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The Effects of Sodium Chloride on Hyalella Azteca, Ameletus Sparsatus, and Tubifex Tubifex

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THE EFFECTS OF SODIUM CHLORIDE ON *HYALELLA AZTECA*, *AMELETUS SPARSATUS*, AND *TUBIFEX TUBIFEX*

by

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INTRODUCTION

Various salts are used every day in domestic, industrial, and municipal establishments. Sodium chloride is one salt which is widely used by virtually everyone. It is not only a useful end-product for some establishments, but it is also a by-product and waste-product for others. Whenever anything is as widely used as sodium chloride, problems are bound to arise in the handling, application, and disposal of that substance.

Sodium chloride, although not always thought of as a potential cause of pollution, should not be overlooked as a possible agent in the actual or potential impairment of the quality of natural waters. Domestic, municipal, industrial, and recreational activities could all suffer if sodium chloride was found to impair water quality significantly.

The major sources of salt pollution vary depending on geographical locations, local industrial operations, and other environmental, municipal, and domestic factors. The increasing use of sodium chloride in water softeners is a common and widespread avenue for the passage of sodium chloride into natural waters. In the northern part of the United States deicing salts are used heavily in the winter. This contributes many tons of sodium chloride along with calcium chloride to the waterways. In areas where there are extensive oil well operations, the brine which is a by-product of these operations contains, along with other salts, high concen-

trations of sodium chloride. These, along with other sources of salt polution should not be taken too lightly in man's concerns over water quality. This is compounded by the fact that presently used water treatment methods do not remove sodium chloride from the water.

Various techniques have been used to evaluate the effects that a susnected pollutant will have on conditions in a body of water. Chemichal analysis is useful, but to get any long term information, many tests must be made over long periods of time. The time and cost involved in these studies are often prohibitive.

Observations on the effects that a suspected pollutant has on the organisms that inhabit a body of water have been made in recent years. If a pollutant alters water quality, this alteration is evident in the organisms that inhabit the body of water. A pollutant which is toxic to some organisms will kill them, allowing one or more tolerant organisms to occupy the position in the community previously occupied by the intolerant organisms. This may lead to a decrease in diversity which may result in only a few species occupying an area. Samples of the organisms in an area can be used as an indication of the past history of the area. Macroinvertebrates are often used in these types of studies because many cannot rapidly move from an area of pollution, they are easily collected, and they are important links in freshwater food webs.

This type of thinking has led to tests to determine if substances are toxic to aquatic organisms, and if they are, how toxic. A test in which the quantity or strength of a material is determined by

an organism's reaction to it is referred to as a bioassay. In these tests, organisms are exposed to varying concentrations of some substance for a particular time period and observations are made on mortality and/or other reactions to the substance.

The purpose of this study was to investigate the effects that sodium chloride has on three species of freshwater invertebrates. These were Hyalella azteca, an amphipod, Ameletus sparsatus, a mayfly, and Tubifex tubifex, an oligochaete. The ultimate purpose of all bioassay studies is to predict the response of native populations of aquatic organisms to changes in their environment. It is hoped that the results of this study may add to the information on the effects that discharges of sodium chloride could have on natural waters of lakes and streams.

LITERATURE REVIEW

Salt Pollution

The problem of salt pollution is not new. Since 1955 one can find references to it scattered throughout the scientific literature. In this literature, concern is expressed for the effects this problem might have on aquatic and terrestrial communities.

Early concerns over salt contamination came from the southwestern sectors of the United States. Concern was prompted by the realization that brine, a by-product of oil operations, was being discarded into the environment. Lewelling and Kaplan reported in 1959 that the oil industry was beginning to look at the problems involved in salt disposal. They reported that in 1956, three billion barrels of oil were produced. For every barrel of oil produced, two barrels of salt water were produced. Few efforts were made at this time to dispose of this brine properly. Companies were, however, beginning to consider methods of disposal other than dumping the brine into the environment. The best, but probably most costly method of disposal, was considered to be the pumping of the brine back into a permeable formation which would accept it. Cations in the brine consist of sodium, potassium, magnesium, and calcium. The anions present consist of chloride, sulfate, and bicarbonate ions. Of these, sodium chloride is most common.

The concern of biologists over the effects of oil brines on the environment began somewhat earlier than that of the oil companies.

This concern prompted Clemens and Jones (1955) to test the toxicity of oil well brines on ten species of fish and five species of invertebrates. Their results showed that the species they were testing were killed by concentrations of brine which were equivalent to those in sections of a stream near oil well sites.

Chipman (1959) also tested the tolerance of several species of fish to brine from oil wells. He found concentrations of brine which approached twenty p.p.t. in an impoundment near New Orleans, Louisiana.

Mathias and Dorris (1968), recognizing the possible danger to the environment from oil well brine, studied the community structure of benthic macroinvertebrates in a stream receiving oil field brines. They found that there were some harmful effects from the brines of oil wells, although they concluded that oil field brines are not as harmful to benthic invertebrates as are other domestic and industrial effluents.

More recently, especially in the northern areas of the United States, concern has been growing over the use of deicing salts by cities, counties, and states. According to Bubeck et al. (1971), the use of salt for deicing has increased sharply in the past few decades. Nationally, the increase in salt utilization for deicing has been nearly exponential, with a doubling time of about five years. In 1940, approximately 100,000 metric tons of salt were used for deicing roads. By 1970, the use of deicing salt had risen to 10,000,000 metric tons. Deicing salt consists of both sodium chloride and calcium

chloride, but sodium chloride is the major constituent (Bubeck et al., 1971). Bubeck and his co-workers reported the build-up of high concentrations of sodium chloride from deicing salts in Irondequoit Bay at Rochester, New York. This salt tended to concentrate in the deeper waters of the bay, with which there was little exchange with Lake Ontario because of the shallow channel connecting the bay and the lake. It should be noted that much of the problem of deicing salts stems from the improper storage of salt rather than the application of it (Conner, 1971).

There has been a great deal of controversy in the last few years as to whether or not deicing salts should or should not be used. Some states have proposed legislation against the use of deicing salts. Such legislation was passed in Minnesota, and considered in Massachusetts, Oaklahoma, and Vermont. Other people and places feel that the benefits of deicing salts far outweigh the detrimental effects they may have on the environment (Conner, 1971).

Thorne and Peterson (1967) reviewed the problems of salinity in United States waters. They discussed the sources of salt in Unites States waters. Natural leaching contributes only minimal amounts of salts to waterways. Mineral springs and irrigation also contribute to salt in natural waters, but their contribution is also minor. Thorne and Peterson (1967) attribute the major salt burden of natural waters to urban and industrial use. The use of water softeners by industries, municipalities, and families has caused an increase in sodium chloride in water supplies. Bunch and Ettinger

(1964) studied five Ohio communities that ranged in population from 1,440 to 292,000. After water had passed through the communities once, they found an increase in salts in effluents of 127 to 312 parts per million (p.p.m.). Two of the five cities without water softening had salinity increments of 127 to 188 p.p.m. Three cities with water softening had salinity increments of 231, 281, and 312 p.p.m.

Rebhun (1965) analyzed the salinity increments in municipal sewage and methods of reducing these. The average increment of chloride in Haifa, Israel, was 170 p.p.m. and total dissolved solids were 352 p.p.m. He stated that for the removal of one gram of hardness, water softeners use three to four grams of salt. These salts are then not affected by conventional treatment processes.

A study by Meron and Ludwig (1963) showed that the Los Angeles County water system had a saline increment of 1875 p.p.m. Of this, 640 p.p.m. were attributed to oil brines, 225 were attributed to domestic use, 350 to commercial and industrial use, and 25 to water treatment plants. Sea water intrusion contributed 635 p.p.m. of the total salinity.

Sea water intrusion into coastal aquifers is becoming an ever increasing problem in coastal areas. It results from the rapid removal of freshwater from the ground by high density coastal populations and the resulting movement of sea water in to take its place. This can also occur from river beds. During low flow periods, sea water often moves up the floor of a river. This has happened in the

Hudson, Delaware, Potomac, and Sacramento Rivers as well as others (Thorne and Peterson, 1967).

The cost of salt pollution to society has not been fully assessed. The maximum salt concentration allowable in drinking water before undesirable tastes are found is 250 p.p.m. (Huling and Hollocher, 1972). The removal of salt from water supplies is costly. Electrodialysis costs were estimated by Thorne and Peterson (1967) to range from 20 to 40 cents for each 1,000 gallons of water. Distillation costs were considered to be even higher.

The costs of salt pollution in terms of environmental damage are also not completely known. Although one finds in the literature work on the responses of some organisms to sodium chloride along with other salts, much needs to be done. Hynes (1963), in talking of pollution by non-toxic salts said, "There is, however, as yet very little information available, this being another aspect of pollution which would repay further study." In the ten years since that statement, more work has been done in this area, but more is yet to be completed.

Salt Tolerance of Organisms

Studies of the tolerance of certain organisms to salt are not only valuable with respect to the effects salt pollution might have on aquatic communities, but such studies are also valuable in explaining the distribution of certain freshwater species. For example, if one species occurs in two adjacent river systems which have no interconnections, it would be valuable to find out if the species could have spread from one river basin to the other through

estuaries or the ocean. Kendall and Schwartz (1964) studied the salinity tolerance of two crayfish, Orconectes virilis and Cambarus bartonii. They found that some individuals of these species could withstand salinities up to 33 p.p.t. for up to 180 hours. Another study of this type was done by Hedgpeth (1968). In California the atyid shrimp, Syncares, is found in lowland streams. Hedgpeth concluded that the salt tolerance of this organism was too low for its distribution in California streams to have been by sea migration. Other studies of this type might add to the information on the distribution of species in coastal areas.

Most of the literature describing the reactions of organisms to waters of varying salinities deals with marine organisms. Many articles deal with variations in salinity as it effects the rate of development, reproduction, and distribution of the organisms studied. These studies, although important to the marine biologist, do little toward answering the questions of the freshwater biologist about the effects salts may have on freshwater communities.

There is much less literature on the tolerances of freshwater organisms to sodium chloride and salts in general. Studies of freshwater fish have shown that many species seem to be quite tolerant of salt in their water (Hynes, 1963). Schwartz (1964) studied the salinity tolerance of some fishes. He found 24 species within twelve families which were tolerant of relatively high salt concentrations.

Doudoroff and Katz (1953) reviewed the literature on the toxicity of industrial wastes and their components to fish. In this review

they covered sodium chloride and its effects on fish. They summarized results of 50 researchers working with many species of fish. They listed tolerance ranges from 1.5 to 20 p.p.t. They suggested that the salinity tolerance of fish depends on the efficiency of their mechanisms for osmotic regulation. Fish are, by far, the best studied organisms with respect to salinity tolerance.

Studies by Lomte and Nagabhushanam (1971) revealed that, in the freshwater mussel Parregsia corrugata, oxygen consumption decreased as the sodium chloride concentration was raised from one to seven p.p.t. Other studies with marine organisms (McLusky, 1969 and Hagerman, 1970) have shown that salinity changes may effect rates of respiration in marine organisms.

Much work has been done with the salinity tolerance of the larvae of mosquitoes. Studies by Noaks (1944), Ivanov (1944), Vogt (1947), and Downs (1951) have shown that larval development will not take place in concentrations of sodium chloride above 0.2 normal. Mortality of larvae will occur in concentrations of 0.275 normal sodium chloride.

Another insect which has been studied in relation to its tolerance of saline waters is the caddisfly. Haage (1968) found that freshly deposited eggs will not develop to hatching in salinities above seven p.p.t., but the larvae died soon after emerging. The larvae themselves had about the same tolerance as the freshly deposited eggs. They died in concentrations of seven p.p.t.

Daphnia was studied with respect to its salt tolerance by El'Tzina (1939) and Anderson (1944 and 1946). El'Tzina found that at

five p.p.t. there was a delay in reaching sexual maturity and at 25 p.p.t. death occurred. Anderson (1944) stated that concentrations of sodium chloride above 30 p.p.t. are deleterious to Daphnia, but below 2.1 p.p.t. there were no observable effects. Anderson (1946) also stated that with Daphnia magna sodium chloride is innocuous until the concentration is high enough to exert unfavorable osmotic stress.

The freshwater crab Parotelphusa hydrodromous was studied by Ramamurthi (1967) in relation to its salinity tolerance. He found that this species could exist in a saline medium up to 100 percent sea water. He found that oxygen consumption was greatest in 100 percent sea water and that variations in patterns of metabolic response to salinity were related to the chloride gradient existing between the blood and the medium.

Crayfish have been studied by Kendall and Schwartz (1964) and Barkman (1970). Kendall and Schwartz found that some individuals of Orconectes virilis and Cambarus bartonii could withstand salinities up to 33 p.p.t. for 180 hours before death occurred. Barkman found evidence with Orconectes rusticus that the animal is capable of cellular osmotic regulation when exposed to a medium containing salts in higher concentrations than they are normally found.

The salinity tolerance of a species of amphipod has been studied by Beadle and Cragg (1940). Gammarus pulex was found to tolerate concentrations of sea water up to fifteen p.p.t. for 24 hours. They found a disproportionate increase in tissue chloride as salinity increased, indicating the passage of chloride into cells. Two mechan-

isms were proposed to explain the wide salinity tolerance. One was the regulation of blood chloride concentration and the second was the control of cell to blood concentration gradients. A study by Schmitz et al. (1968) found evidence that indicated salinity tolerance was limited by the osmotic stress placed on Gammarus pulex.

Clemens and Jones (1955) studied the tolerance of four different invertebrates to brine from oil wells. The snail Physa sp. was found to withstand a salt concentration of six p.p.t. and a mayfly of the family Baetidae withstood brine concentrations up to 6.4 p.p.t. Tubifex sp. could withstand concentrations up to 8.5 p.p.t. and the amphipod Hyalella azteca withstood concentrations of brine up to 6.8 p.p.t.

The knowledge of the effects of salts on freshwater inhabitants is far from complete. Much remains to be done to assess the effects of salt, whether it is from road deicing, water softeners, oil wells, or other industries. This information may be applicable to the analysis of the distribution of organisms in coastal areas and their adaptation to saline conditions.

Bioassay Techniques

Early work with bioassay techniques was done by Doudoroff et al. (1951). Attempts to evaluate the toxicity of industrial wastes prompted Doudoroff and his co-workers to study bioassay techniques. They point out that chemical analysis alone is not enough. A toxic component of a waste product can not usually be identified by chemical analysis. Interactions of components of waste are also important

in toxicity studies and can be properly evaluated with bioassay procedures. Doudoroff et al. (1951) published bioassay procedures in an attempt to standardize bioassay methodology. These since have been modified slightly (Sprague, 1973) in attempts at further standardization. It is desirable that results of various workers can be compared with assurance that there are few differences in the results which might be attributed to different methodology.

