Bulletin 71

# Rotifers as Monitors of Heavy Metal Pollution in Water

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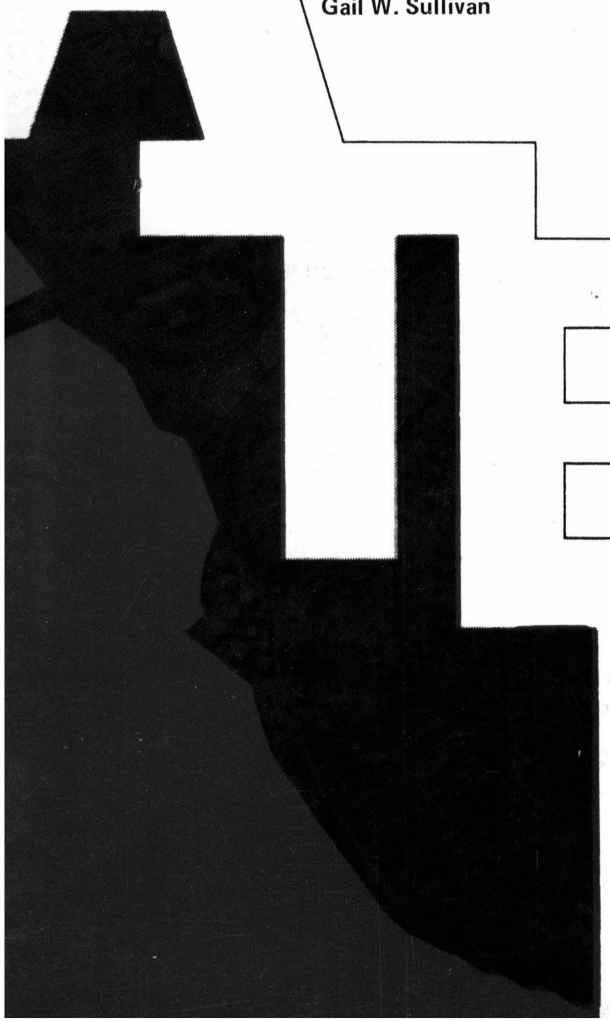

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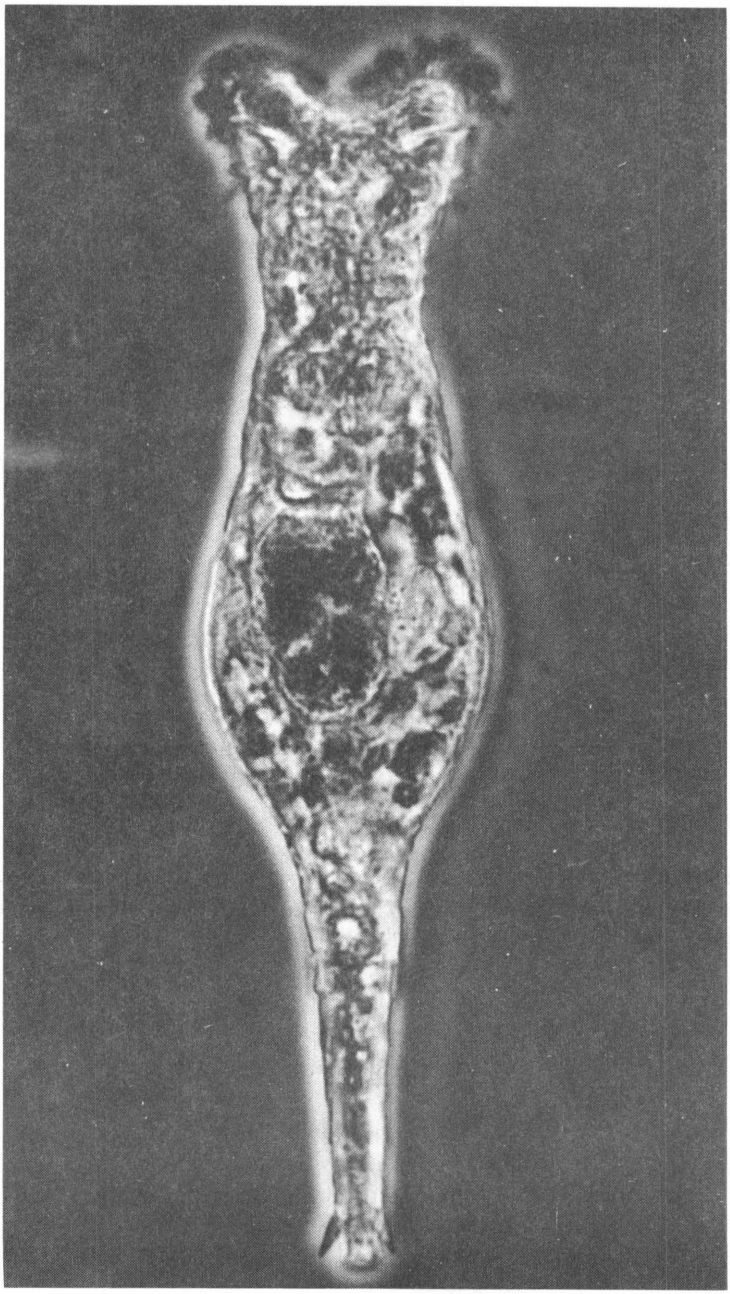

The Rotifer, *Philodina acuticornis*, Magnification approximately 500 X

# PREFACE

Rotifers, visible only through a microscope, live wherever there is fresh water. From the arctic to the tropics, they are present in lakes as important food for young fish, in moss and lichens, and in sewage facilities as vital members of the decomposition community.

Yet, heretofore, these readily available animals have been used very little to monitor water quality. Therefore, the purpose of this research was to examine the suitability of the rotifer as a monitor for heavy metal pollution in water. Another aim of this research was to develop a scaled-down, simplified, and inexpensive monitoring technique that could be used with other studies in which fish serve as monitors for water quality.

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# ABSTRACT

In an assessment of acute bioassay techniques, the following parameters were studied: static versus continuous flow, acclimation to test conditions, methods for making dilution waters, toxicant addition, effect of autoclaving on dilution water hardness, rotifer counting methods, suitability of probit analysis and rotifer culture methodology.

A static bioassay technique is recommended using log phase population of the rotifer, *Philodina acuticornis* and an acclimation period of 48 hours. $EC_{50}$ values were determined by recording the number of dead or withdrawn rotifers in triplicate samples after the specified time period.

This technique was tested by determining the toxicity of cadmium, chromium, cobalt, copper, lead, nickel, mercury, silver, zinc, ammonium chloride, and phenol. After 96 hours in soft water, the most toxic to the least toxic metals were: cadmium, mercury and copper, zinc, silver, nickel (as chloride), chromium, nickel (as sulfate), lead, and cobalt. After 96 hours in hard water, the toxicity of the metals from most to least was: cadmium, copper, mercury, chromium, and lead. The data suggest that zinc will follow mercury toxicity. The sulfate forms of zinc and cadmium were more toxic than the chloride forms. The reverse was true for nickel. *Philodina* were extremely tolerant to ammonium chloride and phenol.

