishes which measured 6.0 cm X 2.5 cm and were covered with a fine mesh nylon cover to confine the organisms in the petri dishes during testing. Ten specimens of each species were placed in the test containers in a random sequence.

An initial 48-hour test was conducted to determine short-term, acute toxic effects of copper sulphate upon these organisms. The

organisms which survived the initial test were placed in aquaria with freshwater containing no added pollutants and survivors were observed daily for three weeks to detect delayed or long-term effects. The water temperature and photoperiod were identical to that of the initial test. During the long-term tests the water was oxygenated with an airstone and the animals were deprived of food.

During the initial 48-hour test observations on mortality were made at 15, 30, and 60 minutes and at 2, 4, 8, 12, 24, 33, and 48 hours. This follows the observation schedule recommended by Sprague (1973). The daily photoperiod for the tests was set for 12 hours beginning at 8 a.m. and illumination was provided by a 40-watt fluorescent bulb.

The temperature of the test water was kept at room temperature of approximately 23°C , and the flow rate was maintained at approximately 2.1 ml/second into each of the test containers, with the volume of the containers being 800 ml. According to the ratio given by Sprague (1973) the replacement time would be approximately 0.1 hour.

The stock solution of copper sulphate was accurately measured to equal 5.0 g/l. Twenty-two milliliters of this stock solution were added to each 220 liters of the test water resulting in an original concentration of 0.50 mg/l. Therefore, the expected concentrations would be 0.50, 0.40, 0.30, 0.20, 0.10, and 0 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /l.

These concentrations are below the level that can be accurately determined with the cuprethol test. Tests run by Miller (1974) with the same diluter using NaCl show the dilutions are within 5% of the expected values. The copper sulphate values are equivalent to 0.125, 0.100, 0.075, 0.050, 0.025, and 0 mg Cu/l.

All calculations of LC_{50} values are by the method of Sprague (1973) and are reported in terms of the initial amount of copper sulphate added to the test water. Since solubility of this salt is greatly influenced by the character of the dilution water, it is possible the concentrations of metal to which the organisms were actually exposed may have been somewhat less than the amount added; however, no precipitates were noted.

The physical and chemical characteristics of the dilution water which were monitored during the 48-hour test include temperature ($^{\circ}C$), pH, dissolved oxygen (ppm), total hardness (ppm $CaCO_3$), total alkalinity (ppm $CaCO_3$) and flow rate (ml/sec). The water source was tap water supplied to the university by the Kalamazoo Municipal Water Department. Changes in the city pumping sites may have caused variations in the physical and chemical characteristics listed above. Sodium thiosulphate was added to each barrel of water to remove all chlorine.

The flow rate was determined by calculation from the time required to fill a 225 ml container with the effluent from a test container.

RESULTS

Determinations of dissolved oxygen, pH, temperature, total hardness, total alkalinity, and flow rate were made during these tests and the values of these characteristics are listed in Table 3. Very little fluctuation was observed in values for the flow rate, total alkalinity, dissolved oxygen, pH, and temperature. The total hardness was the most variable of the parameters tested with a range from 180-210 ppm CaCO_3 . The continuous-flow bioassay tests used in this study were 48 hours in duration and observations on mortality were made at the pre-determined times. The cumulative mortality of the experimental animals after 48 hours is given in Table 4. These numbers represent the cumulative mortality of the animals from all three trials and represent the number dead out of 30 at each concentration.

Observations made during the 48-hour tests indicate that at first exposure to the test concentrations the animals exhibited increased locomotion or activity. This was particularly true for the amphipods, mayflies, and tubificids. The amphipods and mayflies soon became inactive and clung to the nylon cover or rested on the bottom of the containers for the duration of the tests. The tubificids were particularly active during the initial exposure and maintained an active state for the entire test. Many, if not all, of the worms in every concentration, crawled out of the small test containers in each trial. Some even found their way into the test

Table 3. Chemical and Physical Characteristics of Dilution Water

	Temperature (°C)	pH	Dissolved Oxygen (ppm)	Total Hardness (ppm CaCO ₃)	Total Alkalinity (ppm CaCO ₃)	Flow Rate (ml/sec)
Trial 1	23	8.2-8.4	8.0	180-210	240-250	2.1-2.2
Trial 2	23-24	8.2-8.4	8.0	185-200	240-250	2.1-2.2
Trial 3	24-26	8.2-8.4	8.0	180-210	240-250	2.1-2.2

Table 4. Cumulative Mortality After 48 Hours

	Concentration $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ mg/l					
	Control	0.10	0.20	0.30	0.40	0.50
<u>Hyalella azteca</u>	5	6	5	7	4	11
<u>Ameletus sparsatus</u>	4	3	2	7	8	4
<u>Tubifex tubifex</u>	1	6	1	5	7	14
<u>Sphaerium simile</u>	0	0	0	0	0	0

containers of the amphipods and mayflies. Others got caught in the shells of the clams. This made observations of mortality quite difficult for Tubifex. A very interesting and significant observation was made on Sphaerium during the initial 48-hour tests. The clams did not show much activity during the 48-hour tests and after the first 2 to 4 hours, clams tested in all containers except control had tightly closed their shells. Occasionally, the siphons were extended but there was no mortality in any concentration over the 48-hour tests. The copper sulphate concentrations may have already been too weak to affect any mortality among the clams, but, in preliminary studies in much higher concentrations, the same behavior was observed.

Forty-eight hour mortality was so low that the projections on probit scales to determine median lethal concentrations (LC_{50} 's) are very conjectural in this study.

A nomograph (Figure 4) was constructed for correcting the mortality in the tests with the appropriate fraction of control mortality. The 48-hour LC_{50} values for Hyaella azteca, Ameletus sparsatus, and Tubifex tubifex were 10.0, 5.0, and 0.9 mg $CuSO_4 \cdot 5H_2O/l$, respectively. The probability scales and eye fitted projections from which these values were determined are graphed for Hyaella azteca in Figure 5, Ameletus sparsatus in Figure 6, and for Tubifex tubifex in Figure 7. In some instances mortality in the control group equalled or exceeded mortality in the experimental group. In Figures 5, 6, and in Figure 7 the