Sprague (1973) outlined the basic method which has become standard procedure:

(1) a series of test tanks, each with a different but constant concentration of the toxicant;
(2) a group of similar organisms, usually ten, in each container;
(3) observations on mortality that last 48 or 96 hours; and
(4) final results expressed as the concentration which is lethal to the median or average fish.

Two types of assays are in use today. One of these types of assays is referred to as the static bioassay in which the organism is tested in standing water. The second type of test is referred to as the continuous-flow bioassay. In this type of test, the water and the toxic material are continuously flowing through the bioassay container, thus continually renewing the water and the toxic material. The continuous-flow bioassay has certain advantages over the static bioassay, but these advantages do not make the static bioassays inapplicable. In continuously flowing bioassays, there is no need to disturb the test organisms with regular water changes. Also in continuously flowing bioassays, the toxic material is continuously being renewed in the system. The constant addition of a certain concentration of the toxicant keeps the concentration of that material

more nearly constant. In static tests the concentration of the toxic material may fluctuate due to absorption, volatilization, or decomposition.

Brungs (1969) stated that many factors, both experimental and environmental can have significant effects on the results of a bioassay. The quality of the dilution water should be a primary concern for the experimenter. Such things as hardness, pH, dissolved oxygen, and temperature can effect the results of a bioassay. The choice of test organisms is also an important consideration. Various species may demonstrate a range of sensitivity to some compounds of one or two magnitudes. This may be affected by the age, sex, and the condition of the test organisms.

In order to have defined test conditions, steps must be taken to limit the variability among the above factors. These steps include the careful monitoring of the physical and chemical characteristics of the dilution water and the careful selection of the test organisms. The organisms should be randomized during their placement in bioassay containers. Other workers who have made important contributions to this field are Bell and Nebecker (1969), Dimick and Breese (1965), Woelke (1967), Arthur and Leonard (1970), Biesinger and Christensen (1971), Gaufin (1971), Sprague (1969 and 1971), McKim and Benoit (1971), and Eaton (1970).

The advantages in the use of macroinvertebrates in the analysis of the toxicity of various substances has been described many times. Recently Cairns and Dickson (1973) and Weber (1973) have reinforced

these ideas. Benthic macroinvertebrates occupy most trophic levels. They include scavengers, detritus feeders, parasites, grazers, and predators. They are important members of the food chain and their well being is reflected in the well being of organisms such as fish which may occupy higher trophic levels. Many are sensitive to environmental stresses and are, therefore, useful tools in determining the effects of a toxic material on the ecosystem. Their relative immobility, as compared to other forms such as the fishes, means that they can not easily move away from an area of pollution. For these reasons, macroinvertebrates make ideal subjects in pollution analysis.

The Test Organisms

There has been extensive work done with Hyalella azteca. Hyalella azteca is a freshwater amphipod which inhabits most of the permanent bodies of water throughout North America in which the monthly mean temperature exceeds ten degrees centigrade (Mathias, 1971). It inhabits sediment of water up to to two meters deep where the attenuation of light corresponds with a decrease in epibenthic algal production. Hyalella azteca is predominately a deposit feeder. It digests algal and bacterial cells from ingested surface sediment. Chara and its epiphytes are also extensively browsed. It has been reported to feed on freshly killed animal flesh. It is often associated with Anacharis and Myriophyllum (Hargrave, 1970). Hargrave (1971) studied the energy budget of Hyalella azteca. Adult amphipods ingested 0.0525 calories as sediment and microflora per hour. Of this, egested sediment represented a loss of 0.043 calories per hour. Of the calories

assimilated, 49 percent were respired, 39 percent were lost as soluble excretory products and fifteen percent were assimilated as growth, egg materials and moults.

Cooper (1965) studied a population of Hyalella azteca in Sugarloaf Lake, Michigan. He found that fish depend heavily on this amphipod. Of the yellow perch he examined, 99 percent contained Hyalella azteca in their stomachs, with an average of 33.6 ± 4.03 amphipods per stomach. Other fish, including bluegills, were found to feed on Hyalella azteca, but not as frequently as the yellow perch.

Hyalella azteca has been used in other studies of the toxicity of various substances. Bovee (1949) studied the thermal tolerance of Hyalella azteca. It survived from 39,000 minutes to one second over a temperature range of 33 to 50 degrees centigrade.

Clemens and Jones (1955) studied the toxicity of brine from oil wells to various invertebrates and fishes. They found that a concentration of 6.8 p.p.t. was toxic to 50 percent of the individuals of Hyalella azteca over a 96-hour time period.

Another group of invertebrates which were selected for the tests is represented by the species Tubifex tubifex. This group belongs to the freshwater oligochaetes and is considered to show a greater degree of tolerance to many organic materials than do other aquatic macroinvertebrates (Whitten and Goodnight, 1966). A great deal of work has been done on the tolerance of these organisms.

Their classification, physiology, life cycles, and ecology are reviewed by Brinkhurst and Jamieson (1971). Tubificid worms feed on the bottom mud. At depths below one meter the tubificids may be the

dominant form of life. The most concentrated populations are found in bodies of water receiving organic pollution. Most of the true aquatic species are able to thrive in low concentrations of dissolved oxygen. At low oxygen concentrations the familiar waving motion of the worms provide it with oxygen.

Sygnamic reproduction in the family Tubificidae is similar to that in the familiar earthworm. Cocoons containing embryos are deposited on rocks, vegetation, and other debris. Regeneration of lost parts is well developed in this family.

Studies of the tolerance of Tubifex to various substances have been carried out by many workers. Studies of the oligochaetes tolerance of insecticides (Whitten and Goodnight, 1966 and Naqvi, 1973) have shown that Tubifex is more tolerant of insecticides than other macrofauna are. Tubifex was not harmed at concentrations below 100 p.p.m. DDT (Whitten and Goodnight, 1966). In testing the tolerance of Tubifex to fifteen insecticides, maximum concentrations ranging from 0.05 to four p.p.m. failed to cause mortality in 72 hours (Naqvi, 1973).

The effects of heavy metals on Tubifex have also been studied (Whitley and Sikora, 1970). Lead in concentrations of ten to 60 p.p.m. inhibited respiratory processes. No clear cut effects were seen with nickel in concentrations of zero to 60 p.p.m.

A few studies of the salinity tolerance of Tubifex have been made. Styczynska (1972) reported that in sea water surpassing the normal osmotic concentration of the body fluids, egg laying decreases very rapidly and embryonic development stopped. The resistance of

adult animals was higher. The mortality rate was higher than that in the control between five and eight p.p.t.

Clemens and Jones (1955) found brine from oil wells at concentrations of 8.5 p.p.t. was lethal to one-half of the Tubifex tested over a 96-hour period.

Palmer (1968) reported that ten percent sea water (approximately 3.5 p.p.t.) was the highest salinity tolerated by Tubifex tubifex without gradual acclimatization. Above this concentration worm deaths occurred in 24 hours.

Walker (1971) worked with oxygen poisoning in Tubifex. She found that concentrations of sodium chloride between 0.02 molar and 0.05 molar gave protection against oxygen poisoning. This protection was seen if exposure to sodium chloride occurred during or after oxygen exposure.

The third test organism comes from the Ephemeroptera. Work with the mayflies in the area of pollution tolerance has been less than extensive. Work with the genus Ameletus has been almost non-existant in this field. Citations of the tolerance of this group will, therefore, have to be on the Ephemeroptera as a whole.

Gledhill (1958) and Larsen (1968) reported that Ameletus has a one-year life cycle. Larvae emerge from May to August, while hatching of eggs goes from September to February. Ameletus inhabits still or slowly moving water, clambering on green vegetation. (Needham et al., 1969).

Studies of the mayfly tolerance to various algicidal and insecticidal poisons have been made by Wilson and Bond (1969), Macek et al.,

(1972) and Smith (1967). These studies have shown that the mayflies being tested were sensitive to the poisons. In some cases they were found to be more sensitive than other macroinvertebrates.

Studies of the effects of low oxygen concentrations on mayflies have been a major concern for many researchers. Britt (1955), Eriksen (1968), and Nebecker (1972) all studied the effects of low oxygen concentrations on the genus Hexagenia. It was found that this genus must have at least 1.4 p.p.m. of oxygen for normal activity. Substantial reductions in the population of this genus have been reported by Britt (1955) in Lake Erie where oxygen concentrations got as low as 0.70 p.p.m. during summer stagnation.

The toxicity of some heavy metals to aquatic insects was studied by Warnick and Bell (1969). They found that the mayfly Ephemerella subvaria was the insect most sensitive to all metals. Copper was the most toxic to it. It had a 48-hour LC_{50} of 0.32 p.p.m. The notation "LC_{50}" refers to the concentration of a toxicant which kills 50 percent of the test organisms over a specified time period. It is used as a convenient reference point for expressing acute lethal toxicity of a given pollutant to the average or typical organism. The actual safe concentration would be much lower.

Wurtz (1969) studied the effects of heated discharges on freshwater benthos. He found that mayflies were the least tolerant of the organisms selected. The mayflies were not identified.

Kreis and Johnson (1968) studied the response of the macrobenthos to irrigation return water. Irrigation return water carries with it salts, nutrients, silt, and sometimes pesticides. He reported that

a stream receiving this water showed a decrease in the number of mayflies, caddisflies, scuds, and beetle larvae, and an increase in the tubificids present. These were not identified further. The effects of the individual components were not tested, but it was stated that they should be.

Clemens and Jones (1955) found a 96-hour LC_{50} of 6.45 p.p.t. for a mayfly of the family Baetidae for brine from oil wells. Ameletus is a member of this family.

Many other studies have been done with mayflies. Studies of their response to photoperiod, temperature, and oxygen concentration try to gather information on their emergence stimuli. There are relatively fewer studies of this group's tolerance of various toxic materials. This is true with salts, including sodium chloride. Also, relatively little work has been done with the genus Ameletus. Work in this area will add significantly to the knowledge of the Ephemeroptera.

MATERIALS AND METHODS

For determining the effects of sodium chloride on the organisms selected for the tests described here, a bioassay unit was constructed. This bioassay unit is a continuous-flow diluter which was modified from that described by Zillich (1972). He described the use of a continuous-flow diluter by The State of Michigan for monitoring industrial effluents. Modifications of that diluter were made to keep the cost of the apparatus within the budget of this study, to achieve a constant rate of flow in all bioassay containers, and to keep the size of the apparatus within the limits of available laboratory space.

Bioassay containers were constructed from one liter plastic containers which had an internal diameter of eleven centimeters. Standpipes were constructed from eight millimeter internal diameter glass tubing. They were fitted with number three rubber stoppers and inserted into a two centimeter hole drilled in the bottom of each container. This regulated the amount of water in the bioassay containers. The amount of water in the containers in this study was 800 milliliters. Holes were drilled in the lids of the containers to receive the incoming water and to facilitate the taking of water samples. Figure 1 diagrams the bioassay containers in this study. The organisms used were placed inside the bioassay containers in small glass dishes covered with nylon netting.

Concentrations of sodium chloride ranged from 11.2 p.p.t. to zero p.p.t. The diluter was designed to deliver concentrations in

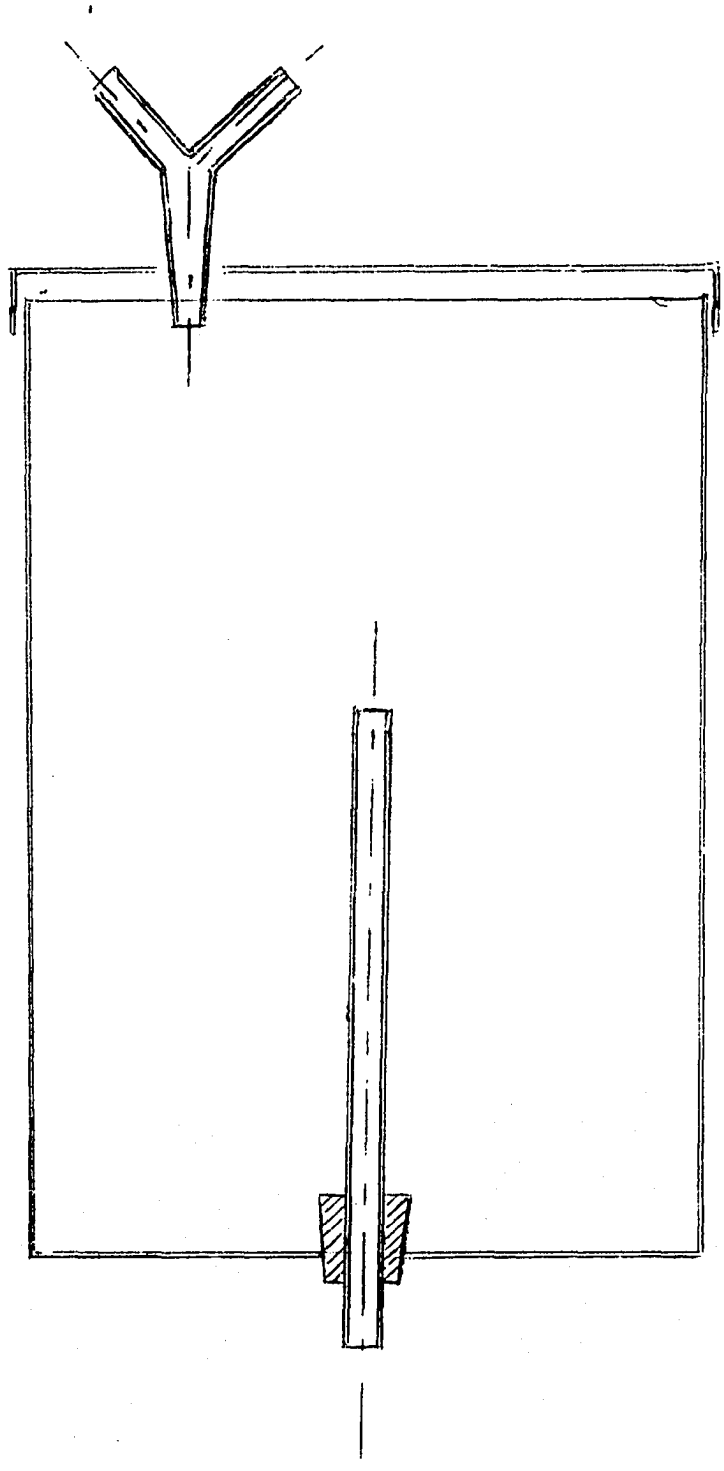

Figure 1. Bioassay containers. One liter plastic containers with a standpipe and inlet from the dilution chambers.

intervals of twenty percent of the stock solution. The proper dilutions were obtained by drilling holes in the bottom of plexiglass tanks and inserting one millimeter internal diameter glass tubing. Two tanks or dilution chambers were used, one received water from the stock solution and the other received dilution water. Water from the stock solution passing through five one millimeter glass tubes delivered 100 percent stock solution. Water from four glass tubes of the stock solution and one glass tube from the dilution water delivered an 80 percent concentration of the stock solution. Three parts of the stock solution and two parts of the dilution water were combined to give a 60 percent concentration of the stock solution. Fourty, twenty, and zero percent concentrations of the stock solution were obtained in a similar manner. Constant flow rates were obtained by having the glass tubes exactly the same length below the plexiglass tanks, maintaining the same water level in both tanks and having water from a total of five glass tubes entering each bioassay container. Figure 2 shows a diluting chamber. Water was collected by funnels secured below the dilution chambers. Plastic tubing carried each solution to the proper bioassay container positioned below the dilution chambers. Intermediate concentrations were mixed at a "Y" connection before entering a bioassay container.