Zinc toxicity decreases as hardness and alkalinity increase, and the effect is most marked when both are considered together. At water hardness $<90$ ppm calcium may be the principal antagonist and $>90$ ppm magnesium may be more important. Zinc was less toxic at 10 C and there were no differences between 20 and 30 C after 96 hours.

Rotifer eggs were very resistant to toxicants, and young rotifers were more sensitive than the adults.

There was no apparent effect of population size on toxicty. The probit analysis may not be an appropriate analysis for populations or organisms which vary in age and size.

# INTRODUCTION

Although there is a considerable amount of data on the toxicity of heavy metals to fish (See reviews by Doudoroff and Katz, 1953; Skidmore, 1964; Jones, 1964; and Anon, 1971), there have been relatively few controlled bioassays conducted with organisms other than fish (Anon, 1971). Sprague (1969, 1970, 1971) reviewed the presently accepted methods and applications for acute and chronic fish bioassays. Most of the invertebrate bioassays have been conducted with *Daphnia*, snails, planarians, protozoans, and economically important marine mollusks and crustaceans (Anon, 1971). There is so much variation in techniques that comparison of data is at best speculative. There have been some studies on the effect of sublethal levels of chemicals on the growth, life span, and reproduction of rotifers (Finesinger, 1926; Lansing, 1942; Gilbert, 1968; and Meadow and Barrows, 1971b) but most have been reports on the effect of added chemicals and other environmental factors, in the field, to rotifer populations (Edmondson, 1944; Hoffman and Olive, 1961; Jones and Moyle, 1963; von Windeguth and Patterson, 1966; Needham, 1966; Prins and Davis, 1966; and Anderson, 1970). There have been few controlled bioassays on the acute toxicity of industrial effluents, such as heavy metals, to rotifers (Allee and Rosenthal, 1949; Sullivan and Buikema, 1972; Sullivan, Buikema, and Cairns, 1973).

As intermediate links in the food web, invertebrates are important members of the aquatic community. By analyzing the gut contents of several common larval fish, such as the bluegill and yellow perch, Siefert (1972) has shown that rotifers form an integral part of a larval fish's diet. In biological waste treatment rotifers are members of the aquatic community responsible for BOD reduction by bioflocculation and sedimentation (Eckenfelder, 1961). In both natural waters and in sewage treatment facilities, rotifers aid in keeping microbial communities in balance by grazing upon bacterial, algal, and protozoan populations. It is conceivable that if a substantial portion of the larval fish food is dead or just not reproducing (especially at a critical time in the fish's life cycle) this could affect fish production. It also seems reasonable that if most of the organisms which are responsible for grazing a bacterial or algal growth are dead or encysted, this could lead to a bloom of fouling microbial organisms.

Bdelloid rotifers of the genus *Philodina* are very common and are found in standing water almost everywhere in the world. *Philodina*, in a dessicated encysted state, can be carried by wind for many miles and still survive. For this reason, single species of this rotifer have colonized over wide geographical ranges. Hyman (1951) reported that some *Philodina* species live in small pools

and some in the littoral zones of large lakes. Some bdelloid rotifers are found in springs up to 46 C and some live in the Antarctic (Hyman, 1951). Some bdelloid rotifer species live in activated sludge (Eckenfelder, 1961) and some have been found in vessels containing only rainwater (Hyman, 1951).

One reason *Philodina* was chosen as a test organism was because it is possible to obtain a large number of uniform test animals for a minimum amount of trouble, time, and expense. *Philodina* is small (about 0.2 to 0.5 mm) but larger than most protozoans. They are large enough to work with under the lower powers of a microscope, but yet are small enough to grow and study in small volume containers. *Philodina* has a high parthenogenetic reproduction rate. It will lay 50 to 53 eggs in its 20 to 22 day life span at 24 C (Meadow and Barrows, 1971a) and the eggs will hatch in 1 to 2 days (Hyman, 1951) as females. By cloning, it is possible to obtain thousands of genetically uniform animals in a relatively short period of time.

The principal objectives of this study were:

a. to examine the feasibility of using an inexpensive and commercially available rotifer as a bioassay organism,

b. to assess difficult simplified acute bioassay techniques, select one technique and study it further, and

c. to test the recommended procedure using different toxicants (especially heavy metals) and to study the effect of various parameters on zinc toxicity.

# LITERATURE REVIEW

## I. Metals

### a. Toxicity to Aquatic Organisms

Metal pollution comes from mine drainage, brines of oil wells, and from industrial effluents of metal processing and chemical plants (Doudoroff and Katz, 1953). The heavy metals were the most toxic to fish when compared to the toxicity of the alkali and alkaline-earth metals (Doudoroff and Katz, 1953; Jones, 1964). Jones (1937, 1940) conducted acute bioassays with the planarian, *Polycelis nigra*, and from these tests he showed that this flatworm, like fish, is more sensitive to the heavy metals than to the other metals. For both the planarian and the stickleback, *Gasterosteus aculeatus*, the toxicity of the metal could be predicted by its solution pressure. The more negative the solution pressure, the more likely that the metal will stay in solution rather than combining with another substance, such as the chemicals making up protoplasm. Thus, the more negative the solution pressure, the less toxic the metal will be. *Daphnia* appear to be as sensitive as, or in some cases more sensitive than, fish to the heavy metals (Anderson, 1948). The snail, *Physa heterostropha*, is more sensitive than the bluegill, *Lepomis macrochirus*, to chromium ($K_2Cr_2O_7$) and zinc ($ZnCl_2$) when tested under comparable chemical and physical conditions (Patrick, Cairns, and Scheier, 1968).

Cairns and Dickson (1970) reported that zinc and copper exposure (24 ppm for 24 hr) reduced the number of species of protozoans in an artificial community, but did not as noticeably affect community density. The species of protozoans present were not stated. Restoration of the different protozoan species was faster after zinc exposure than it was after copper exposure.

### b. Physiological and Behavioral Effects

There are several theories on the cause of death when fish are acutely exposed to the salts of heavy metals. It was observed very early that fish under heavy metal stress show behavioral signs of respiratory distress (Carpenter, 1927). Carpenter also observed that there was a copious secretion of coagulated mucus from the gills and body surface of minnows, *Leuiscus phoxinus*, when exposed to acutely lethal levels of lead nitrate. This coagulated mucus clogged the gills to such an extent that the suggested cause of death was suffocation. When returned to clean water a reasonable time before death, the fish sloughed off the mucus and returned to a normal ventilation pattern. Her theory for the cause of death was reinforced by Jones' (1938) observation that the addition of calcium to the test water had several effects. With

calcium, the fish lived 10 days rather than 10 hours, the fish did not show respiratory distress; and there was less mucus secretion. One physiological effect of calcium is that it will prevent the coagulation of mucus (Jones, 1938). Jones mixed mucus secreted by the eel, *Anguilla anguilla*, with lead nitrate in the presence and absence of calcium nitrate. Where calcium was present there was no coagulation; with the lead salt alone, stringy masses formed.