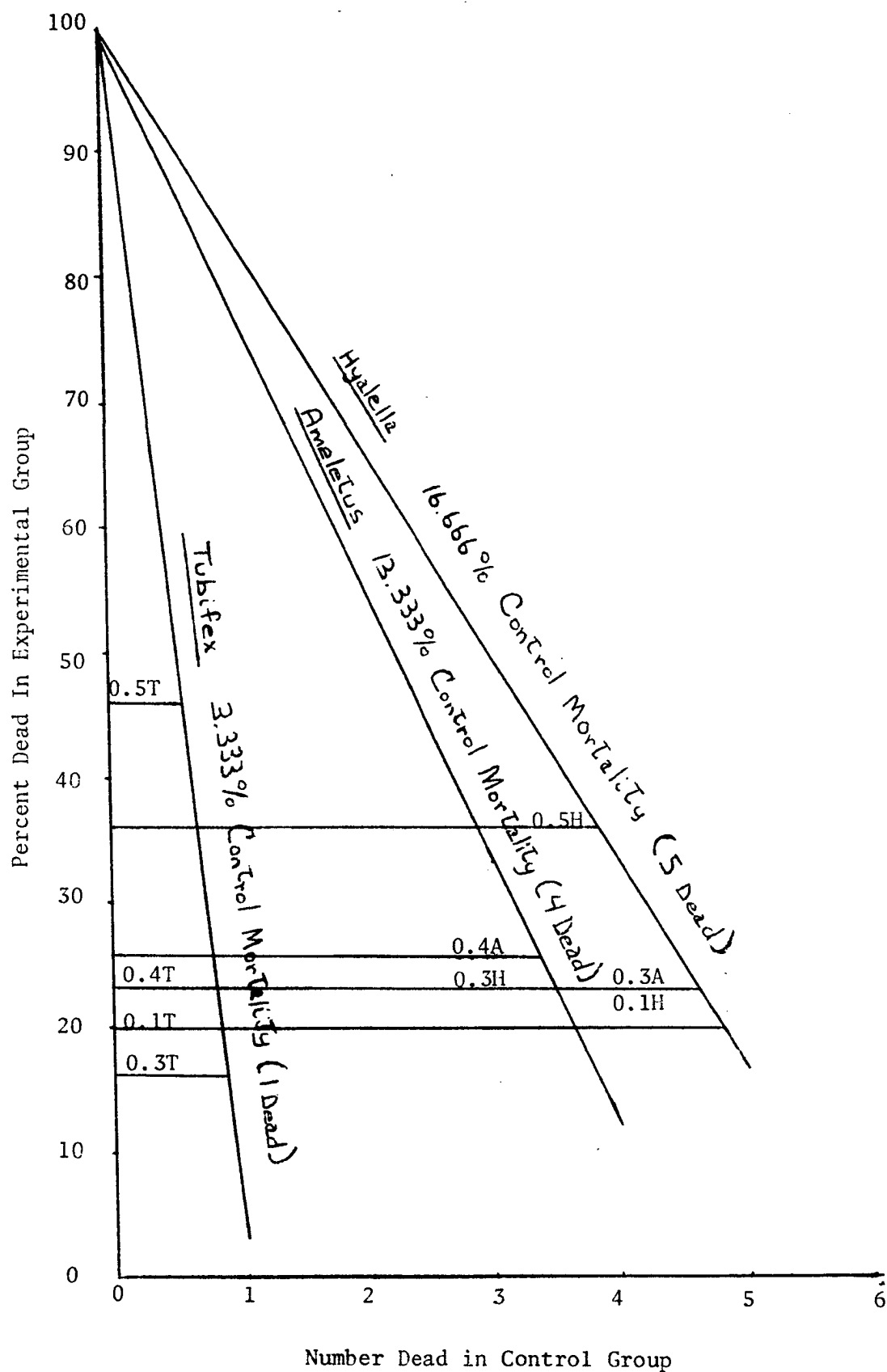


Figure 4. Nomograph for Correcting Mortality

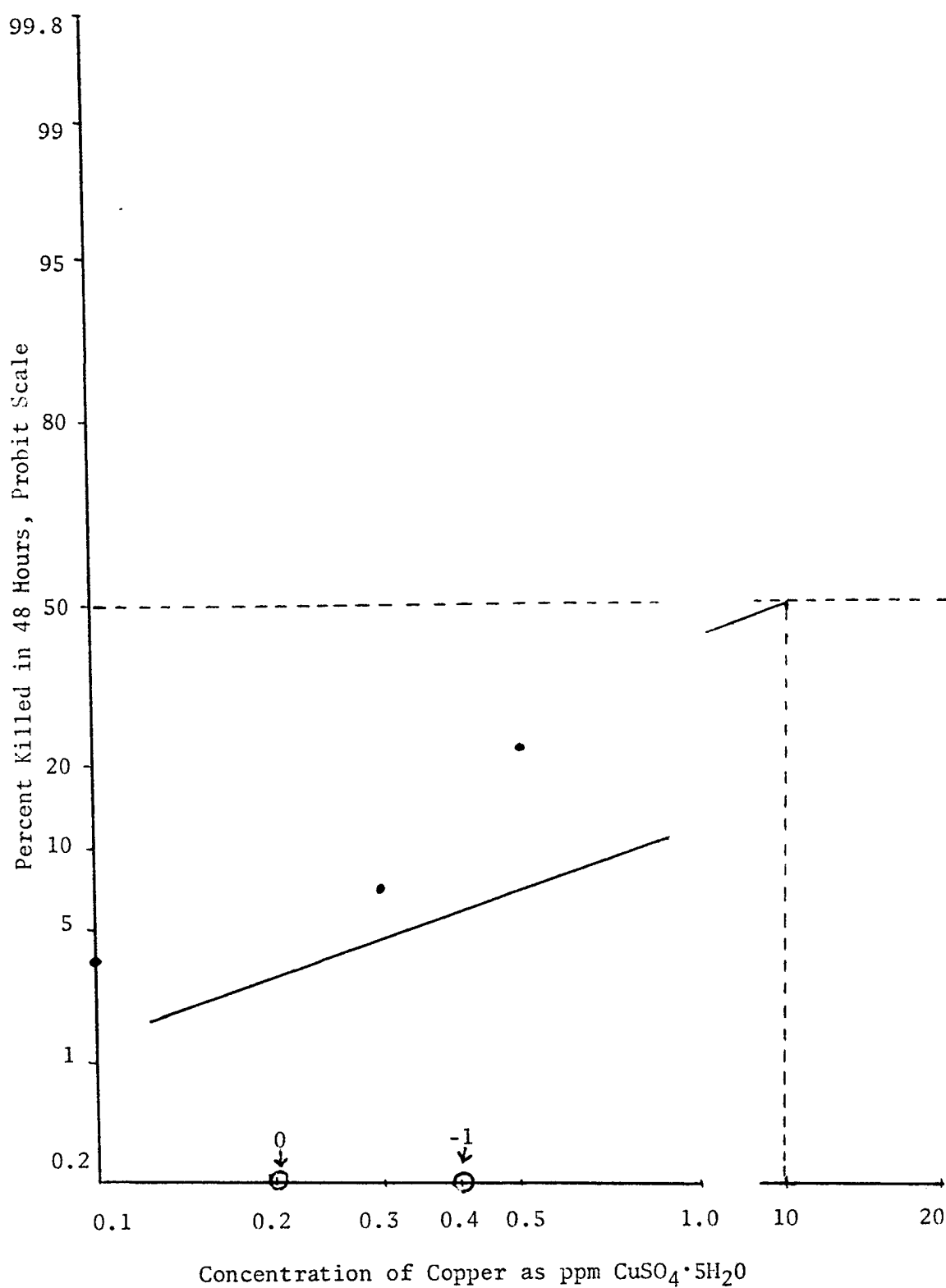


Figure 5. Estimation of the 48-Hour LC_{50} for Hyalella azteca

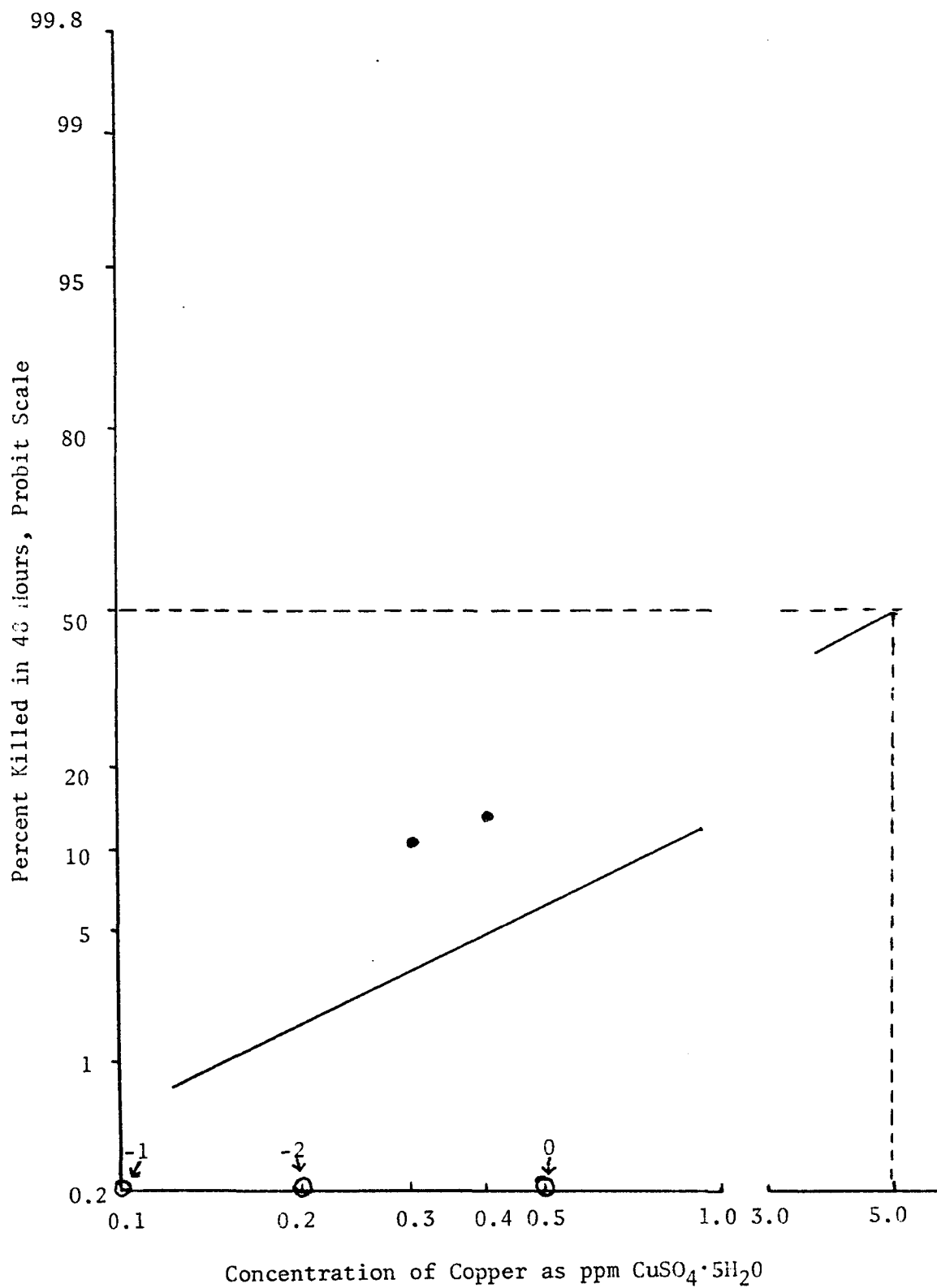


Figure 6. Estimation of 48-Hour LC_{50} for Ameletus sparsatus

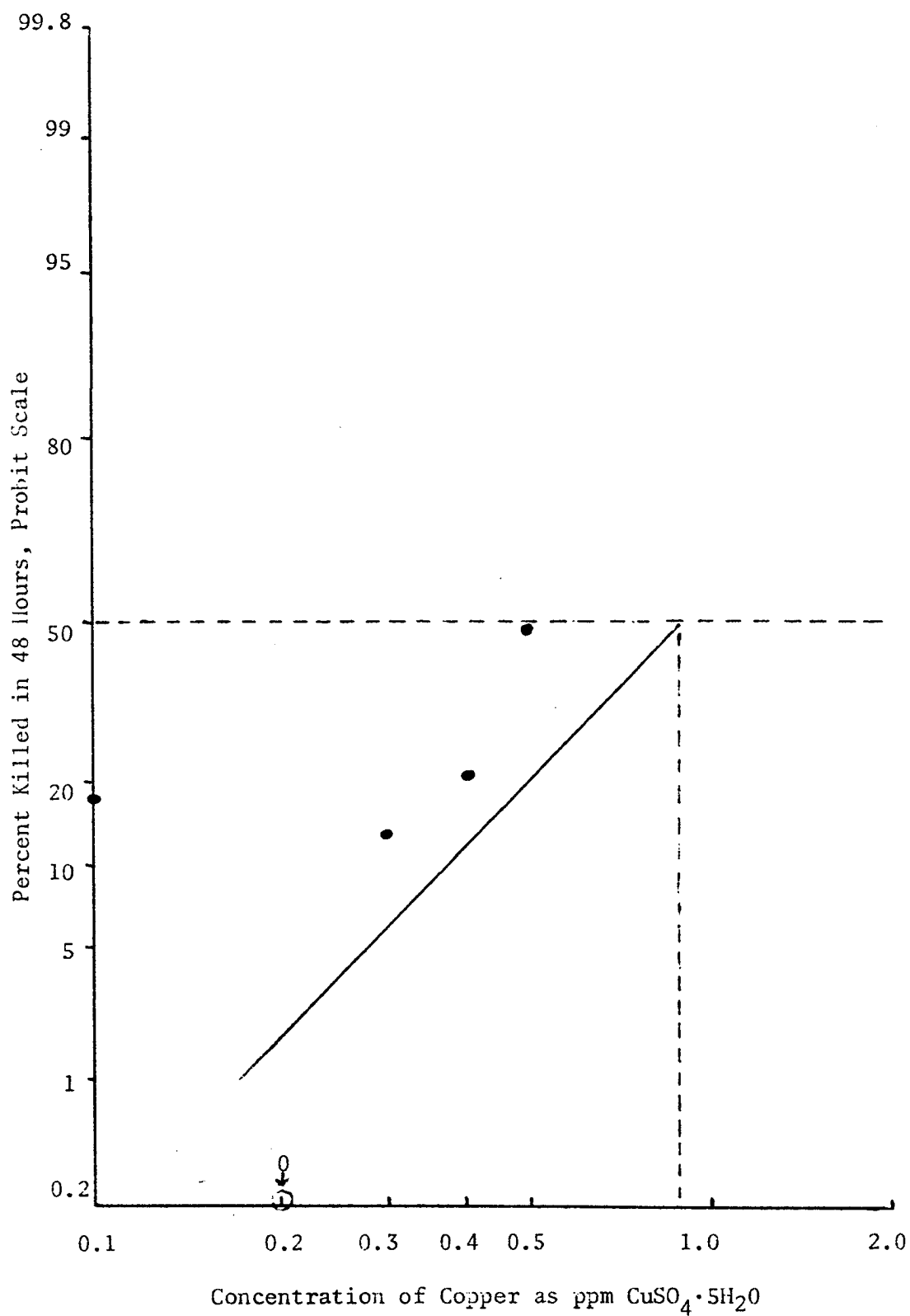


Figure 7. Estimation of 48-hour LC_{50} for Tubifex tubifex

concentrations where this occurred are circled and the number of the experimental group relative to the control group is indicated.

Sphaerium simile exhibited no mortality in the 48-hour tests, therefore, no calculation of the LC_{50} value was possible.

Another method of evaluating the results may be done by calculating the percent mortality in the 48-hour tests. This may facilitate understanding