The stock solution and dilution water were held in two 55-gallon barrels which were set on a platform above the dilution chambers. Barrels were lined with fiberglass resin to prevent metal-water contact. Twelve millimeter internal diameter plastic pipe, inserted in the bottom of the barrels and through the platform, fed water into

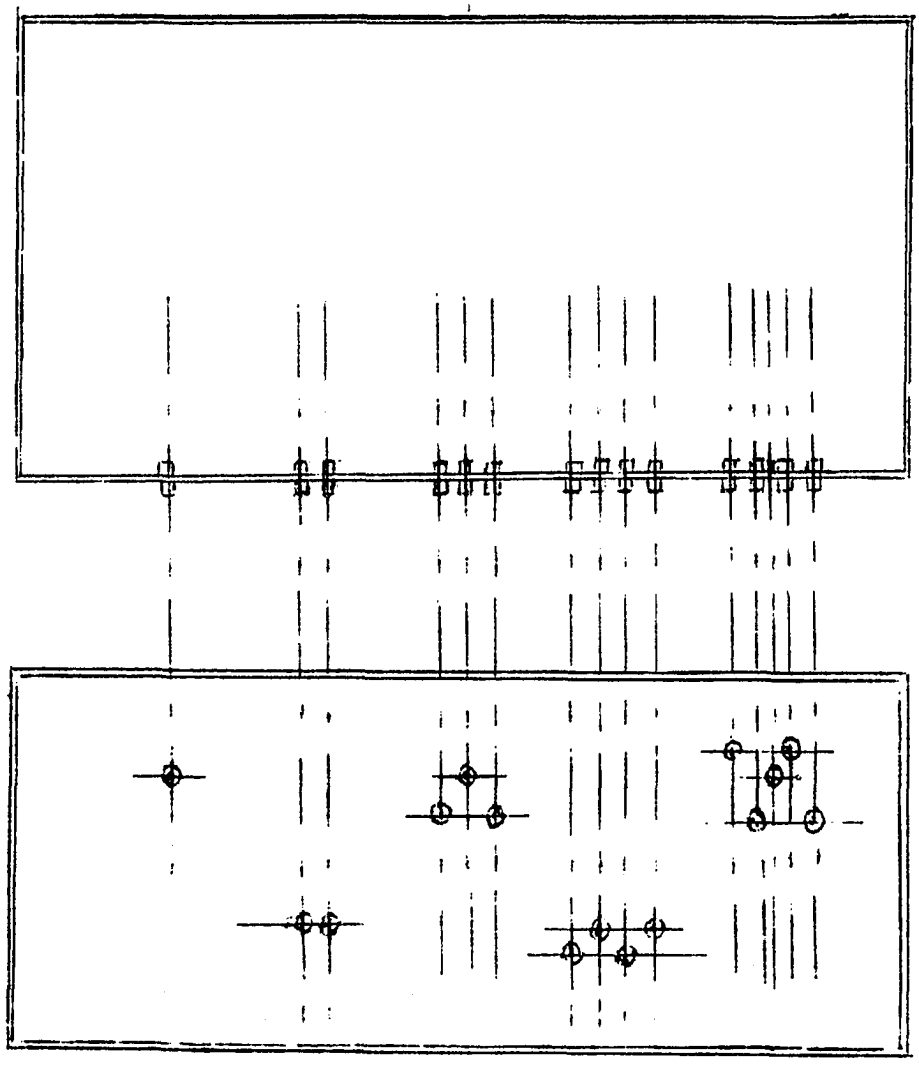

Figure 2. Dilution chambers. One millimeter internal diameter glass tubing in 46 cm. x 24 cm. x 24 cm. plexiglass tanks.

the dilution chambers. The water level in the dilution chambers was regulated by toilet floats fitted with a valve which pressed against the outlet of the plastic pipe. Toilet floats were also coated with fiberglass resin.

Barrels similar to those used for the delivery of the dilution water and the stock solution were lined with fiberglass resin and used for mixing the sodium chloride solution and holding the dilution water prior to their use. When these solutions were needed a submersible pump with plastic impeller was used to pump the dilution water and stock solution into the delivery barrels.

Bioassay containers were placed on a shelf below the dilution chambers. At the time of construction, the positioning of the bioassay containers that were to receive a particular dilution, was randomized so that any position effects would be randomized. These positions were then maintained throughout the tests.

A fluorescent light secured above and to the rear of the bioassay containers illuminated the organisms being studied. This was connected to a timer which was set to turn on and off at twelve hour intervals. This photoperiod approximated the outside photoperiod at the time the tests were made. Figure 3 shows the entire diluter as it was set up in the laboratory.

The sodium chloride used in the tests was processed by the American Salt Company*. It was sold as compacted water softener salt and contained no additives.

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*Mention of products does not constitute an endorsement.

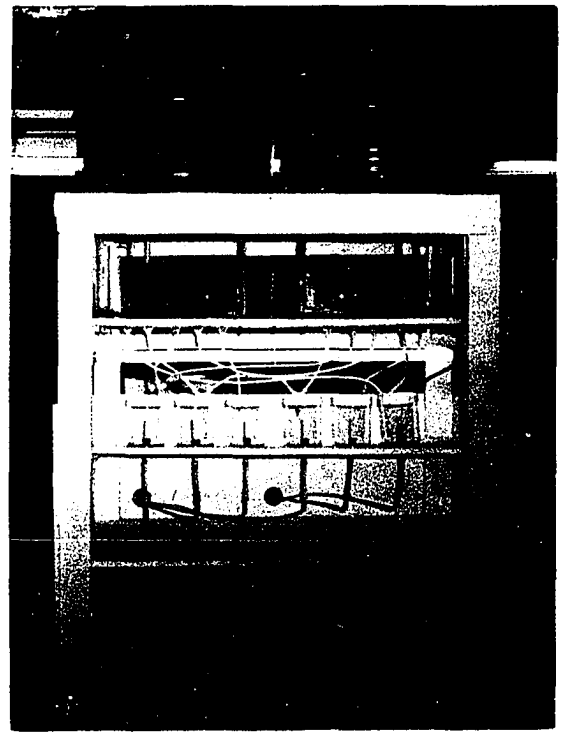

Figure 3. The bioassay unit used in this study.

All organisms used in the tests were collected in the Kalamazoo, Michigan area. The Tubifex tubifex were collected from Portage Creek at the point it crosses Lake Street in Kalamazoo. They were then brought back to the laboratory and maintained as described by Pennak (1953). This consisted of holding them in a pan with dechlorinated water running through the pan. These tubificids were used for all tests. Amphipods were collected from the West Branch of Portage Creek, at the corner of O. N. Avenue and Twelfth Street in Kalamazoo. Collections were made four days prior to each test. Mayflies were collected at the outlet of Limekiln Lake on Brook Drive in Kalamazoo. Collections were again made four days prior to each test.

To insure that the organisms collected were not exposed to high levels of sodium chloride at the time of collection, tests were made of the sodium chloride concentration at the collection sites. Sodium chloride tests were made throughout the experiment with the Argentometric Method of analysis as described in Standard Methods for the Examination of Water and Wastewater, published by the American Public Health Association (1971).

Preliminary static water tests were made to determine the concentration of sodium chloride to use in the tests with the bioassay unit. These were 48-hour tests carried out in flasks, and were made prior to the first run. They were started on December 30, 1973, and January 7, 1974. These consisted of placing organisms in concentrations of sodium chloride ranging from zero to 30 p.p.t. It was decided to use ten p.p.t. as the highest concentration because it was the lowest concentration where there was 100 percent mortality.

Four days before each test the organisms were placed in the dilution water which was to be used in tests. This consisted of tap water which was dechlorinated with sodium thiosulfate. This was designated as the acclimation period. The use of dechlorinated tap water as dilution water has been established by Zillich (1972), and Clemens and Jones (1955). Twenty-four hours before each test, 60 individuals of each of the three species were placed in dishes, ten in each dish. They were placed in dishes randomly with the use of a table of random numbers. Twenty-four hours later the test was started.

Fourty-eight hour exposure periods were used to test the effects of sodium chloride on the invertebrates selected. The placement of dishes in the bioassay containers was randomized with a table of random numbers. The observation periods used in this experiment were those recommended by Sprague (1973). Observations of mortality followed a logarithmic time schedule. A great deal of information is lost if frequent early observations are not made. The observations approximate a logarithmic series in keeping with the apparent logarithmic nature of biological time. For example, an invertebrate exposed to a pollutant for one minute, doubles its exposure time after two minutes; however, after an exposure of one hour, another minute would be insignificant. It would take another hour to double the exposure period. The periods of observation are given below:

minutes = 15, 30, and 60
hours = 2, 4, 8, 14, 24, 33, 48.

Records were made of the numbers of each organism that died within a certain time interval.

At the end of 48 hours, the organisms were removed from the bioassay containers. They were counted, and the dead individuals were removed and preserved in 70 percent ethanol. Live organisms were then placed in aquaria in the same dishes they were held in during the 48-hour exposure period. This began the long-term observations on recovery or mortality of the test organisms. Test organisms were counted daily and those that died were preserved in 70 percent ethanol. Water was changed after the first day and every five days thereafter. The first day was designated as the reverse acclimation period because during this first day mortality may be due to sudden changes in osmotic pressure that resulted from the transfer of the organisms to the aquaria. Data was recorded at the start of day one and daily thereafter for a period of 21 days. A total of three runs was made between March 11, 1974, and April 18, 1974.

Using the zero p.p.t. group as the control group, statistical analysis of the data was used to determine where mortality was statistically different from that in the control group. A corrected Chi-square test was used to test the significance of mortality. A corrected Chi-square test was used because of the small numbers in the expected cells of the two by two contingency table. Using an uncorrected Chi-square, there would be a tendency to reject the hypothesis that mortality in some concentration is not significantly different from that in the control when the hypothesis is true. This correction factor is referred to as the "Yates's correction" (Leabo, 1972). The

small number of deaths in the control group makes this correction necessary.

The Chi-square analysis was carried out with the aid of the computer center of Western Michigan University. Library program number 1.1.3 was used in this analysis. Only data for the 48-hour period was analyzed this way.

The usual method of reporting the results of toxicity studies is with the use of LC_{50} values. They were determined as outlined by Sprague (1971).

Physical and chemical parameters were measured throughout the tests. Alkalinity and hardness were measured each time new solutions were mixed up (approximately once every eight hours). These were measured with the chemicals and procedures of the Hach Chemical Company. Dissolved oxygen was monitored regularly by the Winkler Method of analysis. The pH was monitored with a phenol red indicator. Temperature was monitored with a mercury thermometer, and flow rate was monitored by the timing of the filling of a flask. Chlorine was monitored with orthotolidine to insure that all had been removed by the sodium thiosulfate. Sodium chloride was monitored throughout test periods. At the time of the mixing of each solution, a test of the sodium chloride was made to insure that the solution was at the proper concentration. Twice during each test, samples were taken from each bioassay container to determine the concentration of sodium chloride in each container. In addition to this, each dilution was monitored with the use of a hydrometer to insure that the proper concentrations were present. This was done six times during each run.

RESULTS

Prior to the collection of the organisms used in these tests, an analysis of the chloride concentration at the collection sites was performed. Individuals of Ameletus sparsatus were collected from water which had a chloride content of 29 p.p.m. Individuals of Hyalella azteca were collected from a concentration of chloride of 4.01 p.p.m. Individuals of Tubifex tubifex were collected from water which had a concentration of 41 p.p.m. These concentrations are normal for freshwater and are not considered to be high enough to cause acclimation to chloride on the part of the organisms used.

Chemical data from the tests made during the three runs are listed in Table 1. Hardness varied from 130 to 200 p.p.m. calcium carbonate. Alkalinity varied from 180 to 250 p.p.m. calcium carbonate. Temperature varied from 23 to 25 degrees centigrade. Dissolved oxygen varied from 6.5 to 8 p.p.m. The pH varied from 8 to 8.2, with 8.2 being most common. At no time was any chlorine present in the water running through the bioassay containers.

Table 2 lists the rate of flow through all bioassay containers. Flow rates ranged from 7.031 liters per hour in the twenty percent dilution chamber to 7.380 liters per hour in the 80 percent dilution chamber. This represents a deviation of 4.8 percent from the mean flow rate of 7.205 liters per hour.

Table 3 lists the data on the concentration of sodium chloride in the six bioassay containers. The mean concentration, relative

Table 1. Chemical and physical parameters measured.

Hardness (ppm $CaCO_3$)	Alkalinity	Temp. °C	Dissolved oxygen (ppm)	pH	Chlorine
200	250	25	8	8	0
200	250	25	6.5	8.1	0
180	250	25	6.5	8.2	0
180	240	25	6.5	8	0
180	240	25	7	8.2	0
200	250	25	7	8.2	0
180	250	24	7	8.2	0
190	250	24	7	8.2	0
190	250	24	7	8.2	0
150	230	24	7	8.2	0
180	220	24	7	8.2	0
170	250	24	7	8.2	0
140	180	23	8	8.2	0
150	250	23	8	8.2	0
160	250	23	8	8.2	0
180	210	23	8	8.2	0
160	250	23	8	8.2	0
130	210	23	8	8.2	0

Table 2. Rate of flow through each bioassay container.