In later studies using rainbow trout, *Salmo gairdneri*, as the test fish, Lloyd (1960) observed that with concentrations of zinc that were lethal, there was little secretion of mucus, so he concluded that the fish must be dying of another cause. Using radiolabeled zinc, he observed that while most of the zinc was in the gill tissue, there was also a considerable amount of zinc distributed throughout the body of the fish. Mount (1964) was able to distinguish between fish just exposed to zinc and those killed by zinc, by the ratio of zinc in the gills to zinc in the bones. This method was confirmed by Cairns et al. (1971). Histological examination of the gills of fish exposed to sublethal concentrations of zinc showed little evidence of gill damage (Lloyd, 1960).

A field study of the Kalamazoo River reported that of the 17 genera of rotifers found in the river, *Philodina* was the most resistant to low oxygen concentrations caused by the addition of paper mill effluents and treated sewage into the river (Prins and Davis, 1966). As Jones (1937) points out, a different theory for the cause of death should be proposed for animals which lack gills and withstand low oxygen conditions. Jones suggested that the animal's intracellular protein probably gets fixed into a coagulated matrix, but Skidmore (1964) reports that as yet there is no case where coagulated protoplasm has been observed.

Gardner and Yevich (1970) showed that the initial site of histological damage due to cadmium differed for marine and freshwater fish. Although freshwater fish first evidenced gill damage when exposed to the heavy metals, an acutely exposed estuarine fish, *Fundulus heteroclitus*, showed histological damage in the intestine before gill damage was observed. This difference can possibly be explained by the fact that marine fish tend to drink water; whereas freshwater forms do not.

Lewis and Lewis (1971) observed that both channel catfish, *Ictalurus punctatus*, and the golden shiner, *Notemigonus crysoleucas*, when exposed to acutely lethal concentrations of copper or zinc salts showed a drop in the osmolarity of the blood below 230 MosM before death. When the osmolarity

of the blood did not go this low, the fish regulated its blood osmolarity and survived. When the fish were placed in a more concentrated NaCl solution (235 MosM) rather than tap water, the osmolarity of the blood rose above normal. When the fish were placed into a salt solution plus a heavy metal salt, the fish lived several hours longer than with the heavy metal alone, but still died even though the osmolarity of the blood was equal to or above normal. These results suggest that heavy metals can cause permeability changes in fish, but the principal cause of death is probably not the drop in blood osmolarity.

## II. Factors Affecting Toxicity

a. Hardness

Hardness is the most frequently cited antagonist to heavy metal toxicity (Trama and Benoit, 1960; Skidmore, 1964; Sprague and Ramsay, 1965; Mount, 1966; Sprague, 1970). Only Naumann (1934; in Skidmore, 1964), working with *Daphnia*, reported a case where heavy metal was more toxic in a hard water than a soft water.

Jones (1939a), using the tadpole, *Bufo bufo bufo*, as his test organism, measured the relative ability of the alkaline-earth metals to antagonize copper toxicity. He found that the order of ability to antagonize depended on the level of copper and the level of the hardness ion (calcium, strontium, barium or magnesium). At 0.01N copper nitrate and the peak concentration of the hardness ion for the greatest antagonism (0.1N), the order of chemicals from the greatest to the least antagonism was: strontium, calcium, magnesium, and barium. At 0.002N copper at the same 0.1N hardness level, the order changed to: calcium, strontium, barium, and magnesium). Jones used very high concentrations of both copper and the hardness ions.

There are presently two theories concerned with the mechanism of antagonism between the alkaline-earth metals and heavy metals. Both theories are based on the assumption that competition occurs between the alkaline-earth and heavy metal ions for binding sites on the protein molecules. The two theories differ in the choice of principal site for this competition. The first theory is the permeability theory (Osterhout, 1922; Prosser, 1973). According to this theory, the alkaline-earth metal, by binding with the membrane, reduces the permeability of the cell membrane preventing the influx of other cations such as the heavy metals. The influx of sodium and potassium into the tissues was retarded by the presence of the alkaline-earth metals. The alkaline-earth metals inhibited the entrance of heavy metals to a lesser extent.

Lloyd (Dept. of Sci. Ind. Res., 1958 in Skidmore, 1964) observed that the acute toxicity of zinc will be antagonized equally in fish raised or tested in hard water. His results suggested that calcium antagonizes at the surface of the fish, because no pre-exposure time is necessary to get antagonism. The fact that fish will retain their resistance to zinc when transferred from hard to soft water for acute testing is evidence that the effect of calcium probably is physiological rather than a chemical effect of the dilution water on the zinc.

The second theory, contrary action, is concerned with the coagulation of protoplasm. Heilbrunn (1952) pointed out the importance of calcium in maintaining the integrity of the protoplasmic structure within the cell. He also stated that calcium can be replaced in this structure by other metals. As Jones (1938) showed, calcium did prevent the coagulation of mucus in the presence of the heavy metals. At this time, this theory has not been tested histologically.

b. Solubility and Alkalinity

Lloyd (1960) found that suspended zinc (as $ZnCO_3$) was quite toxic to trout. The toxicity of suspended zinc is further demonstrated by the fact that a rise in pH actually increased the toxicity of zinc to the fathead minnow, *Pimephales promelas*, although it decreased its solubility (Mount, 1966). Lloyd (1960) suggested that suspended zinc carbonate could be toxic to fish if the zinc carbonate particles that got trapped in the gills were redissolved by a local drop in pH caused by the release of carbon dioxide by the gills.

c. Temperature

As Sprague (1970) points out, "No assumptions should be made about temperature effects on toxicity." He reported that the effects of temperature are quite variable for fishes and invertebrates and the data may even vary for each species. For example, Cairns and Scheier (1958) reported that the snail, *Physa heterostropha*, was more resistant to zinc at cooler temperatures but Wurtz (1962) found the opposite effect. Sprague (1970) reviewed the earlier literature on the effects of temperature on fish and its variability.

d. Life Cycles Stage

There have been several studies on the effect of life cycle stage on the toxicity of the heavy metals. Cairns, Scheier, and Loos (1965) showed that the eggs of the zebra danio, *Brachydanio rerio*, were more resistant to potassium dichromate and zinc chloride than the adults. McKim and Benoit

(1971) showed that the hatchability of eggs and subsequent survival of the alvins were adversely affected by long-term exposure of adult brook trout to sublethal levels of copper. Brungs (1969) found that levels of zinc, sublethal to adults, affected the survival of young in the fathead minnow, *Pimephales promelas*. Both McKim and Benoit (1971) and Brungs (1969) found that the eggs hatched prematurely on exposure to these metals. Mount (1968) with copper and Brungs (1969) with zinc found that spawning was the most sensitive stage in the life cycle of the fathead minnow. With both metals there was a great decrease in the total number of eggs spawned at sublethal levels of these metals.

e. Body Size and Population Size

Although body size affected the toxicity of some chemicals to the bluegill sunfish, *Lepomis macrochirus*, size did not affect the toxicity of either potassium dichromate or zinc chloride (Cairns and Scheier, 1959b).