other figures in this report. The percent mortality of the experimental animals in the 48-hour tests is given in Table 5. To determine any delayed or long-term effects of the original exposure to copper sulphate on the experimental animals, observations were continued for a maximum of 21 days and observations on mortality were made daily. After the original 48-hour exposure the surviving animals were transferred to freshwater aquaria and were maintained and observed for a maximum of 21 days. The experimental conditions were identical to the 48-hour test with the exception that the water was artificially oxygenated and no copper was added.

The 21-day observations on mortality are graphed for Hyalella azteca in Figure 8, for Ameletus sparsatus in Figure 9, and for Tubifex tubifex in Figure 10, and for Sphaerium simile in Figure 11. The graphs obtained for the long-term observations are constructed from the cumulative mortality of all the animals in all concentrations in the three tests. The total mortality in the clams is probably strongly biased by one trial. In the total of the three trials, 88.2% (60 individuals) of the entire mortality resulted from Trial 2. Each of the other trials contributed 2.9% (2 individuals) to the total mortality.

Table 5. Percent Mortality After 48 Hours

	Concentration $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ mg/l					
	Control	0.10	0.20	0.30	0.40	0.50
<u>Hyalella azteca</u>	16.6	20.0	16.6	23.3	13.3	36.6
<u>Ameletus sparsatus</u>	13.3	10.0	6.6	23.3	26.6	13.3
<u>Tubifex tubifex</u>	3.3	20.0	3.3	16.6	23.3	46.6
<u>Sphaerium simile</u>	0.0	0.0	0.0	0.0	0.0	0.0