	Concentration of sodium chloride (parts per thousand)					
	0	2.32	4.2	6.84	8.9	11.2
Flow rate (ml/sec.)	1.95	2.14	2.14	2.14	1.8	2.01
	1.97	2.0	1.97	1.97	1.95	2.05
	2.03	2.03	2.03	2.07	1.97	2.07
	2.03	2.03	2.05	1.97	1.97	2.02
	1.97	2.05	2.05	2.03	2.07	2.08
	1.97	2.03	2.04	2.03	1.95	2.05
mean	1.973	2.050	2.047	2.036	1.953	2.045
mean (l/hr.)	7.103	7.380	7.369	7.320	7.031	7.362
relative error	4%	7%	8%	8%	14%	3%

Table 3. The concentrations of sodium chloride delivered from each dilution

Theoretical dilution (percent)	0	20	40	60	80	100
Concentration of sodium chloride (p.p.t.)	0	2.3	4.0	6.6	8.83	11.4
	0	2.31	4.3	6.61	8.85	11.4
	0	2.28	4.0	6.45	9.35	11.0
	0	2.4	4.2	6.8	8.6	11.0
	0	2.3	4.1	7.0	9.0	11.3
	0	2.35	4.4	7.1	9.0	11.2
	0	3.35	4.4	7.2	9.1	11.2
Total	0	16.29	29.4	46.8	62.43	78.5
Mean	0	2.23	4.2	6.84	8.9	11.2
Relative error (percent)	0	3.45	4.7	2.34	4.5	1.8
Actual dilution (percent)	0	21.2	37.5	61	79.5	100

error, and actual percent dilutions are listed. The relative error of the Argentometric Method of analysis of sodium chloride is 1.7 percent.

Tables 4 through 6 list the numbers and percent dying for the three species tested. Data is recorded as the number and percent dying since the start of the run. There was a total of 30 of each organism in each of the test concentrations. There was no mortality in the first fifteen minutes of the tests. After 30 minutes mortality began to appear in the higher concentration in the tubificids (Table 6). At two hours, mortality had begun in the amphipods (Table 4) and mayflies (Table 5) that were in the higher concentrations. At this time all the tubificids had died in the highest (11.2 p.p.t.) concentration. Mortality gradually increased with time, but it increased more slowly in the lower concentrations.

Table 7 lists the Chi-square values from the tests. The 0.05 level (Chi-square = 3.84) was chosen for rejection of the null hypothesis. A total of fifteen Chi-square tests were performed.

Table 7 shows that mortality for Hyalella azteca in the 2.32 and 4.2 p.p.t. concentration was not statistically different from that in the control group. Mortality of Hyalella azteca in concentrations of 6.84, 8.9, and 11.2 p.p.t. sodium chloride was significantly higher than that in the control group.

Mortality of Ameletus sparsatus was not significantly different from the control group in the 2.32, 4.2, and the 6.84 p.p.t. concentrations. Mortality was significantly higher than that of the control group in the 8.9 and 11.2 p.p.t concentrations of sodium chloride.

Table 4. Cumulative number and percentage of Hyalella azteca dying over a 48-hour period. There were a total of 30 individuals in each concentration.

Time (min)	Concentration of sodium chloride (p.p.t.)						
	0.0	2.32	4.2	6.84	8.9	11.2	
0	0	0	0	0	0	0	Number dying
	0	0	0	0	0	0	Percent dying
15	0	0	0	0	0	0	
	0	0	0	0	0	0	
30	0	0	0	0	0	0	
	0	0	0	0	0	0	
60	0	0	0	0	0	2	
	0	0	0	0	0	6.6	
120	0	0	0	0	0	4	
	0	0	0	0	0	13.3	
240	1	6	0	3	5	10	
	3.3	19.9	0	9.9	16.6	33.3	
480	1	6	2	4	10	18	
	3.3	19.9	6.6	13.3	33.3	60	
840	2	6	2	8	18	23	
	6.6	19.9	6.6	26.3	60	76.6	
1440	3	9	5	13	27	27	
	9.9	29.9	16.6	43.3	89.9	89.9	
1980	3	10	5	17	27	29	
	9.9	33.3	16.6	56.6	89.9	96.6	
2880	4	11	5	23	28	29	
	13.3	36.6	16.6	76.6	93.3	96.6	

Table 5. Cumulative number and percentage of Ameletus sparsatus dying over a 48-hour period. There were a total of 30 individuals in each concentration.

Time (min)	Concentration of sodium chloride (p.p.t.)						
	0	2.32	4.2	6.84	8.9	11.2	
0	0	0	0	0	0	0	Number dying
	0	0	0	0	0	0	Percent dying
15	0	0	0	0	0	0	
	0	0	0	0	0	0	
30	0	0	0	0	0	0	
	0	0	0	0	0	0	
60	0	0	0	0	0	0	
	0	0	0	0	0	0	
120	0	0	0	1	2	0	
	0	0	0	3.3	6.6	0	
240	1	2	0	1	2	1	
	3.3	6.6	0	3.3	6.6	3.3	
480	1	3	1	1	3	7	
	3.3	9.9	3.3	3.3	9.9	23.3	
840	1	5	1	9	11	13	
	3.3	16.6	3.3	29.9	36.6	43.3	
1440	2	6	2	10	18	20	
	6.6	19.9	6.6	33.3	60	66.6	
1980	4	6	4	10	18	25	
	13.3	19.9	13.3	33.3	60	83.2	
2880	9	7	5	10	24	30	
	29.9	23.3	16.6	33.3	73.3	100	

Table 6. Cumulative number and percentage of Tubifex tubifex dying over a 48-hour period. There were a total of 30 individuals in each concentration.

Time (min)	Concentration of sodium chloride (p.p.t.)						
	0	2.32	4.2	6.84	8.9	11.2	
0	0	0	0	0	0	0	Number dying
	0	0	0	0	0	0	Percent dying
15	0	0	0	0	0	0	
	0	0	0	0	0	0	
30	0	0	0	0	1	4	
	0	0	0	0	3.3	13.3	
60	0	0	0	0	2	11	
	0	0	0	0	6.6	36.6	
120	0	0	0	2	14	30	
	0	0	0	6.6	46.6	100	
240	0	0	1	6	17	30	
	0	0	3.3	19.9	56.6	100	
480	0	0	1	7	19	30	
	0	0	3.3	23.3	63.3	100	
840	1	0	1	10	19	30	
	3.3	0	3.3	33.3	63.3	100	
1440	1	0	2	10	19	30	
	3.3	0	6.6	33.3	63.3	100	
1980	1	0	2	11	19	30	
	3.3	0	6.6	36.6	63.3	100	
2880	2	1	3	11	19	30	
	6.6	3.3	9.9	36.6	63.3	100	

Table 7. Corrected Chi-square values for the 48-hour time period. Values greater than 3.84 indicate a significant difference between the experimental and control mortality at the 0.05 level.

Organism	Concentration of sodium chloride				
	2.32	4.2	6.84	8.9	11.2
Hyalella azteca	3.2	0.0	21.81	35.42	38.79
Ameletus sparsatus	0.85	.838	0.00	9.0	29.3
Tubifex tubifex	0.00	0.00	6.28	18.75	48.8

Mortality of Tubifex tubifex in the 2.32 and 4.2 p.p.t concentrations was not significantly higher than mortality in the control group. Mortality in the 6.84, 8.9, and 11.2 p.p.t. concentrations of sodium chloride was significantly higher than mortality in the control group of Tubifex tubifex.

In determining the LC_{50} values, the relatively high mortality in the control groups, especially for Ameletus sparsatus, suggests that the percentage dying in each concentration should be corrected for death which occurred due to non-experimental causes. A nomograph was used to determine a correction factor. Percent mortality in the control is plotted against percent mortality in the experimental groups. Lines for control mortality are drawn. The correction factor is read from the intersection of experimental mortality with the line for control mortality. This correction factor is subtracted from the actual percentage dying. In cases where experimental mortality was less than control mortality, the higher value was used to determine the correction factor. Figure 4 shows the nomograph used.

Figures 5 through 7 show the 48-hour LC_{50} for the three species. The percentage of each organism killed is plotted on a probability scale on the "Y" axis, and the concentration of sodium chloride is plotted on a logarithmic scale on the "X" axis. The best fitting straight line is drawn through the points and the LC_{50} is read from where the line intersects the 50 percent mortality level. This type of analysis is similar to that recommended by Sprague (1973). A concentration of about 5.2 p.p.t. of sodium chloride was required

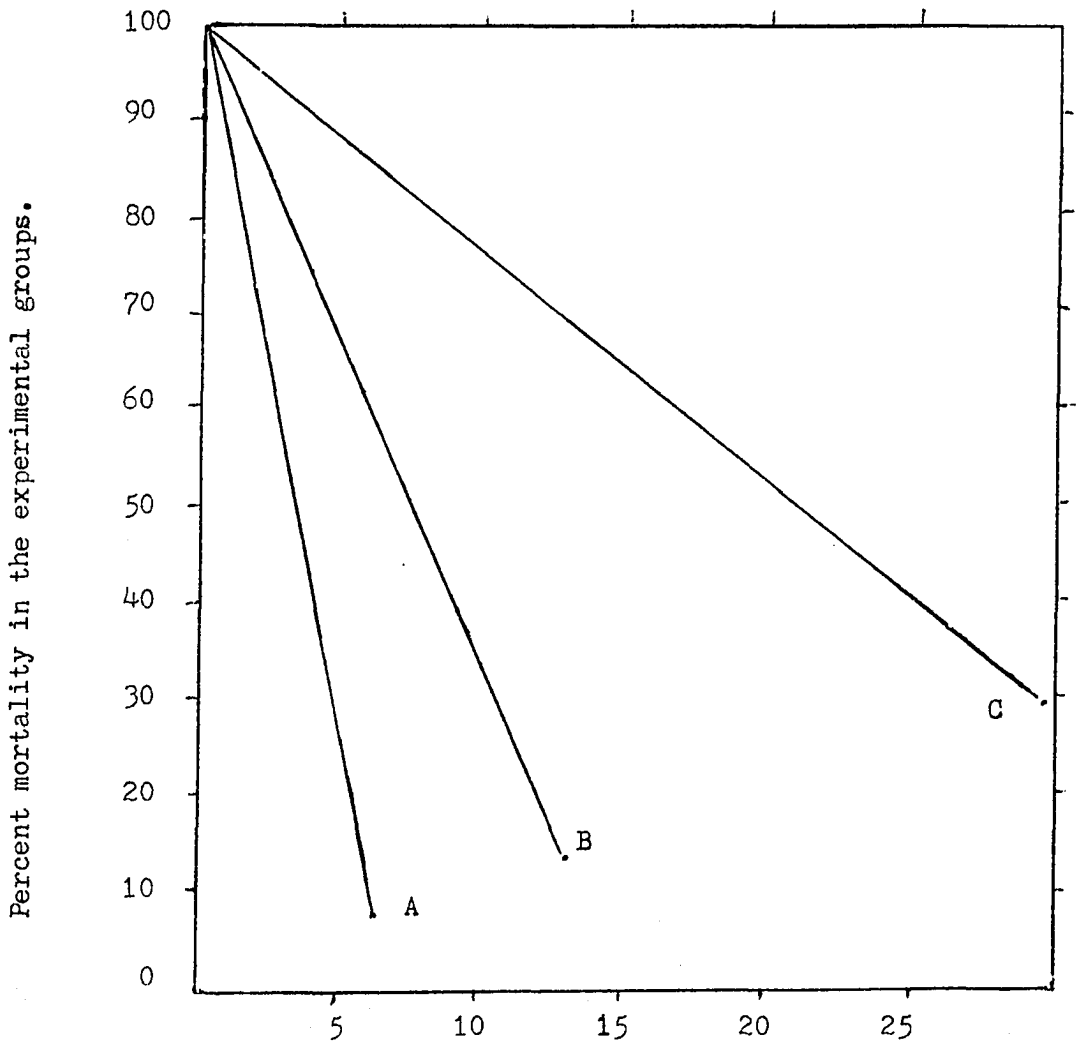

Figure 4. Nomograph used in determining the correction factor for LC_{50} determinations. A. Control mortality for Tubifex tubifex. B. Control mortality for Hyalella azteca. C. Control mortality for Ameletus sparsatus.

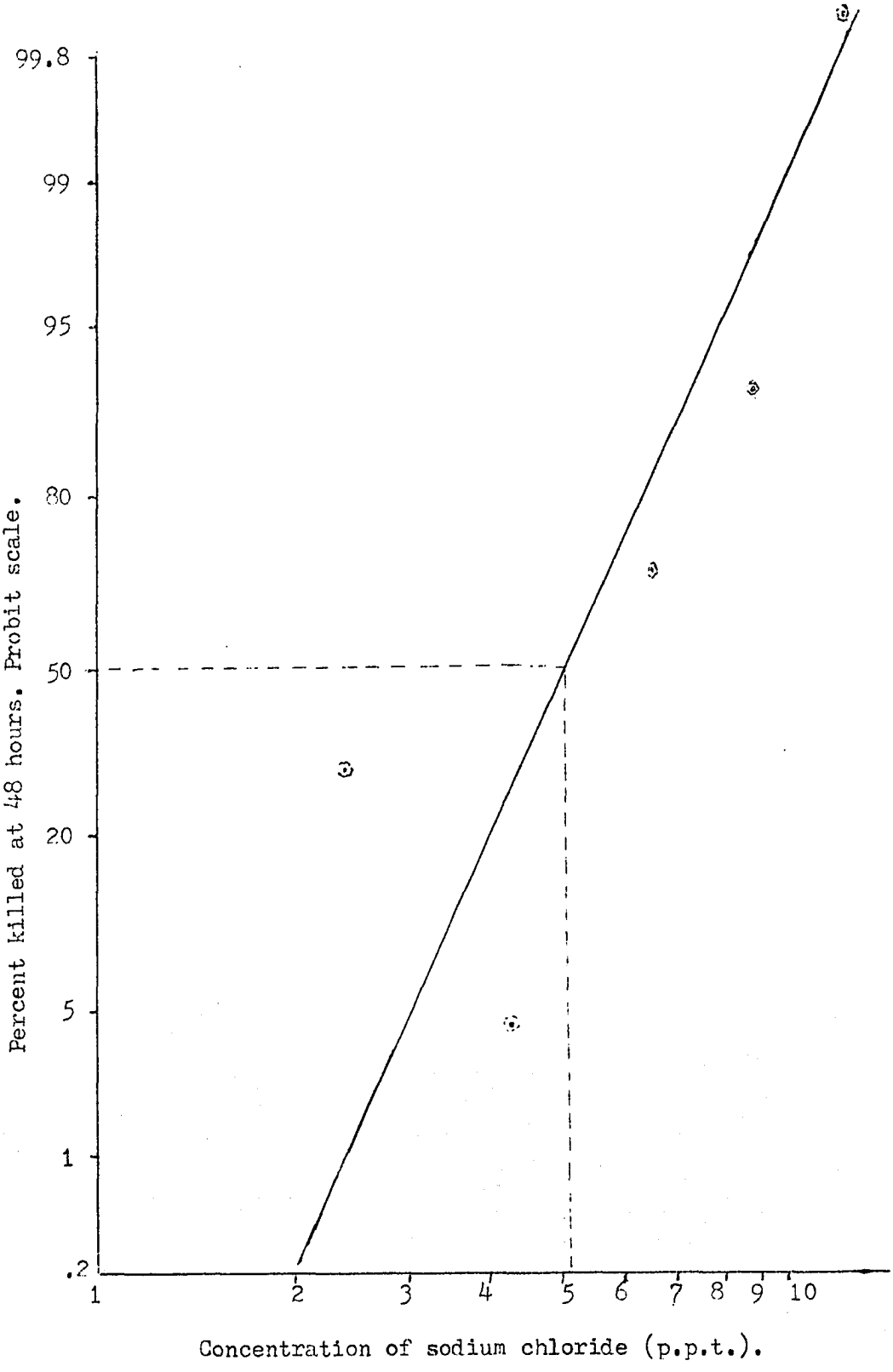

Figure 5. Fourty-eight hour LC_{50} for Hyalella azteca.