Carpenter (1927, 1930) found that as the ratio of the mass of fish to the amount of lead nitrate increased, the toxicity decreased. Allee and Bowen (1932) reported that the toxicity of colloidal silver to goldfish decreased with the presence of more animals in the same volume. Allee and Rosenthal (1949) found that the rotifer, *Philodina roseola*, had a greater resistance to acutely lethal levels of copper sulfate when grouped than when isolated.

# MATERIALS AND METHODS

During the development and assessment of bioassay techniques, various experimental methods were employed. Table 1 summarizes the experimental conditions which were used in this study. This table should be referred to during the discussion of the materials and methods.

## I. Culturing

Rotifers have been cultured in boiled pond water with suspended algae, grass, lettuce, and milk media (Meadow and Barrows, 1971a; Lindner, Goldman, and Ruzicka, 1961). It was an objective of this project to culture and test the animals in a defined medium, so that possible influences of the dilution water on toxicity could be determined more readily. A dilution water similar to that used for fish bioassays was studied so that the results could be compared (Cairns and Scheier, 1957). The methods used in these assays were modified for the following experiments to allow for acclimation of the animals to the test water, and so that fewer dead rotifers and rotifer eggs were transferred to the test solution.

The rotifers were cultured in a soft artificial dilution water (modified from Cairns and Scheier, 1957; Table 1, this paper) made with doubly distilled water or deionized water. The dilution water was bubbled with air for at least 24 hours.

The culture medium was prepared from the dilution water by adding a fine suspension of trout food (Trout Chow #3, Ralston-Purina Co., St. Louis, Mo.) to the dilution water. In experiments A, B, and C trout food was added to 800 ml of doubly distilled water and blended for one minute in a food blender. This suspension was passed through a fine bolting cloth and one part of the filtrate was added to 19 parts of dilution water. This dilution trout food medium was autoclaved and stored in the refrigerator. Before adding to the cultures, 100 ml of the dilute trout food medium was swirled in a one-liter Erlenmeyer flask to saturate it with air.

The rotifer cultures were maintained in gallon jars or in 250-ml Erlenmeyer flasks. The gallon jar cultures were filled with three liters of culture medium; the Erlenmeyer flasks were filled with 150 ml of culture medium. In experiments A, B, and C the cultures were lightly aerated and distilled water was added when needed to replace water lost through evaporation. In experiments D, E, and F, one half of the culture medium was removed every two to three days and replaced with fresh, sterile trout food medium. The

Table 1

Chemical Composition and Characteristics of Dilution Waters.*

| Experiment | Dilution Water | Hardness (ppm $CaCO_3$) Ca | Mg | Total | Alkalinity (ppm$CO_3$) Carbonate | Total | pH |
|---|---|---|---|---|---|---|---|
| A. | O** | – | – | 0 | – | 0 | 5.4 |
| | Basic* | – | – | 0 | – | 14 | 7.8 |
| | 43 | – | – | 43 | – | 19 | 8.8 |
| | 103 | – | – | 103 | – | 23 | 6.9 |
| | 154 | – | – | 154 | – | 16 | 6.7 |
| | 205 | – | – | 205 | – | 16 | 6.6 |
| | 256† | – | – | 256 | – | 235 | 7.7 |
| | 291† | – | – | 291 | – | 225 | 7.7 |
| | Culture†† | – | – | 40–50 | 0–2 | 55–80 | 8.0–8.3 |
| B. | All Mg | 0 | 162 | 162 | 4 | 50 | 8.8 |
| | All Ca | 162 | 0 | 162 | 0 | 35 | 7.4 |
| | All $CO_3$ | 77 | 77 | 154 | 4 | 75 | 8.2 |

Table 1
(Continued)

| Experiment | Dilution Water | Hardness (ppm $CaCO_3$) Ca | Mg | Total | Alkalinity (ppm$CO_3$) Carbonate | Total | pH |
|---|---|---|---|---|---|---|---|
| B. | All $SO_4$ | 81 | 81 | 162 | 0 | 15 | 7.7 |
| | All ions (hard) | 81 | 81 | 162 | 0 | 36 | 8.0 |
| | Basic* | 0 | 0 | 0 | 0 | 14 | 7.8 |
| | Culture†† | – | – | 40–50 | 0–2 | 55–80 | 8.0–8.3 |
| C. | $MgSO_4$ | 0 | 100 | 100 | 0 | 14 | 7.6 |
| | $MgCO_3$ | 0 | 92 | 92 | 5 | 96 | 8.3 |
| | $CaSO_4$ | 100 | 0 | 100 | 0 | 14 | 7.5 |
| | $CaCO_3$ | 92 | 0 | 92 | 6 | 91 | 8.1 |
| | Basic* | 0 | 0 | 0 | 0 | 16 | 8.2 |
| | $NaHCO_3$ | 0 | 0 | 0 | 10 | 97 | 8.5 |
| | Culture†† | – | – | 40–50 | 0–2 | 55–80 | 8.0–8.3 |

Table 1
(Continued)

| Experiment | Dilution Water | Hardness (ppm $CaCO_3$) Ca | Mg | Total | Alkalinity (ppm $CO_3$) Carbonate | Total | pH |
|---|---|---|---|---|---|---|---|
| D. | $MgSO_4$ | 6 | 70 | 76 | 0 | 32 | 6.8 |
| | $MgCO_3$ | 6 | 57 | 63 | 0 | 103 | 6.6 |
| | $CaSO_4$ | 77 | 3 | 80 | 0 | 39 | 4.9 |
| | $CaCO_3$ | 83 | 3 | 86 | 0 | 77 | 8.4 |
| | $Na_2SO_4$ | 6 | 3 | 9 | 0 | 34 | 7.2 |
| | All $SO_4$ | 46 | 26 | 72 | 0 | 27 | 7.1 |
| | All $CO_3$ | 65 | 22 | 87 | 2 | 122 | 8.4 |
| | All ions (soft) | 30 | 7 | 37 | 0 | 24 | 7.8 |
| | All ions (hard) | 80 | 18 | 98 | 0 | 67 | 7.4 |
| E. | $MgSO_4$ | – | – | – | 0 | 34 | 7.4 |
| | $MgCO_3$ | 27 | 80 | 107 | 0 | 110 | 8.0 |
| | $CaSO_4$ | 94 | 10 | 104 | 0 | 37 | 7.8 |
| | $CaCO_3$ | 102 | 15 | 117 | 0 | 106 | 8.1 |
| | Basic* | 0 | 6 | 6 | 0 | 32 | 7.8 |