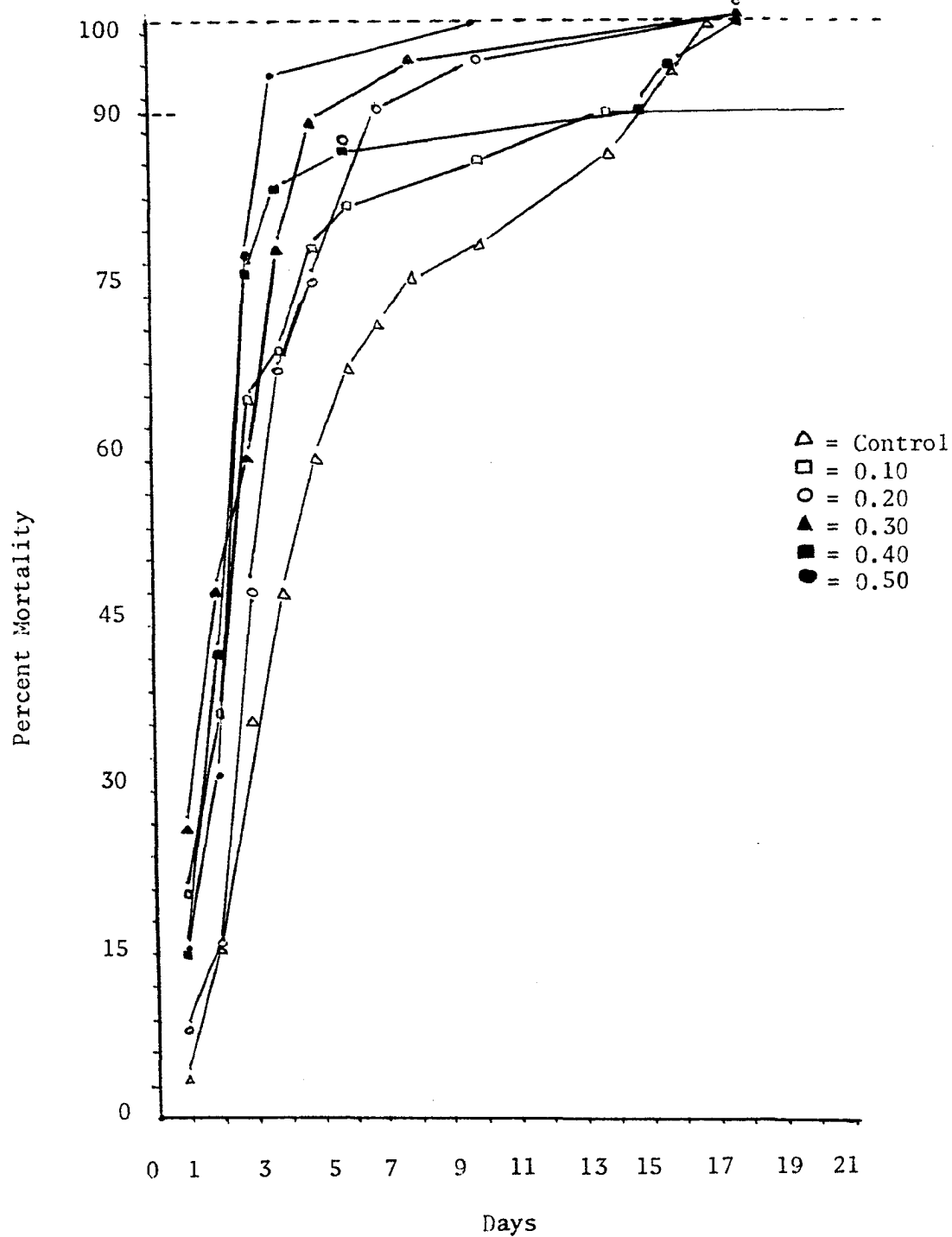


Figure 8. Long-term Observation of *Hyalella azteca*

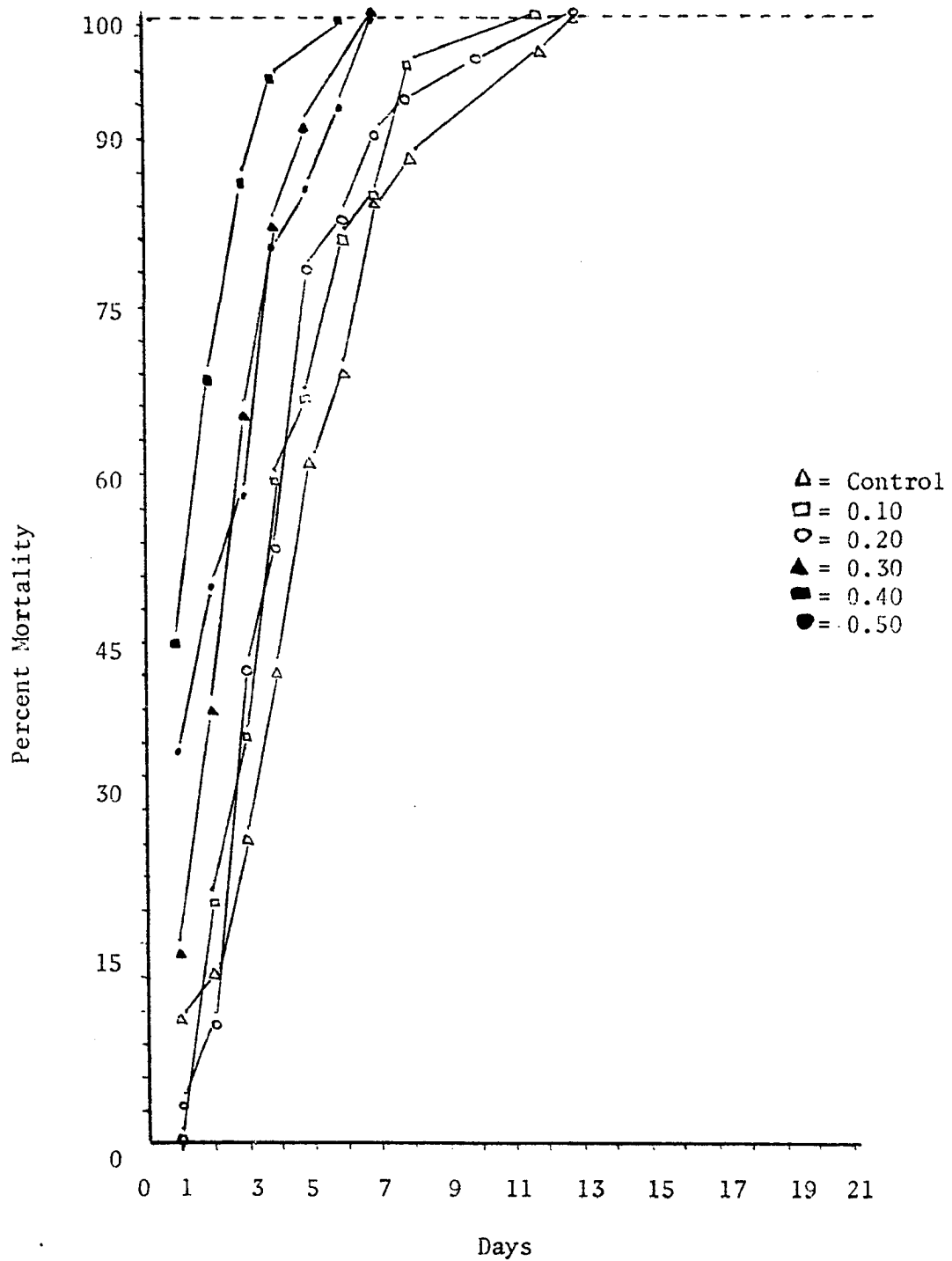


Figure 9. Long-term Observation of *Ameletus sparsatus*

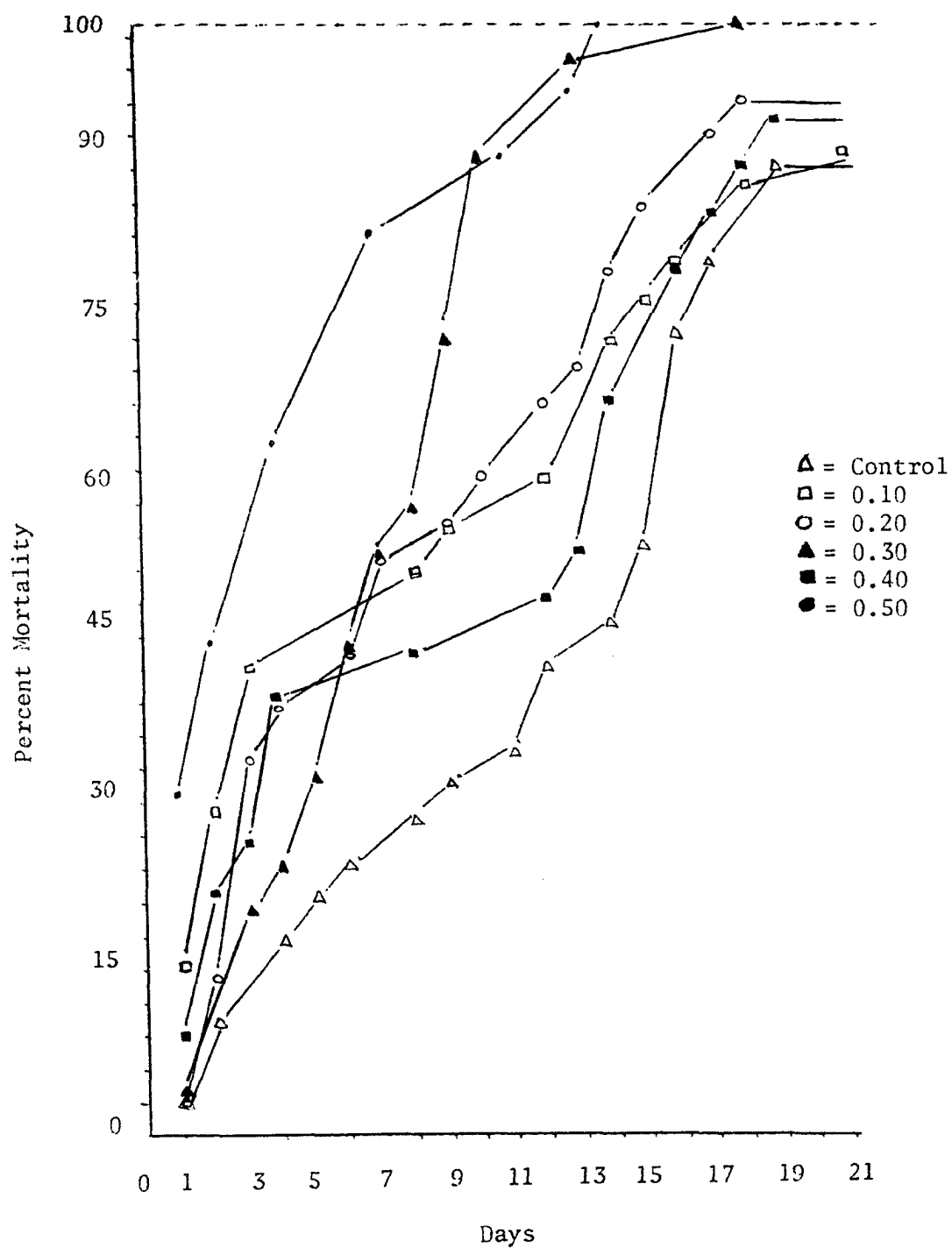


Figure 10. Long-term Observation of *Tubifex tubifex*

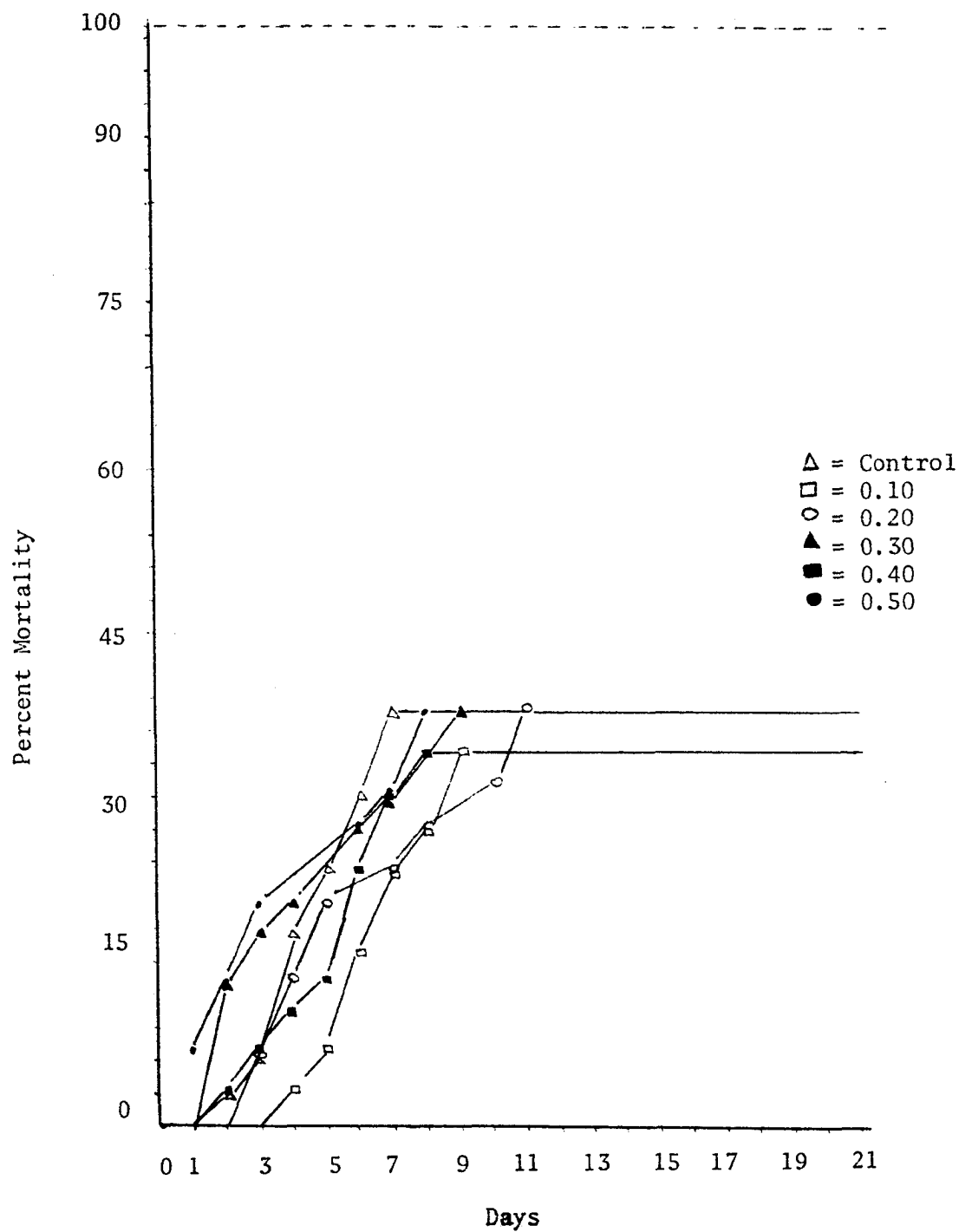


Figure 11. Long-term Observation of *Sphaerium simile*

The results of the long-term observations of the other animals show consistent results in the three trials. The number of survivors at each concentration at four day intervals during the first two weeks of the long-term trials is given in Table 6.

There is a positive correlation between percent mortality and the original concentration of copper sulphate in which they were tested. In all tests the animals which were originally exposed to the higher concentrations died sooner than the animals exposed to the lower concentrations.

Long-term observations of Hyalella azteca indicated that the amphipods exposed to the lower concentrations of copper sulphate had a lower mortality for the first 14 days than did those exposed to the higher concentrations. The percent mortality after seven days for these groups, control, 0.10, 0.20, 0.30, 0.40, and 0.50 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}/\text{l}$ concentrations are 72, 83, 92, 93, 88, and 98 percent, respectively. One-hundred percent mortality in the 0.50 group occurred in ten days, and in the 0.20 group in eighteen days.

Long-term observations on Ameletus sparsatus also show very well that those mayflies tested in the higher concentrations of copper sulphate exhibited more rapid mortality than those mayflies tested in the lower concentrations. Generally, the mayflies seem to be more sensitive than the amphipods because the total mortality in each group of mayflies occurred sooner than in the corresponding group of amphipods. However, this shift in sensitivity in the mayflies may be attributed to factors not considered in this study, e.g. tolerance to lack of food. The latter is indicated by comparison of control groups.