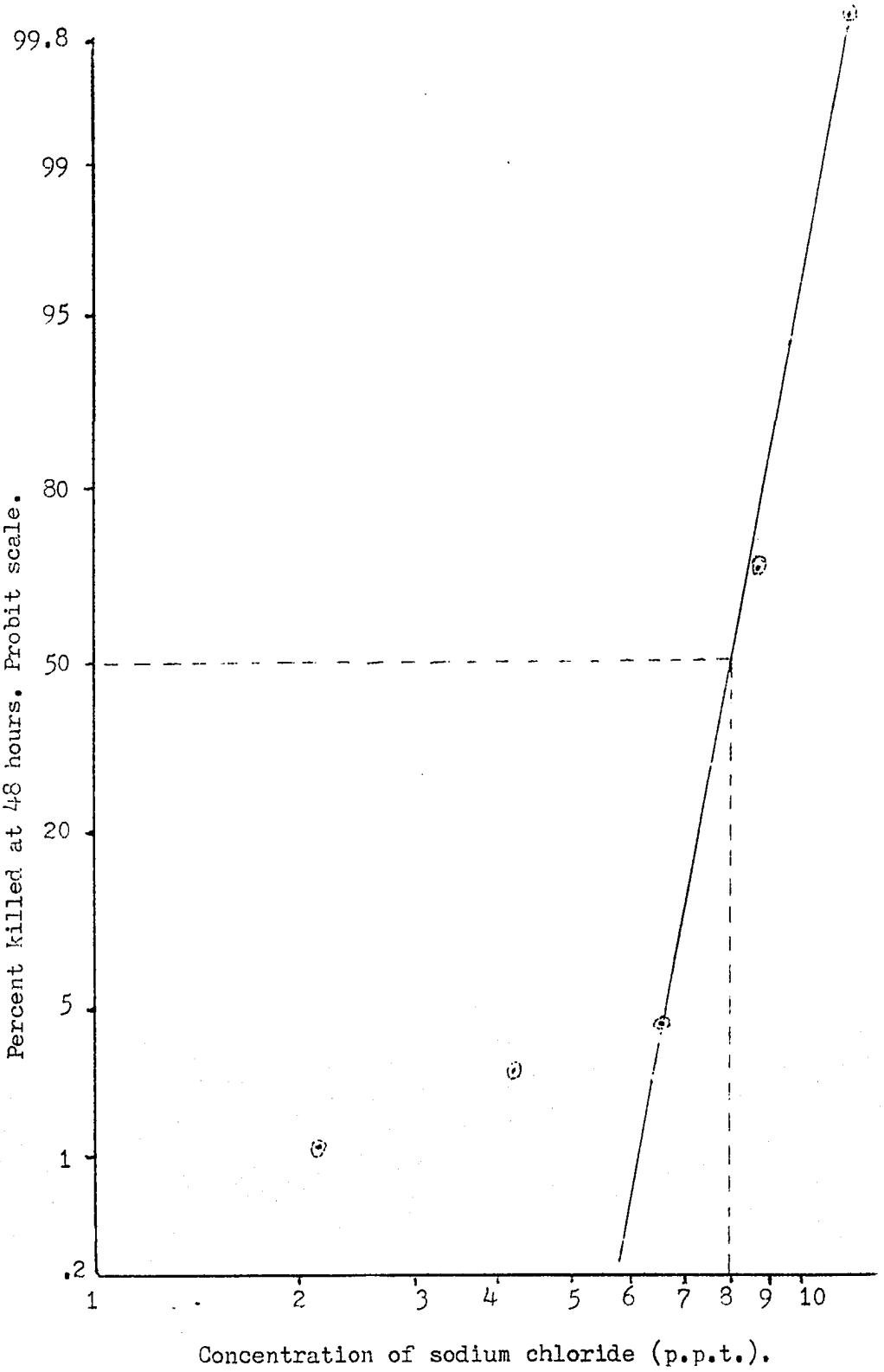

Figure 6. Fourty-eight hour LC_{50} for Ameletus sparsatus.

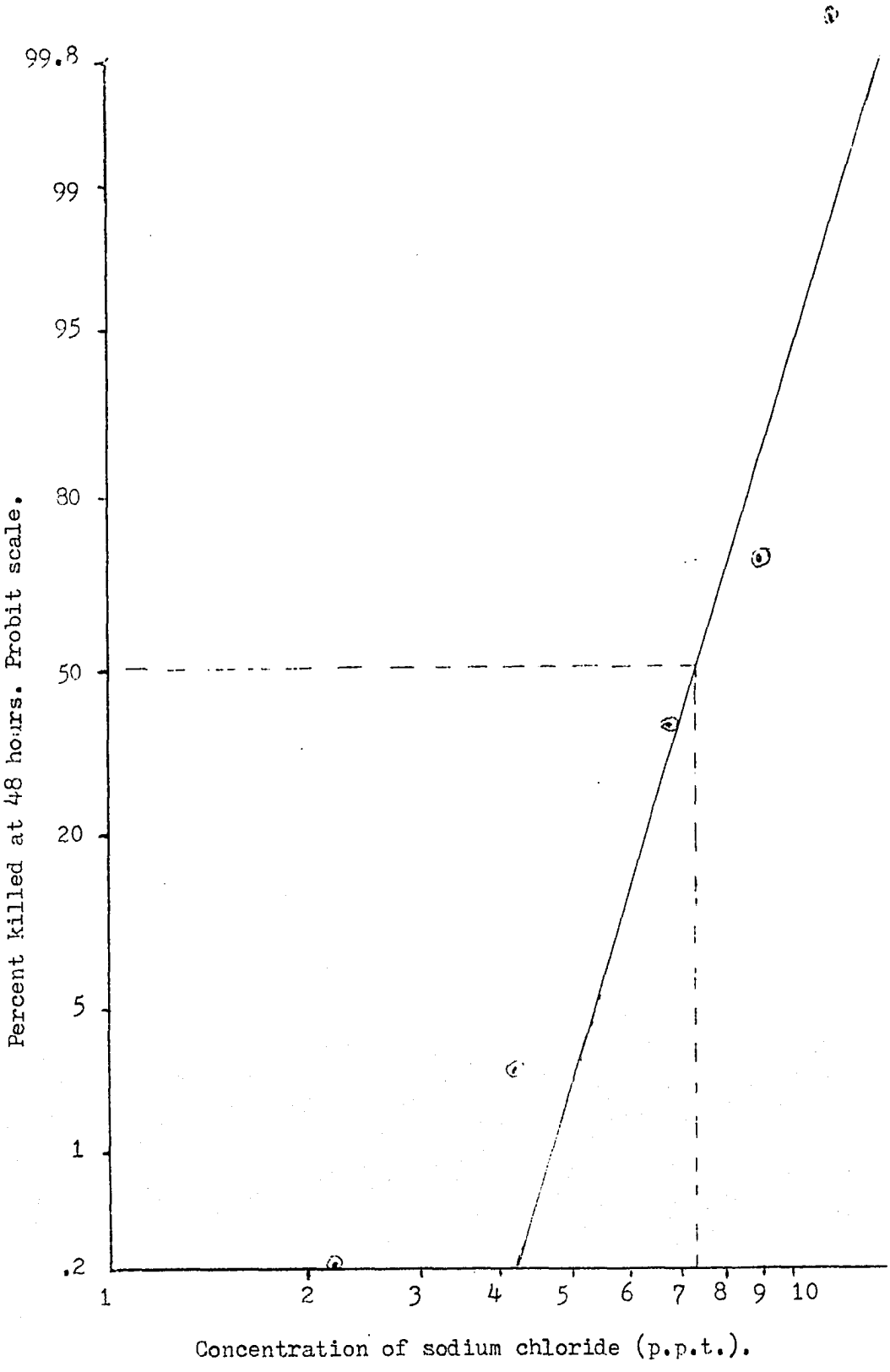

Figure 7. Fourty-eight hour LC_{50} for Tubifex tubifex.

to kill 50 percent of the individuals of Hyalella azteca (Figure 5). A concentration of about 7.9 p.p.t. of sodium chloride was necessary to kill 50 percent of the mayfly Ameletus sparsatus (Figure 6). For Tubifex tubifex a concentration of about 7.2 p.p.t. was required to produce 50 percent mortality (Figure 7). From this data there is an indication that Hyalella azteca is less tolerant of sodium chloride than is either Tubifex tubifex or Ameletus sparsatus.

Figures 8 through 10 are graphs of percent mortality versus time for each of the concentrations used, and for each of the three organisms. The slope of the line indicates the rate of mortality, the higher the slope, the faster the mortality rate. An increasing slope of the line indicates that the mortality rate is increasing and flattening of the line or a decreasing slope indicates that the mortality rate is slowing down.

Figure 8 presents time versus mortality data for Hyalella azteca. The linear nature of the 11.2 and 8.9 p.p.t. concentrations indicates that the rate of mortality in both of these concentrations was constant. The similar slopes of these two lines indicate that the rates of mortality are very similar. The main difference in mortality in these two concentrations lies in the fact that it took longer for mortality to begin in the 8.9 p.p.t. concentration. Mortality in the 6.84 p.p.t. concentration started out slowly at first, but after 500 minutes it increased abruptly, with a rate similar to that in the 11.2 and 8.9 p.p.t. concentration. In the 4.2 p.p.t. concentration and in the 2.32 p.p.t. concentration, mortality was slower, as evidenced by the flattening of the curves. Here it should be

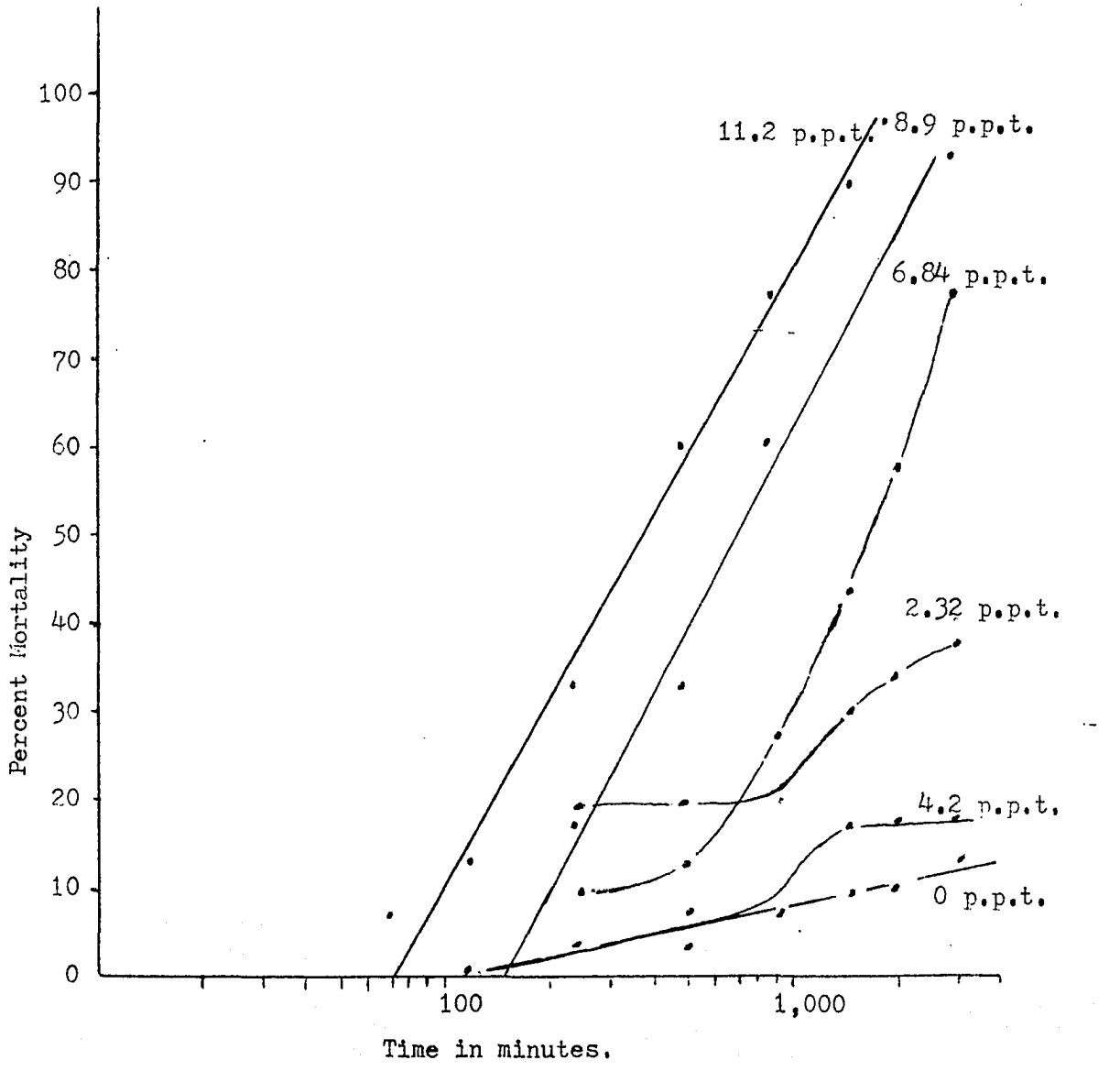

Figure 8. Mortality rates during a 48-hour exposure to sodium chloride for Hyalella azteca.

remembered that the mortality after 48 hours in these two concentrations was not statistically different from that of the control group. The linear nature of mortality of the control group as opposed to the curvature in the 2.32 and 4.2 p.p.t. concentrations indicates that the sodium chloride may, nevertheless, be influencing the rates of mortality in the two experimental groups.