Table 1
(Continued)

| Experiment | Dilution Water | Hardness (ppm $CaCO_3$) Ca | Mg | Total | Alkalinity (ppm $CO_3$) Carbonate | Total | pH |
|---|---|---|---|---|---|---|---|
| E. | $NaHCO_3$ | 19 | 7 | 26 | 2 | 121 | 8.2 |
| | All ions (soft) | 39 | 8 | 47 | 0 | 59 | 7.8 |
| | All ions (hard) | 53 | 28 | 81 | 0 | 54 | 7.8 |
| F. | Test | 17 | 8 | 25 | 0 | 24 | 7.4–7.9 |
| | Culture†† | 32 | 8 | 40 | 0 | 59 | 7.2–7.9 |

Table 1
(Continued)

Chemical Quantities Added x $10^{-1}$ gram/liter

| Experiment | $CaCO_3$ | $CaSO_4 2H_2O$ | $MgCO_3 nH_2O$ | $MgSO_4 7H_2O$ | $NaHCO_3$ | $Na_2SO_4$ | HCl |
|---|---|---|---|---|---|---|---|
| A. | – | – | – | – | – | – | – |
| | – | – | – | – | – | – | – |
| | 0.82 | – | – | 0.37 | – | – | – |
| | 2.18 | – | – | 1.00 | – | – | 0.25 |
| | 3.27 | – | – | 1.62 | – | – | 0.35 |
| | 4.36 | – | – | 2.25 | – | – | 0.45 |
| | – | – | – | – | – | – | – |
| | 1.23 | – | – | 1.48 | – | – | 0.11 |
| | 0.10 | 0.17 | – | 0.12 | – | – | – |
| B. | – | – | 1.34 | 2.91 | – | – | 0.70 |
| | 6.56 | 2.50 | – | – | – | – | 0.60 |
| | 7.08 | – | 1.38 | – | – | – | 1.50 |

Table 1
(Continued)

Chemical Quantities Added x $10^{-1}$ gram/liter

| Experiment | $CaCO_3$ | $CaSO_4$ $2H_2O$ | $MgCO_3$ $nH_2O$ | $MgSO_4$ $7H_2O$ | $NaHCO_3$ | $Na_2SO_4$ | HCl |
|---|---|---|---|---|---|---|---|
| B. | – | 2.00 | – | 2.59 | – | – | – |
| | 7.61 | – | – | 2.91 | – | – | 0.80 |
| | – | – | – | – | – | – | – |
| | 0.10 | 0.17 | – | 0.12 | – | – | – |
| C. | – | – | – | 2.46 | – | – | – |
| | – | – | 0.84 | – | – | – | – |
| | – | 1.72 | – | – | – | – | – |
| | – | – | – | – | – | – | – |
| | – | – | – | – | – | – | – |
| | – | – | – | – | 1.68 | – | – |
| | 0.10 | 0.17 | – | 0.12 | – | – | – |

Table 1
(Continued)

Chemical Quantities Added x $10^{-1}$ gram/liter

| Experiment | $CaCO_3$ | $CaSO_4$ $2H_2O$ | $MgCO_3$ $nH_2O$ | $MgSO_4$ $7H_2O$ | $NaHCO_3$ | $Na_2SO_4$ | HCl |
|---|---|---|---|---|---|---|---|
| D. | – | – | – | 2.46 | – | – | – |
| | – | – | 0.84 | – | – | – | – |
| | – | 1.72 | – | – | – | – | – |
| | 1.00 | – | – | – | – | – | – |
| | – | – | – | – | – | 1.42 | – |
| | – | 1.20 | – | 0.74 | – | – | – |
| | 0.70 | – | 0.25 | – | – | – | – |
| | 0.10 | 0.17 | – | 0.12 | – | – | – |
| | 0.40 | 0.69 | – | 0.49 | – | – | – |
| E. | – | – | – | 2.46 | – | – | – |
| | – | – | 0.84 | – | – | – | – |
| | – | 1.72 | – | – | – | – | – |
| | 1.00 | – | – | – | – | – | – |
| | – | – | – | – | – | – | – |

Table 1
(Continued)

Chemical Quantities Added x $10^{-1}$ gram/liter

| Experiment | $CaCO_3$ | $CaSO_4$ $2H_2O$ | $MgCO_3$ $nH_2O$ | $MgSO_4$ $7H_2O$ | $NaHCO_3$ | $Na_2SO_4$ | HCl |
|---|---|---|---|---|---|---|---|
| E. | – | – | – | – | 1.68 | – | – |
| | 0.10 | 0.17 | – | 0.12 | – | – | – |
| | 0.40 | 0.69 | – | 0.49 | – | – | – |
| F. | 0.10 | 0.17 | – | 0.12 | – | – | – |
| | 0.10 | 0.17 | – | 0.12 | – | – | – |

*See text for the methods of preparation of the dilution waters. The dilution waters were made with the basic soft water which had the following composition: dissolved in distilled water: KCl (1.46 x $10^{-2}$ g/l), $NH_4NO_3$ (3.60 x $10^{-3}$ g/l), $NaHCO_3$ (2.30 x $10^{-2}$ g/l), $H_2SiO_3$ (1.00 x $10^{-2}$ g/l), and Fe citrate (2 x $10^{-5}$ g/l).

**Made with distilled water.

†Made with natural hard water.

††Variation in the chemical composition of the culture media are due to the influence of the trout food.

rotifer cultures contained bacteria, protozoans (including *Chilomonas paramecium, Cyathomonas truncatus,* and *Bodo sp.*), and algae on which the rotifers could graze.

The cultures were maintained at room temperature, 20 ± 2 C, on a 16L:8D photoperiod in all experiments, except experiment C in which the cultures were maintained at 15 C in the dark.

**II. Static versus Continuous Flow**

The research below was developed for static tests. Preliminary studies were conducted using continuous flow in modified Sykes-Moore chambers (Sykes and Moore, 1960). The major problem was flow which washed animals out of the chamber. It was not possible to maintain a steady minimum flow rate. The animals also swam upstream and out of the culture chamber. When one side of the chamber was replaced with a nucleopore membrane filter (General Electric), young rotifers swam through the holes. Also the holes became clogged with food organisms and particulate metal compounds and flow was diminished.

Periodic renewal of the assay compound was not possible because live rotifers would also be removed and the loss of live rotifers would affect the results. Meadows and Barrows (1971b) anesthetized this rotifer with chlorobutanol but the potential interaction of chlorobutanol and the toxicants was too great to be considered for this research.