Table 6. Number of Survivors During Long-term Tests

		Concentration mg CuSO ₄ ·5H ₂ O/l																	
		Control			0.10			0.20			0.30			0.40			0.50		
Days		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0	A	9	8	8	8	10	6	8	10	7	9	8	6	8	9	9	9	5	5
	M	8	9	9	7	10	10	8	10	10	5	10	8	6	8	8	8	9	9
	T	10	9	10	10	8	6	10	10	9	8	9	8	10	3	10	6	4	6
	C	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
4	A	9	1	3	6	1	0	6	0	2	4	0	1	4	0	0	1	0	0
	M	6	3	6	4	5	2	4	5	4	0	3	1	1	1	0	1	1	3
	T	9	5	10	9	0	5	9	2	7	8	5	6	9	0	5	4	0	2
	C	9	6	10	10	9	10	10	7	9	10	4	10	10	7	10	9	5	10
8	A	5	0	1	4	0	0	2	0	0	1	0	0	3	0	0	1	0	0
	M	3	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
	T	9	2	10	8	0	4	8	0	6	4	4	3	9	0	4	3	0	0
	C	9	0	10	10	2	10	10	2	9	10	1	10	10	0	10	9	0	10
12	A	4	0	1	3	0	0	1	0	0	1	0	0	3	0	0	0	0	0
	M	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
	T	8	1	8	7	0	3	6	0	4	2	0	1	8	0	4	2	0	0
	C	9	0	10	10	0	10	10	0	9	10	0	9	10	0	10	9	0	10

A = amphipod (Hyalella azteca)M = mayfly (Ameletus sparsatus)T = tubificid (Tubifex tubifex)C = clam (Sphaerium simile)

1 = First Trial 4-18-74 to 5-9-74

2 = Second Trial 4-25-74 to 5-16-74

3 = Third Trial 4-29-74 to 5-20-74

The mortality after five days in the control, 0.10, 0.20, 0.30, 0.40, and 0.50 mg/l groups was 51, 65, 66, 90, 97, and 85 percent, respectively. Complete mortality in the 0.50 group occurred in seven days while complete mortality in the 0.10 group occurred in twelve days.

The long-term observations on Tubifex tubifex also indicate that worms tested in the lower concentrations died at a slower rate than those worms tested in the higher concentrations of copper sulphate. The graphs indicate that generally the tubificid worms lived longer than either the amphipods or mayflies tested. But again, this shift is probably due to longer life under starvation conditions as indicated by the control group.

The percent mortality after eight days for these groups, control, 0.10, 0.20, 0.30, 0.40, and 0.50 mg/l are 28, 51, 53, 61, 43, and 77 respectively. Complete mortality occurred in 14 days for the 0.50 group and in 17 days for the 0.30 group. No other group of worms experienced complete mortality for the duration of the test.

Statistical analysis of the 48-hour tests was performed by employing a computerized Chi-square test of a two by two contingency table. The program for the test was supplied by the Statistics Department of Western Michigan University. This program provided values for the Chi-square and also for the corrected Chi-square. Since the values being used in the analysis were so small the corrected Chi-square value was used. According to Leabo (1972) when the expected frequencies are too small, the correction factor might be used and this reduces the observed frequencies by one-half. This

has a negligible effect when frequencies are large, but when the cells contain too few frequencies, ignoring the correction factor might lead to excessive rejection of the null hypothesis because the computed Chi-square is overstated. In this statistical analysis, the hypothesis that the concentration of copper sulphate in which the animals were tested determines the extent of mortality in the different groups will be accepted if the corrected Chi-square value is equal to or greater than 3.84. This value is based on 95% confidence level with one degree of freedom. The values for the Chi-square tests and the corrected Chi-square tests are given in Table 7.

It can be seen that in only one concentration, namely the 0.50 Tubifex, was the corrected Chi-square value significant, although the 0.40 Tubifex was very close to being significant. In all of the other concentrations the corrected Chi-square value was lower than the value for significance. In light of this fact and since there was no mortality among the Sphaerium during the 48-hour test it can be concluded that these concentrations of copper sulphate are not clearly demonstrated to be toxic to most of the animals tested under the experimental conditions. The results of the long-term test suggest that there is an effect. Perhaps a large number of replicates would establish toxicity for some concentrations in the 48-hour test.

Another statistical test of the long-term graphs can be performed by observing where the majority of points of the experimental line lie relative to the majority of points of the control line. One would expect a 50-50 chance of values falling either higher or

Table 7. Statistical Analysis of 48-Hour Tests

	Concentration mg CuSO ₄ /l	Chi-Square	2X2 Corrected Chi-Square
<u>Hyalabella azteca</u>	0.50	3.06318	2.13068
	0.40	0.13072	0.00000
	0.30	0.41667	0.10417
	0.20	0.00000	0.12000
	0.10	0.11132	0.00000
<u>Ameletus sparsatus</u>	0.50	0.00000	0.14423
	0.40	1.66667	0.93750
	0.30	1.00186	0.44527
	0.20	0.74074	0.18519
	0.10	0.16160	0.32480
<u>Tubifex tubifex</u>	0.50	15.02222	12.80000
	0.40	5.19231	3.60577
	0.30	2.96296	1.66667
	0.20	0.00000	0.51724
	0.10	4.04313	2.58760

lower than this line and the more lines that are located predominantly higher than the control, the stronger is the correlation that the treatment is affecting mortality. In each of the long-term tests, except for Sphaerium, where results are obscure for reasons stated previously, the experimental groups were consistently located higher than the control group, thus strengthening the correlation. Therefore, statistically the probability of two lines both being located higher than the control would be 0.5×0.5 which equals 0.25. However, in these tests all experimental groups were located higher than (to the left of) the control. The probability of this is 0.5^5 or 0.03125. Thus we can say with confidence that there was a delayed effect on mortality in the experimental group.

This shows that the initial 48-hour exposure to copper sulphate concentrations is responsible for delayed effects resulting in mortality. In addition, the animals which were initially exposed to the higher concentration of toxicant exhibited a more rapid rate of mortality in the long-term tests.

DISCUSSION

Test Conditions

Any laboratory experiment is subject to the criticism that conditions are different from those experienced by the organism in its natural environment. These changes may produce differences in survival, behavior, or physiological responses. But the variations due to laboratory conditions should be uniformly experienced by all test organisms. Differences due to individual variations should be masked by random positioning of test organisms in the experiment. Thus differences that show a systematic or statistically significant correlation with the response of the test species to the experimental variable should be due to that variable, in this case copper sulphate concentration.

The chemical and physical characteristics of the dilution water which were monitored during this bioassay were within acceptable levels as compared to standards set forth by the Aquatic Life Advisory Committee (Cleary, J., 1954). Variations in the physical and chemical characteristics of the water used in this test would not be expected to cause significant effects on the results obtained, because all groups tested, including the control groups would have had the same exposure.

The dissolved oxygen values were determined by using the Winkler method and since levels were always high, the water was probably saturated. The construction design of the continuous-flow device resulted in adequate oxygenation and therefore, artificial oxygenation

was not necessary. According to the Aquatic Life Advisory Committee (Cleary, J., 1954) the dissolved oxygen level for warm-water fish should not fall below 5 ppm. Therefore, the oxygen levels were probably not a critical factor for the invertebrates being tested since levels remained at 8 ppm.

The flow rate was maintained fairly constant throughout the tests to meet the designed purposes of the continuous-flow diluter. These purposes are to provide exposure to a constant concentration of toxicant, to remove waste products and to provide an adequate supply of dissolved oxygen. According to the ratio given by Sprague (1973) the time required for 99% replacement of water in the containers would be approximately one hour.

The pH of the dilution water varied little over the course of the experiments and was within the limits given by the Aquatic Life Advisory Committee. Therefore, it is assumed that pH is not a variable in these tests. The values obtained in these tests were high but these are very close to the pH found in the natural waters of the locality. However, many experimenters have indicated that concentrations of copper salts as well as most other heavy metals are reduced in toxicity at a high pH. Even within the normal pH range (6.0 to 8.0), pH has considerable influence on many poisons (Hynes, 1963 and DuPlessis and DuBurger, 1971). The toxicity of the copper sulphate used in my tests may have been reduced somewhat because the pH value of the dilution water was high.