Figure 9 shows the data for time versus mortality for Ameletus sparsatus. The rate of mortality was constant in the 11.2 p.p.t. concentration of sodium chloride. Mortality did not start until after 200 minutes had passed. Mortality in the 8.9 p.p.t. concentration started more slowly, but then reached a rate similar to the rate of mortality in the 11.2 p.p.t. concentration although it is not as constant. In the 6.84 p.p.t. concentration, mortality started slowly and then increased abruptly as it did in the 8.9 p.p.t. concentration. However, instead of continuing to increase as the mortality did in the 8.9 p.p.t. concentration, mortality leveled off and stopped at 33.3 percent. Although the rates of mortality were different in the control group and the 6.84 p.p.t. concentration, the total numbers dying after 48 hours were not statistically different. Something similar to this was seen with the 2.32 p.p.t. concentration, but was not as pronounced. The mayflies in the 4.2 p.p.t. concentration had a mortality rate very similar to that of the control group.

Figure 10 shows the data on percent mortality versus time for Tubifex tubifex. In the 11.2 p.p.t. concentration of sodium chloride mortality started rapidly and was very fast. In the 8.9 p.p.t. con-

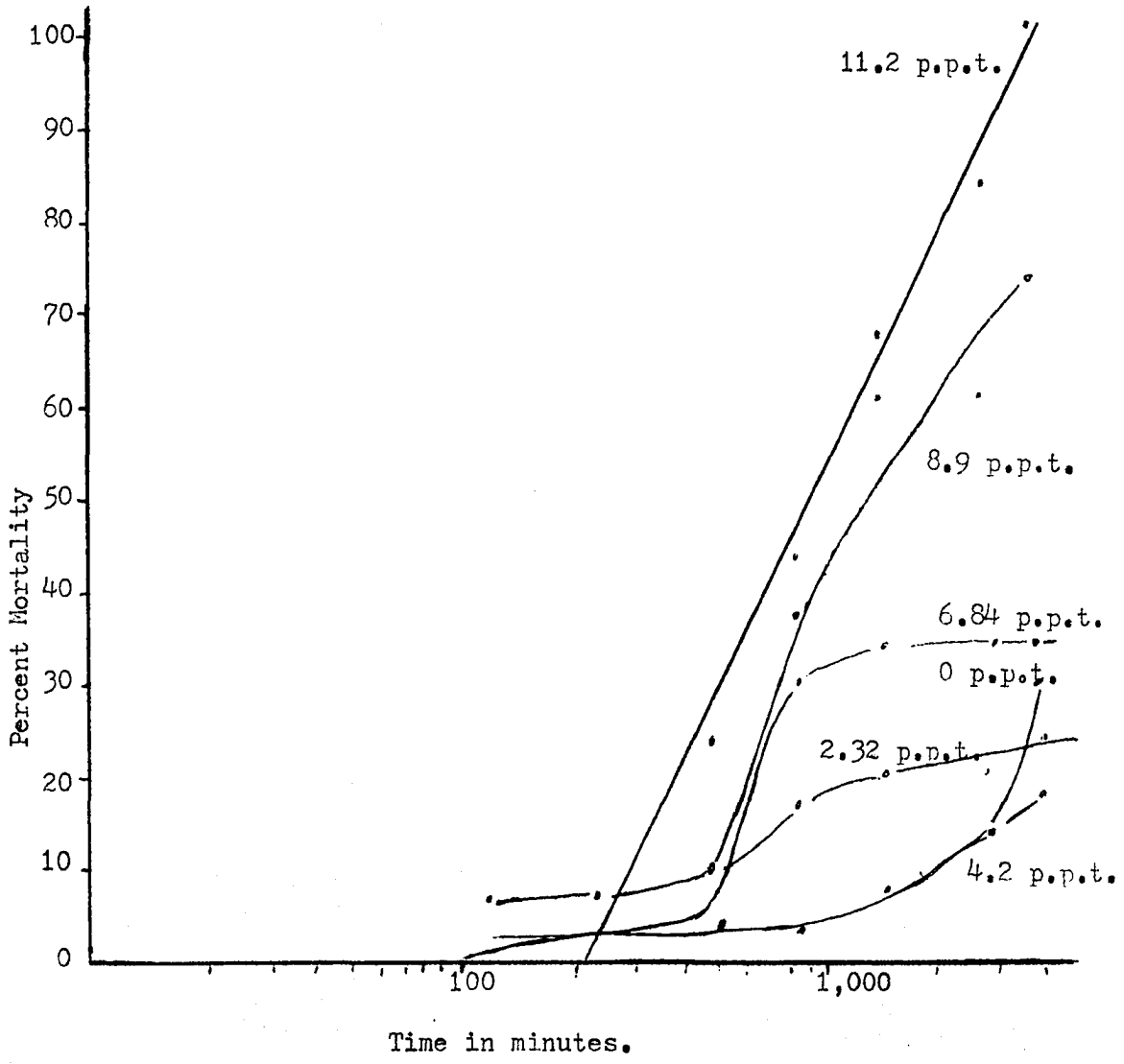

Figure 9. Mortality rates during a 48-hour exposure to sodium chloride for Ameletus sparsatus.

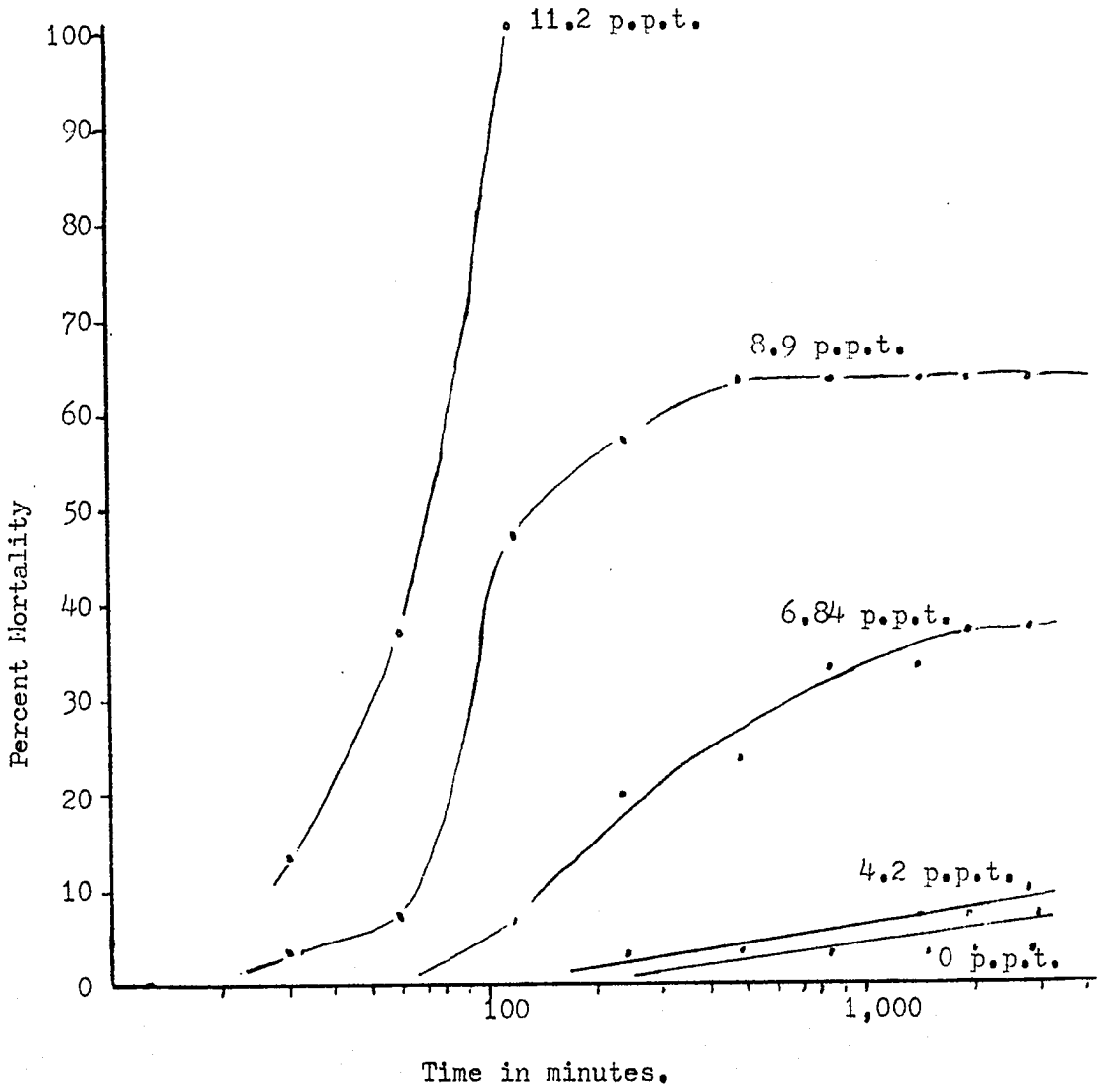

Figure 10. Mortality rates during a 48-hour exposure period to sodium chloride for Tubifex tubifex.

centration mortality started out a little slower, but increased rapidly between 60 minutes and 240 minutes and then leveled off at 63 percent. In the 6.84 p.p.t. concentration the mortality started around 100 minutes. The rate of mortality increased at first, but then started to level off as 48 hours passed. The rates of mortality as well as mortality after 48 hours were very similar in the 0, 2.32, and 4.2 p.p.t. concentrations.

In an effort to determine if there were any differences in mortality of the test organisms after exposure to sodium chloride was terminated, the organisms were maintained in aquaria for a period of three weeks. Table 8 lists the starting numbers and the numbers that survived through time periods up to 21 days. The possibility exists that mortality in the first day was the result of the transfer from the sodium chloride test solution to the aquaria. By allowing one day for reverse acclimation, mortality due to changes in osmotic conditions should be minimized. The data at day one, therefore, were taken as the starting point rather than day zero.

Table 8 shows a few differences in mortality rates between the control group and test organisms. Mortality in the amphipods in the 6.84 p.p.t. solution seems to be greater than that in the control. For the Tubifex tubifex that were in the 6.84 p.p.t. concentration there was less mortality than there was in the control group. The numbers of organisms for these observations are not large enough for statistical treatment of data.

In Table 9, the data for four day intervals was converted into percent mortality. This shows that mortality for the Hyalella azteca

Table 8. Twenty-one day observations on mortality. Numbers alive.

Time (days)	Concentration of sodium chloride (p.p.t.)					
	0	2.32	4.2	6.84	8.9	
	26	19	24	7	2	*Hyalella azteca*
0	21	23	25	17	6	*Ameletus sparsatus*
	28	19	27	19	11	*Tubifex tubifex*
	24	19	20	6	1	
1	20	19	22	11	3	
	28	29	26	19	11	
	21	18	17	3	1	
2	19	17	18	11	2	
	18	19	13	17	5	
	19	15	14	2	1	
3	17	14	11	11	2	
	28	28	23	17	5	
	19	15	14	2	0	
	11	12	9	11	1	
4	24	28	23	17	5	
	18	15	14	2	0	
5	10	9	6	8	1	
	24	27	22	15	5	
	14	14	11	1	0	
6	9	7	5	7	1	
	23	25	20	15	5	
	14	14	8	1	0	
7	7	6	2	7	1	
	22	20	20	15	5	
	14	14	7	1	0	
8	5	6	2	5	1	
	19	19	19	15	4	
	12	13	7	1	0	
9	2	4	2	5	0	
	18	16	19	15	4	

Table 8 continued.

day	0	2.32	4.2	6.84	11.2	
	11	12	7	1	0	Hyalella azteca
10	2	3	1	3	0	Ameletus sparsatus
	17	15	18	14	4	Tubifex tubifex
	10	9	7	1	0	
11	2	2	1	3	0	
	16	15	18	14	4	
	9	9	7	1	0	
12	2	2	1	3	0	
	14	15	18	14	4	
	8	9	7	0	0	
13	1	1	1	3	0	
	11	14	14	14	4	
	8	8	6	0	0	
14	0	1	1	2	0	
	7	14	11	13	4	
	7	7	6	0	0	
15	0	1	1	1	0	
	7	13	9	13	4	
	7	6	5	0	0	
16	0	1	0	1	0	
	5	12	9	12	4	
	7	3	4	0	0	
17	0	1	0	0	0	
	3	10	9	12	3	
	7	3	3	0	0	
18	0	0	0	0	0	
	3	9	9	12	1	
	4	3	1	0	0	
19	0	0	0	0	0	
	2	9	8	12	1	
	4	3	1	0	0	
20	0	0	0	0	0	
	2	4	7	10	1	
	4	2	0	0	0	
21	0	0	0	0	0	
	2	4	7	7	1	

Table 9. Percent mortality at four day intervals.

Time (days)	Concentration of sodium chloride (p.p.t.)					
	0	2.32	4.2	6.84	8.9	
2-5	25.0	21.0	22.2	66.6	100	H. azteca
	50.0	52.6	72.8	27.2	66.6	A. sparsatus
	14.3	6.8	15.3	21.0	54.5	T. tubifex
6-9	50	31.6	65.0	83.4	100	
	90	78.9	90.9	54.4	100	
	35.7	44.8	26.9	21.0	63.6	
10-13	66.6	52.7	70.0	100	100	
	95.0	94.7	95.5	72.7	100	
	67.8	51.7	46.1	26.3	63.6	
14-17	70.8	84.2	80.0	100	100	
	100	94.7	100	100	100	
	89.3	65.5	65.5	36.8	72.7	
18-21	83.3	89.5	100	100	100	
	100	100	100	100	100	
	92.8	86.2	73	63.1	91.0	

that were in the 6.84 p.p.t. concentration of sodium chloride was greater than that in the control group. With the Tubifex tubifex that were in the 6.84 p.p.t. concentration, mortality in the control groups and experimental groups do not markedly differ.

Figures 11 through 13 show data for percent mortality and time in graphical form. Percent mortality and time are plotted on linear scales.

Figure 11, for Hyalella azteca, shows that the rates of mortality in the 0, 2.32, and 4.2 p.p.t. concentrations were all similar. In the 6.84 p.p.t. concentration, however, the mortality rate was rapid in the first five days, and then began to slow down a little.

Figure 12 shows that no pronounced differences in mortality were seen between the control Ameletus sparsatus and those in different concentrations of sodium chloride. Ameletus sparsatus evidently recoveres from the test exposures.

Figure 13 shows that mortality in the Tubifex tubifex that were in the 0, 2.32, and 4.2 p.p.t. concentrations was similar. In the 8.9 p.p.t. concentration mortality increased rapidly at first, then slowed down and then increased again. The percent alive at the end of 21 days was similar to that in the 0, 2.32, and 4.2 p.p.t. concentrations. In the 6.84 p.p.t. concentration the mortality rate was slow during the first sixteen days and then increased rapidly. The percentage alive after 21 days was markedly greater than that for the other concentrations.