**III. Acclimation**

In experiments A, B, and C, the test photoperiod and temperature conditions were the same as the culturing conditions. (Exceptions: in experiment C, in the Basic, $MgCO_3$ and $NaHCO_3$ water tests, the animals came from warmer cultures (24 ± 2C)). In these three tests, the acclimation temperature was higher than the test temperature because the environmental chambers failed.) In experiments A, B, and C the rotifers were not acclimated to the test dilution water. In experiments D, E, and F the animals were acclimated to the test photoperiod (12L:12D, intensity less than 100 foot-candles) and temperature (20 ± 1 C) by transferring the cultures to Sherer, Model Cel 4-4 environmental chambers (Sherer Div., Kysor Ind. Corp., Marshall, Mich.) for 48 hours preceding the bioassay. Although there is no recommended acclimation period for this rotifer in the literature, Prosser (1973) states that temperature acclimation for most aquatic animals is complete in a few days. Because *Philodina* are small and appeared to tolerate abrupt changes in light,

temperature, and dilution water in the laboratory without apparent adverse effects, the short 48-hour acclimation period was selected. In experiments D and E the animals were acclimated to the test dilution water in two stages. Six days before the bioassay, the rotifer cultures were emptied down to the 100-ml mark on the culture flasks and refilled to the 225-ml mark with dilute trout food medium made with the test dilution water. This was repeated 48 hours before the bioassay. The resulting culture medium then contained 86 percent of the new dilution water and 14 percent of the old culture medium. In experiment D the test culture medium was autoclaved before it was added to the cultures. Autoclaving was eliminated when it was observed that it was lowering the hardness of the dilution waters. In experiment E, the test medium was made just before addition to the cultures and was not autoclaved.

### IV. Test Dilution Water

See Table 1 for a description of the dilution waters. The dilution waters for experiment A were made by adding the hardness salts to either the basic soft dilution water or to a hard natural water. One percent HCl was added until the desired hardness was obtained as measured with a Hach Chemical Kit (Hach Chemical Co., Ames, Iowa). In experiment B, one hardness salt was added until a hardness of 75 ppm (equiv. ppm $CaCO_3$) was obtained as measured with a Hach Chemical Kit; the second salt was added until the total hardness reached $\approx$ 150 ppm. One percent HCl was added with carbonates to facilitate their solution.

The dilution water for experiments C, D, and E was made by adding the hardness salts to the basic soft water. Those dilution waters which contained carbonates were bubbled for one hour with carbon dioxide. All the dilution waters were bubbled with air for one day just before use to saturate them with air and to remove excess carbon dioxide. The dilution water for experiment F was prepared the same way as the culture media were. The differences in chemical characteristics are due to the trout food.

### V. Bioassay Procedure

Just prior to experimentation, the toxicant solutions were prepared. In the experiments A, B, and C the toxicant solutions were prepared at the desired test strength; in experiments D, E, and F the toxicant solutions were prepared at a concentration five times that desired in the final test samples.

In the experiments A, B, and C, five ml of the toxicant solution or test dilution water (for the controls) were pipetted into a small plastic beaker

(Scientific Products, Columbia, Md.). Each toxicant concentration was set up in triplicate. A separate bioassay was prepared for each time period. The rotifers were added to the test solution by placing two or three pieces of trout food from the culture into the toxicant solution. The sample sizes ranged from 20 to 150 animals per sample. There was no apparent effect of sample size on toxicity.

In experiments D, E, and F, three four-ml samples of the culture containing rotifers were pipetted into a small plastic culture dish (Bellco Glass Co., Vineland, N.J.). The population sizes ranged from 20 to 450 rotifers per sample with an average of about 70 per sample. Again there was no apparent effect of sample size on toxicity. One ml of a specific 5X toxicant solution was added to each sample. One ml of test dilution water was added to the control samples. At least seven toxicant concentrations which spanned the $EC_{50}$ range, as predicted from preliminary tests or from reports on tests with other aquatic organisms, were used for each bioassay. A separate bioassay was conducted for each $EC_{50}$ determination. To help insure uniformity of toxicant composition and uniformity in culturing, the $EC_{50}$ values for a single toxicant were obtained simultaneously, whenever feasible.

Each culture plate or small plastic beaker containing the bioassay samples was placed on a moistened towel in a tray. The tray was covered with a clear cover and placed into the original environmental chamber in experiments C, D, E, and F or kept in the room in experiments A and B under the conditions above.

**VI. Chemical Measurements**

See Table 1 for the chemical characteristics of the dilution waters and cultures.

The pH values were measured at the initiation of the bioassay with a pH meter.

Water hardness was determined at the beginning of the bioassays with a Hach Chemical Kit (Hach Chemical Co., Ames, Iowa) in the experiments A and B. Hardness was measured by EDTA titration (Amer. Public Health Assoc., 1965) in experiment C. Hardness was measured with a Unicam Model SP90 atomic absorption spectrophotometer in experiments D, E, and F. In experiment F the hardness of membrane filtered (Gelman VM, 0.45 micron pore-size) control samples were measured at both 0 and 48 hours with the atomic absorption unit.

In experiment D, hardness was corrected for the effects of autoclaving with trout food and the change in hardness incurred from the incomplete replacement when adding the test trout food medium to the cultures. These mathematically corrected values for hardness were the values used in the regression analyses.

The hardness values in experiment E were corrected for the effects of membrane filtration on the atomic absorption readings. The hardness values used in the regression analyses are the mathematically corrected values.

The alkalinity of the cultures and dilution waters was determined at the beginning of the bioassay by sulfuric acid titration (Amer. Public Health Assoc., 1971). The reported alkalinities in experiments D and E were the expected alkalinities when four parts of culture were added to one part dilution water.

In all the experiments the toxicant levels used in the $EC_{50}$ calculations were the calculated amounts introduced.

### VII. $EC_{50}$ Determination

$EC_{50}$ was defined as the expected concentration of toxicant that would affect 50 percent of the animals after a specified length of time. The criterion for "affected" was no visible internal or external motion when exposed to bright light. (Unaffected animals usually became quite mobile when exposed to bright light.) This criterion was selected because a withdrawn animal (even if not dead) is removed functionally from the community because it is neither feeding nor reproducing. In the majority of cases the animals were dead or effectively immobilized.

In experiments A, B, and C at the end of the bioassay periods the numbers of rotifers affected and alive were counted in each sample by first lightly staining the rotifers with the vital stain methylene blue and then spreading the sample out on slides and small petri dishes.

To determine the percent affected in experiments D, E, and F at the end of the assay period, the number affected in each sample was counted, formalin was added to the sample, and the total number in each of the triplicate samples counted.

The results for the triplicates for each test concentration were added together for the $EC_{50}$ determination. The $EC_{50}$ values for experiments C, D, E, and F

were calculated by probit analysis using the BMD computer program (Dixon, 1970) and/or reading the values directly from the plotted data.