The influence of temperature on the survival time of organisms in toxic solutions of heavy metals has been investigated by various

experimenters: Hynes (1935), Lloyd (1960, 1965), and Skidmore (1963). The temperature of the dilution water did not change appreciably from the 23° starting temperature during the 48-hour tests. The survival times of the organisms in these tests should not have been influenced by temperature since temperature fluctuations were slight.

Many experimenters have indicated that the toxicity of heavy metal salts is lessened by an increase in hardness and alkalinity of the water. Both total alkalinity and total hardness were quite high during my bioassay tests and this may have significantly reduced the toxicity of copper sulphate to the aquatic invertebrates being tested. Total alkalinity varied little throughout the tests and was not a source of concern. The values of the alkalinity were similar to the alkalinity of the natural waters of this area, therefore, this was not expected to influence the tolerance of the experimental organisms. Both total alkalinity and total hardness were determined by the techniques set forth by the Hach Chemical Company.

The total hardness of the water was the most variable of the parameters measured, with values ranging from 180-210 ppm CaCO_3 . This variation was not expected to have detrimental effects upon the test organisms.

The possible detrimental effects of the sulphate ion was not considered in this test. Since the amount of sulphate used was less than commonly encountered in the natural waters, it was not expected to contribute to toxicity.

Short-term LC_{50} Tests

In copper toxicity tests the LC_{50} value is a common parameter used in comparing acute toxicity among different groups of animals. It is a reliable criterion to use, assuming the experimental conditions are identical.

A thorough review of the literature was made and few reports can be found describing the toxicity of copper sulphate to the aquatic invertebrates used in this study. Therefore, reference to other aquatic organisms which have had studies made of toxicity of copper sulphate are included in this report.

The 48-hour LC_{50} value for Hyalella in my bioassay test was 10.0 mg $CuSO_4 \cdot 5H_2O/l$. In a similar study the 96-hour TL_m value for the amphipod Gammarus pseudolimnaeus was reported as 0.02 mg/l total copper* (0.08 mg $CuSO_4 \cdot 5H_2O/l$) by Arthur and Leonard (1970). The 48-hour LC_{50} for Daphnia magna tested without food was reported as 0.009 mg/l total copper (0.036 mg $CuSO_4 \cdot 5H_2O/l$) by Biesinger and Christensen (1972).

The LC_{50} value determined for Ameletus in this test was 5.0 mg $CuSO_4 \cdot 5H_2O/l$. In a related study Warnick and Bell (1969) reported a 48-hour TL_m of 0.32 mg/l copper for the mayfly Ephemerella subvaria.

When the results of Arthur and Leonard (1970) are compared with the results reported here it appears there is a large difference between Hyalella and Gammarus. But comparison of the early days of

*Ionic concentrations of copper may be converted to copper sulphate concentrations ($CuSO_4 \cdot 5H_2O$) by multiplying by 4.

the long-term tests indicate the test concentrations used were all producing an increased mortality. Thus time duration seems to be a very important factor. The results for both Hyalella and Ameletus seem to demonstrate this. The chitinous cuticular covering common to these forms may be less susceptible to the effects of heavy metals than the protoplasmic membranes of the respiratory surfaces of fish. Thus the effects of copper poisoning on arthropods may be delayed until the internal concentrations reach toxic levels and 48-hour LC_{50} 's may be rather meaningless measures.

It should be noted that the experimental conditions for the studies cited were not always mentioned, therefore, the significance of the differences between the LC_{50} 's might be reduced.

Tubifex was reported to have a LC_{50} of 0.9 mg/l in this test. Fowler and Goodnight (1965) reported that a 1 ppm solution of copper sulphate was not lethal to Tubifex. Tubifex is well known for its adaptation to low dissolved oxygen levels. High levels of oxygen have been reported to cause stress in Tubifex (Walker, 1971), thus the oxygen levels of the test conditions may not have been optimal for Tubifex when general recommendations for bioassay oxygen levels are maintained (Sprague, 1969).

Although the 0.50 concentration had a statistically significant lethal effect in 48 hours, the projection to the LC_{50} is conjectural but undoubtedly is the lowest of the organisms tested. The covering of the arthropods and the behavior of the clams are protective mechanisms not found in Tubifex. Long-term tests indicate Tubifex had a sensitivity in the same range as the arthropods.

Long-term Observations

A significant result of this bioassay is the detection of delayed effects on the test organisms. In every trial, organisms exposed to the copper sulphate concentrations showed a more rapid rate of mortality than the control groups during the long-term tests. There is also a positive correlation showing that those organisms exposed to a higher concentration of toxicant in the short-term test experienced a greater rate of mortality in the long-term tests. These tests suggest that a consistent rise in mortality curves of long-term observations results from exposure to even the lowest concentration of copper sulphate ($0.10 \text{ mg CuSO}_4 \cdot 5\text{H}_2\text{O/l}$).

This same observation was noted by Hubschman (1967) in a study of the crayfish Orconectes rusticus. He showed conclusively that mortality resulted long after initial exposure, thus making it difficult to define levels of acute toxicity accurately.

In these experiments the mortality curves seem to give a clear indication of the toxic effects of all concentrations in the first week of the long-term observations.

The levels producing mortality are less than one-tenth those of the LC_{50} projections. Thus the practice of setting permissible dosages at one-tenth values determined by short-term tests is a dubious one needing much further investigation. Delayed mortality is a significant factor to consider in establishing acceptable limits of copper that could be safely added to the aquatic environment. It

is of little significance to state that an organism can survive a certain concentration of copper for 48 hours if delayed effects result in significant mortality.

SUMMARY

In this continuous-flow bioassay test, copper sulphate concentrations ranging from 0.10 to 0.50 ppm were tested on four aquatic invertebrates, Hyalella azteca, Ameletus sparsatus, Tubifex tubifex, and Sphaerium simile. Forty-eight hour LC₅₀'s were reported based on the initial amount of copper sulphate added to the the test water and long-term delayed effects were investigated. The LC₅₀ values projected for Hyalella azteca, Ameletus sparsatus, and Tubifex tubifex are approximately 10.0, 5.0, and 0.9 mg CuSO₄·5H₂O/l, respectively. Sphaerium simile exhibited no mortality in the 48-hour tests, therefore, no calculation of the LC₅₀ value was possible.

The initial copper sulphate concentrations could not be shown to be statistically significant in toxicity to the experimental organisms other than Tubifex, over the 48-hour exposure period. However, long-term delayed effects conclusively showed that organisms which were exposed to the higher test concentrations experienced a more rapid rate of mortality than those exposed to lower concentrations.

This work indicates that long-term delayed effects must be taken into consideration in setting limits for "safe" levels of copper sulphate addition to natural waters.

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