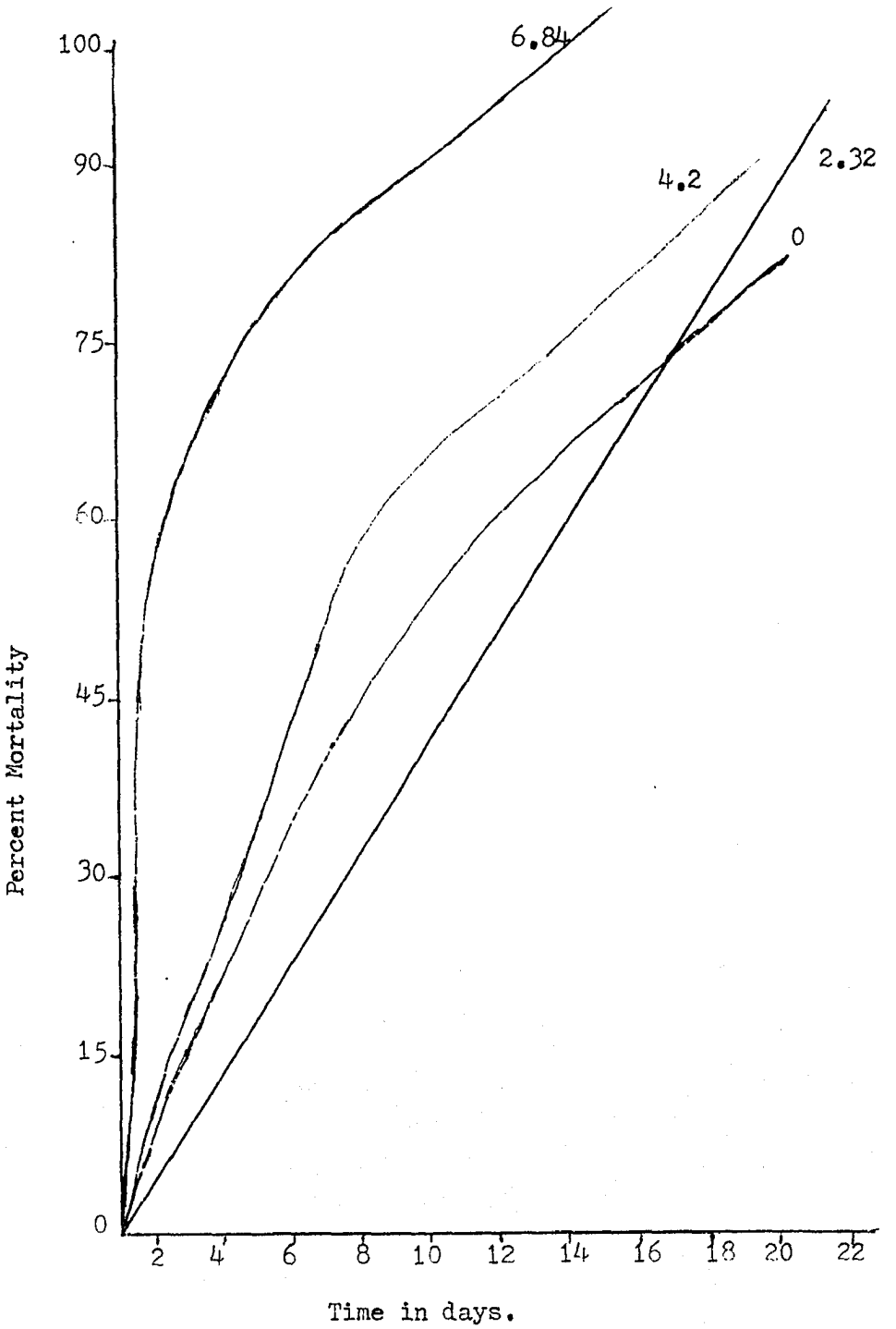

Figure 11. Twenty-one day observations on mortality in Hyalella azteca following exposure to sodium chloride.

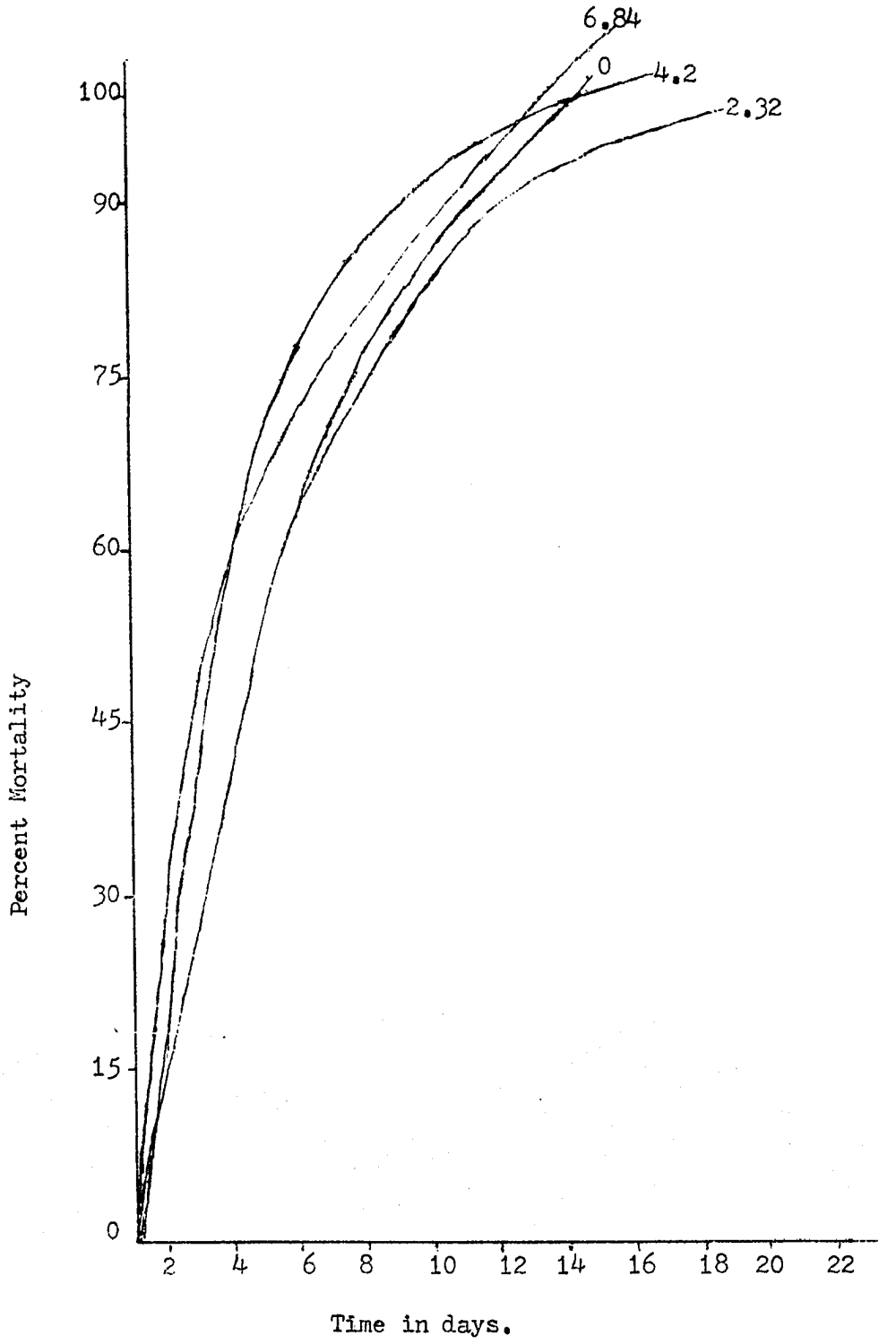

Figure 12. Twenty-one day observations on mortality in Ameletus sparsatus following exposure to sodium chloride.

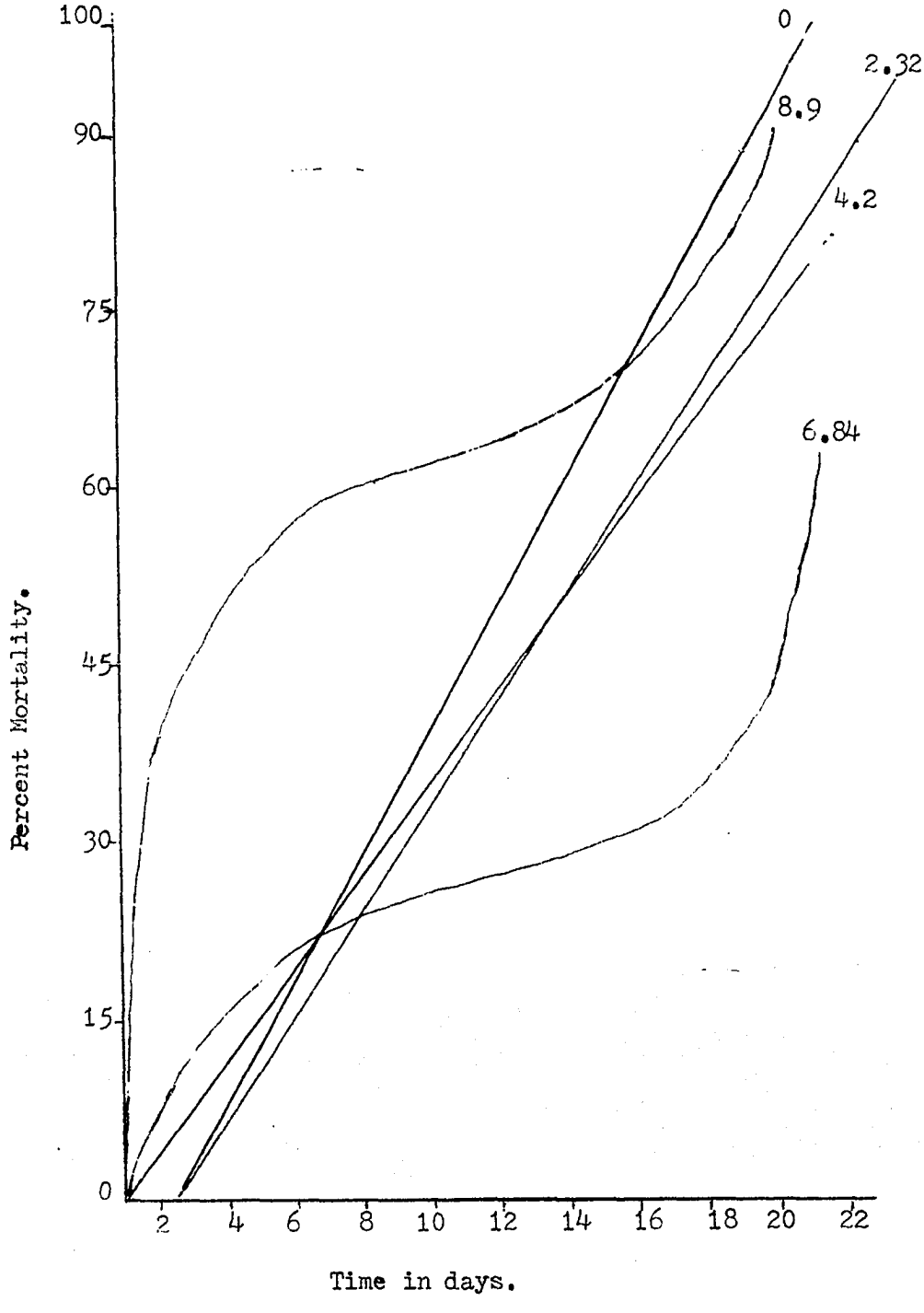

Figure 13. Twenty-one day observations on mortality in Tubifex tubifex following exposure to sodium chloride.

DISCUSSION

The Chemical and Physical Data

The chemical and physical data show that these parameters should not be influencing mortality significantly. Dissolved oxygen during the test periods was relatively constant (Table 1), and at no time did it drop to a level which would be limiting for the organisms being tested. The bioassay system adequately oxygenated the water as the water ran from the delivery barrels to the bioassay containers.

The temperature of the water was also relatively constant. Water was held at the laboratory temperature and remained there throughout the tests. Although the temperature is higher than what would be experienced in the outside environment, temperature can not explain the differences in mortality between the control and test organisms.

The pH of the dilution water did not vary significantly. Toxicity of certain materials is known to be influenced by the pH of the medium. Whitley and Sikora (1970) found that pentachlorophenol was more toxic to tubificid worms at a pH of 6.5 than at a pH of 10.5. Because of this, it is important that the pH of the water remains constant. The pH of the flowing water did remain constant without having to be adjusted artificially.

Alkalinity and hardness were the most variable physical parameters measured. Alkalinity varied from 180 to 250 p.p.m calcium carbonate. Hardness varied from 130 to 200 p.p.m. calcium carbonate.

This variability may reflect the switching of the water pumping sites by the city of Kalamazoo during the testing periods.

The toxicity of some substances may vary with the hardness of the water used (Anderson, 1944 and Sprague, 1973). The variability of the hardness of the water in these tests may have influenced mortality of the organisms tested. Although this variability was not great, it makes the test conditions a little less defined. All organisms, however, received water of comparable hardness and alkalinity at one time. This probably would reduce the variability in mortality due to variations in hardness and alkalinity for the organisms in the different concentrations.

The flow rates in the bioassay containers were relatively constant. Sprague (1973) has indicated that the replacement time for the water in flowing bioassay systems is not directly proportional to the volume of water in the tank and the rate of flow. According to his data, the time for a 99 percent replacement of water in the bioassay tanks used in this study would be between one and one and one-half hours. He recommends that replacement should occur at least every 24 hours, or preferably every twelve hours.

Hyalella azteca

Few studies have been made on Hyalella azteca's tolerance of sodium chloride. A study of the toxicity of oil well brines by Clemens and Jones (1955) revealed that the 96-hour LC_{50} for Hyalella azteca was 3.8 percent of the original brine by volume. This would be equivalent to a concentration of salts of 6.8 p.p.t. Most of this

brine consisted of sodium chloride, with calcium, magnesium, and potassium salts making up substantial, but lesser proportions of the brine.

A few studies of the salt tolerance of freshwater amphipods other than Hyalella azteca have been made. Beadle and Cragg (1940) studied Gammarus pulex in relation to the concentration of sea water it could tolerate. They found that it could tolerate, for a period of 24 hours, concentrations of sea water ranging from zero to 40 percent. This would be equivalent to a concentration of salts from zero to approximately fourteen p.p.t. They provided evidence which indicated that this species could withstand a sudden transfer to a salt concentration of approximately seven p.p.t. They indicated that these animals suffered a disproportionate increase in tissue chloride as salinity increased, indicating the passage of chloride into the cells.

Schmitz et al. (1968) also worked with the tolerance of Gammarus pulex to sea water. They concluded that survival depended primarily on the osmotic stress which the cytoplasm could tolerate.