Probit analysis was chosen as the method of data analysis for two reasons. Because this method is employed for fish bioassays, these results may be compared with fish data. Also, this method of analysis puts the emphasis on the median values and therefore stresses the concentrations that will affect most animals while deemphasizing atypical responses. However, some of the data deviated markedly from a probit line and the probit analysis is probably not the best analysis to use (Dr. F. Calhoun, per. comm.). Consequently, some values were presented both ways for comparison purposes.

Some $EC_{50}$ values were determined by the straight line estimation method (Amer. Public Health Assoc., 1971) because there were not enough X-values to use the probit analysis. These values are identified in the appropriate tables.

### VIII. Regression Analysis

The regression analyses for Figures 15, 16, 17, and 18 were determined by Fortran computer program (A 12; Sokal and Rohlf, 1969). The data in Figure 17 were transformed to logarithms to obtain a straight line.

In the regression analysis of the effect of the relative concentrations of magnesium and calcium on the $EC_{50}$ (Figure 1) 60 ppm was selected as the lowest total hardness. Below this value all solutions that had comparable hardness, also had similar Ca:mg ratios. The data for the values above a total hardness of 60 ppm were split into two categories (60 to 89 ppm and 90 to 119 ppm) because there appeared to be an apparent split at 90 ppm.

# RESULTS AND DISCUSSION

## I. Bioassay Techniques

The best method for handling the rotifers was that used in experiment F. In this experiment, the animals were fed a specific concentration of food and the culture medium was changed two to three times per week. This maintained the rotifer culture in a log growth phase. While changing the culture medium, it was also possible to acclimate the rotifers to the test dilution water by stages. Acclimation to test conditions was done by placing the cultures in the appropriate conditions 48 hours prior to the beginnings of a bioassay. This method maintains a large swimming population which can be pipetted into a test chamber. Consequently, the inadvertent transfer of trout food particles, rotifer eggs, and dead rotifers was minimized. Four ml of rotifer culture and one ml of toxicant (5X the desired concentration) were placed in a five-ml container, placed under the appropriate test conditions and observed for the duration of the bioassay. After a specified period of time, the affected (dead or withdrawn) rotifers could be counted. Subtraction of the original affected count from the total was considered the number of live animals.

This method makes bioassays with rotifers quite feasible but the method does sacrifice some certainty about the composition of the test medium. The 5 ml test volume used was too small to obtain chemical and physical measurements. These values were calculated from analyses of the culture medium and the toxicants. The trout food extracts used as protozoan food vary in chemical composition.

There is no control over the number of animals per test condition. Population size does affect toxicity of fishes and higher invertebrates. A regression analysis of $EC_{50}$ values as a function of population size for various chemicals was not significant. The effect of population size was negligible.

This method also is subject to the problems of static bioassays and the advantages of continuous flow currently are not attainable.

## II. Control Mortality

The present mortality of the controls varied between 1 and 16 percent after 96 hours. The mean mortality for each bioassay was usually less than 10 percent, the limit suggested by EPA and Standard Methods. A control mortality exceeding 10 percent may not reflect poor experimental procedure.

When testing populations of animals which have a short life span and which have a rapid reproductive cycle, a larger mortality would be expected.

### III. Toxicity Compared to Other Aquatic Organisms

a. Introduction

The results are summarized in Table 2 and Figures 1 to 14. Two $EC_{50}$ values usually are presented for each metal for each time. The first value was read directly from the data plotted on probit paper (Figures 1 to 14) and the second value was an estimated value from the probit analysis (Dixon, 1970). On occasion, it was not possible to compute a probit analysis and these values were determined from the Standard Methods straight line interpolation; e.g., nickelous sulfate. There was no attempt to assign 95 percent confidence limits to the $EC_{50}$ values because of the large heterogeneity (Figures 1 to 14). The probit analysis may not be adequate to describe data obtained from populations that have varying age classes. (Dr. F. Calhoun, pers. comm.).

The discussion below comparing the toxicity data for *Philodina* to other organisms is speculative. There are considerable variations in bioassay techniques, organism size, and the chemical and physical conditions of the test media. All these factors are known to affect toxicity and these differences prevent detailed comparisons. Further, most bioassay data are for animals of known age or size and stage or reproductive cycle. These data are not for populations of animals whose age and stage of reproduction are variable as is the case for a short-lived, rapidly reproducing organism like *Philodina acuticornis*.

In the following discussion common names will be used as much as possible in discussing toxicity data for fish. These names are:

| | |
|---|---|
| bluegill | *Lepomis macrochirus* |
| goldfish | *Carrassius auratus* |
| fathead minnow | *Pimephales promelas* |
| guppy | *Lebistes reticulatus* |
| rainbow trout | *Salmo gairdneri* |
| brown trout | *Salmo trutta* |

Table 2

The Toxicity of Heavy Metals, Salts, Ammonium Chloride, and Phenol to the Rotifer *Philodina acuticornis*

I. Soft Water

| | 24 hr | | 48 hr | | 96 hr | |
|---|---|---|---|---|---|---|
| Chemical | $EC_{50}$ from Data* | $EC_{50}$ from Probit* | $EC_{50}$ from Data* | $EC_{50}$ from Probit* | $EC_{50}$ from Data* | $EC_{50}$ from Probit* |
| Cadmium Chloride | 11.5 | 6.2 | 1.4 | 1.5 | 0.5 | 0.5 |
| Cadmium Sulfate | 8.2 | 5.2 | ≈2.0 | 0.5 | 0.2 | 0.1 |
| Cobalt Chloride | 32.0 | 27.8 | + | 183.0 | + | 59.0 |
| Cupric Sulfate | 1.5 | 1.9 | 0.8 | 1.0 | 0.6 | 0.7 |
| Lead Chloride | 50.3 | 56.2 | 50.5 | 47.4 | 50.4 | 40.8 |
| Mercuric Chloride | 2.0 | 1.0 | 1.4 | 1.3 | 0.8 | 0.7 |

Table 2
(Continued)

| Chemical | 24 hr | | 48 hr | | 96 hr | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ from Data* | $EC_{50}$ from Probit* | $EC_{50}$ from Data* | $EC_{50}$ from Probit* | $EC_{50}$ from Data* | $EC_{50}$ from Probit* |
| Nickelous Chloride | 7.2 | 7.6 | 4.0 | 4.1 | 2.9 | 2.6 |
| Nickelous Sulfate | 7.2 | 7.1† | 7.2 | 7.1† | 7.4 | 7.2† |
| Potassium Dichromate | 42.0 | 31.6 | 50.0 | 31.2 | 3.1 | 3.1 |
| Silver Nitrate | 3.8 | 5.3 | + | 15.7 | 1.4 | 1.7† |
| Zinc Chloride | 4.2 | 3.7 | 3.0 | 2.4 | 1.5 | 1.3 |
| Zinc Sulfate | 1.4 | 1.2 | 1.4 | 0.9 | 1.2 | 1.2 |
| Ammonium Chloride | 1175.0 | 1140.0 | 1150.0 | 1110.0 | 980.0 | 934.0 |
| Phenol | 325.0 | 282.0 | 205.0 | 202.0 | 260.0 | 248.0† |