Lim and Williams (1971) worked with the tolerance of Astrochiltonia subtenuis to sea water. This amphipod is common in freshwater, but is also found in slightly salty waters of Australia. It was found to withstand salinities from 0.02 p.p.t. to concentrations up to 29.1 p.p.t. They also indicated that there was no difference in mortality between male and female individuals.

Data from the tests in this study indicate that Hyalella azteca has a slightly lower tolerance for sodium chloride than it did for oil well brines and lower tolerance of sodium chloride than other species did for sea water.

The 48-hour LC_{50} for Hyalella azteca was 5.2 p.p.t. sodium chloride. This is a lower tolerance level than was established by Clemens and Jones (1955) for oil well brine. This indicates that possibly sodium chloride alone is more toxic than it is in combination with other salts.

Cooper (1965) found while working with natural populations of Hyalella azteea that the mortality rate of individuals was size specific. Older individuals, which tend to be larger, died sooner than smaller individuals in natural populations. If this same idea applies with respect to salt tolerance, some errors could arise if all individuals were not the same size. This error should be minimized in this study, however, because of the randomization procedure used in placing individuals in various concentrations.

Figure 8 shows that the rates of mortality in the 6.84, 8.9, and 11.2 p.p.t. concentrations of sodium chloride were very similar. The primary difference between the three concentrations is that the higher the concentration, the sooner the mortality began. If as Schmitz et al. (1968) suggest, mortality was due primarily to osmotic stress, one would expect that at lower concentrations, mortality would take longer to occur. This is shown in Figure 8; however, if it were entirely osmotic, it would seem that the rates of mortality in concentrations lower than 11.2 p.p.t. would be less than that in the 11.2 p.p.t. concentration. This was not shown in the 6.84 and 8.9 p.p.t. concentrations, but it does seem to be true in the 2.32 and 4.2 p.p.t. concentrations. Mortality in the 2.32 p.p.t. concentration was higher than mortality in the 4.2 p.p.t. concentration. This difference which

was statistically insignificant, is thought to be the result of random mortality not due to sodium chloride. If it was due to sodium chloride it may indicate a threshold for a protective metabolic response that was not met as readily at low concentrations.

In Figure 11, mortality over the 21-day period for those individuals in the 6.84 p.p.t. concentration was rapid during the first five days, then began to slow down. This indicates that the amphipods that were in this concentration were either weakened to the point that recovery was much slower or nonexistent, or that with this concentration there are toxic effects which persist past the initial exposure to sodium chloride. The latter seems likely since it fits a projection of that concentration from Figure 8. After the first five days in the 6.84 p.p.t. concentration, the rate of mortality is similar to that in the 0, 2.32, and 4.2 p.p.t. concentrations. This would indicate that a delayed recovery may have occurred for Hyalella azteca in this concentration.

Ameletus sparsatus

Figure 6 shows the LC_{50} for Ameletus sparsatus. This mayfly was able to withstand considerably greater concentrations of salt than could Hyalella azteca. A concentration of 7.9 p.p.t. was lethal for 50 percent of the individuals of Ameletus sparsatus over a 48-hour period.

Mortality after 48 hours in the 6.84 p.p.t. concentration was not different significantly from that in the control. Comparing the mortality curves of the 6.84 and zero p.p.t. concentrations indicates

that even though the mortality after 48 hours was no different, they got there in different ways. Mortality in the control was low, but gradually increased up to the end of the 48-hour period. In the 6.84 p.p.t. concentration, mortality increased rapidly after 480 minutes until 1,000 minutes into the run then leveled off and stopped. Those individuals which died rapidly in the 6.84 p.p.t. concentration may represent the weaker individuals which would have died anyway. The stress of the sodium chloride may have caused those weaker individuals to die sooner than they would have without the added stress, but the stronger individuals may have been able to withstand the sodium chloride and possibly represent those that survived in the control. Something similar to this was evident in the 2.32 p.p.t. concentration, but was less pronounced. The mortality curve for the 4.2 p.p.t. concentration is almost identical to the control mortality curve. Ameletus sparsatus was able to recover after the exposure to sodium chloride was terminated.

Very little data is available in the literature on the tolerance of any mayfly for sodium chloride or other salts. Clemens and Jones (1955) found that a concentration of sodium chloride of 6.84 p.p.t. caused 50 percent mortality in 96 hours in a mayfly of the family Baetidae. Ameletus sparsatus is in that same family. The 48-hour LC_{50} of 7.9 p.p.t. is somewhat higher than that found by Clemens and Jones, but, because of the different time period, that is expected. Since the species tested by Clemens and Jones is not known, accurate comparisons of the two studies can not be made. Other mayflies have been shown to be intolerant of many types of adverse conditions (Britt,

1955; Smith, 1967; Eriksen, 1968; Warnick and Bell, 1969; Nebecker, 1972; Macek et al., 1972). This does not seem to be true with Ameletus sparsatus in the case of tolerance to sodium chloride. It can withstand high levels of sodium chloride for 48 hours.

Tubifex tubifex

Studies of Tubifex tubifex have shown these oligochaetes to be able to tolerate a greater quantity of many types of pollutants than other freshwater organisms (Whitten and Goodnight, 1966). Of the three invertebrates tested here, Tubifex tubifex was the most tolerant to all but the highest concentrations of sodium chloride.

Mortality of Tubifex tubifex in the higher concentrations of sodium chloride was more rapid than the other two invertebrates tested. In the 11.2 p.p.t. concentration there was 100 percent mortality after 120 minutes. At this time in the 11.2 p.p.t. concentration four individuals of Hyalella azteca had died and no individuals of Ameletus sparsatus had died. A similar situation was seen in the 8.9 p.p.t. concentration up to the 480 minute time period. After this period of time mortality of Tubifex tubifex in the 8.9 p.p.t. concentration stops and mortality of Hyalella azteca and Ameletus sparsatus approaches and then surpasses mortality of Tubifex tubifex.

It is possible that the low initial tolerance of higher concentrations of sodium chloride by Tubifex tubifex as compared to the other two species, was due to the soft body wall of Tubifex tubifex. The soft body wall may have been more permeable to water or sodium chloride than was the body wall of Hyalella azteca or Ameletus sparsatus. High-

er permeability would be likely to increase the osmotic stress when it was exposed to high sodium chloride concentrations.

In the lower concentrations of sodium chloride mortality was not statistically significant below 6.84 p.p.t. The 48-hour LC_{50} of 7.2 p.p.t. indicates that, even though at higher concentrations mortality is rapid, Tubifex tubifex was very resistant to sodium chloride.

Figure 10 shows these results clearly. Mortality was rapid in the 11.2 p.p.t. concentration. In the 8.9 and 6.84 p.p.t. concentrations mortality starts out rapidly (more so in the 8.9 than in the 6.84 p.p.t. concentration), but then slows down and stops. This indicates that there may have been some acclimation to these concentrations after about 1,000 minutes into the tests. Another possibility is that the individuals dying first represent the weaker individuals, and the hardier individuals were able to survive with little mortality.

Neither of these explanations are entirely satisfactory. From the time mortality starts in the 8.9 p.p.t. concentration, until it levels off at 63 percent, there is an elapsed time of 470 minutes (approximately eight hours). From the time mortality starts in the 6.84 p.p.t. concentration until it begins to level off, there is an elapsed time of about 700 minutes (11.6 hours). If acclimation was occurring in these concentrations, it seems unlikely that it would occur faster in the higher concentration than it did in the lower concentration.

The other possibility which is mortality as a result of the presence of weaker individuals, is also not an entirely satisfactory explanation. This would help explain the slower rate of mortality in

the 6.84 p.p.t. concentration; the weaker individuals were not affected as fast or in the same degree as those in the higher concentration. However, in the 8.9 p.p.t. concentration mortality stops abruptly after 480 minutes into the test. It seems unlikely that mortality would completely stop if mortality was a result of weaker individuals dying. It would be more likely that mortality would continue, but at a slower rate.

Table 9 shows that mortality in the 6.84 and 8.9 p.p.t. concentrations for the 21-day observations was different from that of the control group, while mortality in the 4.2 and 2.32 p.p.t. concentrations was little different from that in the control. This is also shown in Figure 13. Mortality of the Tubifex tubifex that were in the 6.84 p.p.t. concentration was considerably lower than mortality of either the control, the 2.32, or the 4.2 p.p.t. groups. It started out similar to the others, but slowed down after six days, and then began to increase again after seventeen days. Walker (1971) found that concentrations of sodium chloride between 1.196 and 2.99 p.p.t. gave Tubifex tubifex protection from oxygen poisoning. It increased worm survival even when administered as much as twelve hours after oxygen exposure. She concluded that the osmotic concentration of the environmental medium is an important factor in the response of Tubifex tubifex to oxygen. She also stated that increased salinity may be effective in protecting this organism from other stressors. Something similar to what Walker observed may be happening in the 21-day observations. Exposure to salt prior to the stress of being held without food being supplied, may have protected Tubifex tubifex.

Although survival is greatest in the individuals that were in the 6.84 p.p.t. concentration, survival was also slightly greater in the 2.32 and 4.2 p.p.t. concentrations. Since no statistical tests could be run on these, it is not known if this difference is statistically significant.

The above possibility is based on two ideas which are as yet unproved. First, Walker (1971) showed that exposure to sodium chloride during or after oxygen stress increased the survival of Tubifex tubifex. It has not been shown that exposure to sodium chloride prior to oxygen stress would increase survival. Second, Walker worked only with oxygen stress. Whether or not this is applicable to other types of stressors should be investigated further.

In the 8.9 p.p.t. concentration, mortality started out rapidly, slowing down after six days. It ended at a level similar to that in the 0, 2.32, and 4.2 p.p.t. concentrations. The mortality of individuals in the 8.9 p.p.t. concentration continued after the initial exposure period. Recovery after six days shows that the increased mortality does not continue indefinitely. The small starting number for this group leaves the results open to question. Styczynska (1972) examined the fecundity, survival, and haemolymph concentration of Tubifex tubifex in relation to salinity of the medium. He found that in sea water surpassing the normal osmotic concentration of the body fluids (four p.p.t.) egg laying decreased and embryonic development stopped. Resistance of adults was higher. They withstood concentrations between five and eight p.p.t. Concentrations between five and eight p.p.t. resulted in mortality such that he called this the critical zone.

Clemens and Jones (1955) studied the tolerance of Tubifex to brine from oil wells. They found that the 96-hour LC_{50} for Tubifex in oil wells brine was 8.5 p.p.t. Palmer (1968) reported that ten percent sea water (a concentration of salt of about 3.5 p.p.t.) was the highest salinity tolerated by Tubifex without gradual acclimation. Above this concentration worm deaths occurred within 24 hours. These studies indicate similar levels of tolerance of Tubifex in sea water and oil well brine as was found in this study with sodium chloride.

The similarity in the tolerance of Tubifex to sodium chloride, oil well brine, and sea water indicates that the toxicity of these salts may be directly related to the osmotic stress which Tubifex can withstand. Styczynska (1972) stated that egg laying and embryonic development are more sensitive to saline conditions than are adults. Levels of sodium chloride which could be withstood by populations of Tubifex tubifex would, therefore, be expected to be lower than those tolerated by adult individuals.

The LC_{50} values for the three organisms tested are in a similar relationship as the concentrations of sodium chloride at the collection sites. This suggests that the species differences could be due partially to acclimation.

It is expected that most, if not all, organisms would show variations in tolerance to pollutants with variations in stage of life cycle, season, and environment. The organisms used in this study were selected from natural populations from single locations during a short time period to minimize those variables.

The levels of sodium chloride used in these tests are considerably higher than those present in most natural waters. Freshwaters can have natural salinities as high as 0.5 p.p.t., although salinities well below 0.1 p.p.t. are most common (Prosser, 1973). Extreme cases of salt pollution have been recorded which could cause mortality in the organisms tested here. Clemens and Jones (1955) found salinities up to 9.487 p.p.t. in a stream receiving brine from oil wells. Wiebe et al. (1934) found salinities up to two p.p.t. from oil brines. These are extreme cases of salt pollution. More common values for salt pollution from industrial and domestic use (Lewelling and Kaplan, 1959) and from road deicing (Bubeck et al., 1971) approach 0.3 p.p.t.

Sprague (1971) reported that safe levels of a tested material should be between 0.01 and 0.4 of the lethal concentration. This means that a concentration of sodium chloride which would be considered safe for Hyalella azteca would be between 0.052 and 2.08 p.p.t. For Ameletus sparsatus the safe levels would be between 0.079 and 3.16 p.p.t. For Tubifex tubifex, safe levels would be between 0.072 and 2.88 p.p.t.

The acute or short-term effects of a pollutant are important in determining the immediate effects a toxic material will have on the environment. Chronic effects are also basic to an understanding of this. Before it is possible to fully assess the impact of salt pollution on the organisms tested here, long-term tests should be made.

SUMMARY

The contamination of freshwaters by various salts is a problem which should not be overlooked in man's concern for the environment. Sodium chloride is one of the commonest salts which finds its way to freshwater supplies. The sources of this salt pollution vary from one locality to another but include domestic, municipal, industrial, and geologic sources. This problem is compounded by the fact that conventional water treatment practices do not remove salt from water supplies.

The use of bioassays in the evaluation of the effects a suspected pollutant would have on the environment is a common practice. Bioassays do this by observing an organisms reaction to a toxic material.

A bioassay using a continuous-flow diluter was performed with three invertebrates to determine the effects that sodium chloride would have on them. Hyalella azteca, an amphipod, Ameletus sparsatus, a mayfly, and Tubifex tubifex, a freshwater oligochaete were used in this study.

Hyalella azteca was found to be the most sensitive of the three to sodium chloride. An LC_{50} of about 5.2 p.p.t. was considerably less than that for either Ameletus sparsatus (7.9 p.p.t.) or Tubifex tubifex (7.2 p.p.t.). After the initial exposure to sodium chloride, mortality of Hyalella azteca was higher in the 6.84 p.p.t. concentration than in the 0, 2.32, and 4.2 p.p.t. concentrations. The mayfly Ameletus sparsatus showed recovery from the initial exposure to sodium chloride. Mortality after exposure was little different from that in the control

group. For the Tubifex tubifex, mortality after the initial exposure in the group that was in the 6.84 p.p.t. concentration was less than it was in the 0, 2.32, and 4.2 p.p.t. groups. Mortality was highest in the group that was exposed to 8.9 p.p.t. sodium chloride. Evidence from another investigation suggests the possibility that in Tubifex tubifex, exposure to sodium chloride may increase this organisms tolerance for other stressors.

Levels of sodium chloride in most natural waters, unless extreme salt pollution occurred, probably would not cause any acute mortality in the organisms tested here. Other unfavorable effects, such as objectionable taste, would probably occur first.

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