Table 2
(Continued)

II. Hard Water

| Chemical | 24 hr | | 48 hr | | 96 hr | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ from Data* | $EC_{50}$ from Probit* | $EC_{50}$ from Data* | $EC_{50}$ from Probit* | $EC_{50}$ from Data* | $EC_{50}$ from Probit* |
| Cadmium Chloride | – | – | – | – | – | – |
| Cadmium Sulfate | 4.5 | 4.8 | 0.8 | 1.4 | 0.3 | 0.3 |
| Cobalt Chloride | – | – | – | – | – | – |
| Cupric Sulfate | 5.8 | 6.4 | 5.8 | 5.4 | 1.1 | 1.1 |
| Lead Chloride | ← ← ← ← ← ← ← greater than 150 ppm → → → → → → → → → | | | | | |
| Mercuric Chloride | 2.8 | 2.3 | 2.0 | 1.7 | 2.1 | 1.6 |
| Nickelous Chloride | – | – | – | – | – | – |

Table 2
(Continued)

| Chemical | 24 hr | | 48 hr | | 96 hr | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ from Data* | $EC_{50}$ from Probit* | $EC_{50}$ from Data* | $EC_{50}$ from Probit* | $EC_{50}$ from Data* | $EC_{50}$ from Probit* |
| Nickelous Sulfate | – | – | – | – | – | – |
| Potassium Dichromate | 23.0 | 22.0† | 19.0 | 21.0† | 15.0 | 15.0† |
| Silver Nitrate | – | – | – | – | – | – |
| Zinc Chloride | – | – | – | – | – | – |
| Zinc Sulfate | 4.1 | 2.6 | 2.4 | 2.0 | – | – |
| Ammonium Chloride | – | – | – | – | – | – |
| Phenol | – | – | – | – | – | – |

*All data are in ppm of the metal ion.

**Survival was less than 50% on all concentrations tested.

†Standard Methods – Straight line graphical interpolation.

| | |
|---|---|
| brook trout | *Salvelinus fontinales* |
| lake trout | *Salvelinus namaycush* |
| channel catfish | *Ictalurus punctatus* |
| stickleback | *Gasterosterus aculeatus* |

b. The Metals

The toxicity of the metals varied among the metals, between salts and time (Table 2). After 96 hours, the most to least toxic of the metals (in ppm) tested in soft water were: cadmium, mercury and copper, zinc, silver, nickel (chloride) chromium, nickel (sulfate), lead, and cobalt. For those metals tested in hard water, the order of toxicity from most to least after 96 hours was: cadmium, copper, mercury, chromium and lead. Copper may be slightly more toxic than mercury in hard water. The 48 hour $EC_{50}$ data for zinc tested in hard water (Table 2) and the other data for zinc (Table 3) suggest that zinc will follow mercury in relative toxicity when tested in hard water.

Comparing the 48 hour $EC_{50}$ values reported as the molarity of the metal, the order of sensitivity of *Philodina* to some of these salts from the most toxic to the least toxic would be: cadmium, mercury, copper and zinc, lead, chromium. Jones (1940) obtained a similar order when measuring the threshold toxicity at 48 hours for salts of these metals to the flatworm, *Polycelis nigra*. The order that he obtained from the most toxic to the least toxic was: mercury, copper, cadmium, zinc, chromium, lead. The only major disagreement in the two orders is the position of cadmium. The toxicity of cadmium may be more dependent on the size of the organisms (see the discussion of the effect of body size on toxicity). The effect of species differences cannot be excluded. However, if there is an effect of body size, cadmium should be more toxic to the rotifer than to the larger flatworm.

According to their solution pressures, the order of toxicity of these metal salts should be: mercury > copper > lead > cadmium > chromium > zinc (Jones, 1940). Except for the high toxicity of mercury and copper to *Philodina*, this rotifer did not show a comparable pattern for the other metals. In the rotifer bioassays the lower than expected toxicity of lead can be explained possibly by the low solubility of lead in the dilution water. Caution should be taken when comparing the position of chromium in these two studies because it was introduced as the dicrhomate ion in the *Philodina* bioassay and as the chromic ion in Jones' experiment.

Table 3

Summary of Factors Affecting the Toxicity of Zinc Sulfate to the Rotifer *Philodina acuticornis*

| Total Hardness (in ppm $CaCO_3$) | Ca:Mg (in ppm $CaCO_3$) | Alkalinity (in ppm $CaCO_3$) | pH | Photoperiod L:D (in hrs) | Temperature °C | $EC_{50}$ (in ppm zinc++) 24 hr | 48 hr | 96 hr |
|---|---|---|---|---|---|---|---|---|
| 0(distilled water) | – | 0 | 5.4 | 16:8 | 20± 2 | 0.3 | 0.3 | 0.3 |
| 0(basic) | – | 14 | 7.8 | 16:8 | 20± 2 | 0.1 | 0.1 | 0.1 |
| 43 | – | 19 | 8.8 | 16:8 | 20± 2 | 2.8 | 3.2 | 3.2 |
| 103 | – | 23 | 6.9 | 16:8 | 20± 2 | 1.4 | 2.4 | 1.1 |
| 154 | – | 16 | 6.7 | 16:8 | 20± 2 | 5.7 | 4.3 | 3.7 |
| 205 | – | 16 | 6.6 | 16:8 | 20± 2 | 9.2 | 2.3 | 2.4 |
| 256 | – | 235 | 7.7 | 16:8 | 20± 2 | 33.0 | 34.5 | 31.1 |
| 291 | – | 225 | 7.7 | 16:8 | 20± 2 | 28.5 | 31.0 | 31.0 |

Table 3
(Continued)

| Total Hardness (in ppm $CaCO_3$) | Ca:Mg (in ppm $CaCO_3$) | Alkalinity (in ppm $CaCO_3$) | pH | Photoperiod L:D (in hrs) | Temperature °C | $EC_{50}$ (in ppm zinc++) 24 hr | 48 hr | 96 hr |
|---|---|---|---|---|---|---|---|---|
| 9 | 6:3 | 34 | 7.2 | 12:12 | 20± 1 | 1.8 | 1.3 | – |
| 9 | 6:3 | 69 | 7.6 | 12:12 | 20± 1 | 0.9 | 0.9 | – |
| 63 | 6:57 | 103 | 6.6 | 12:12 | 20± 1 | 1.1 | 0.6 | – |
| 72 | 46:26 | 27 | 7.1 | 12:12 | 20± 1 | 3.2 | 3.1 | – |
| 76 | 6:70 | 32 | 6.8 | 12:12 | 20± 1 | 1.0 | 1.3 | – |
| 80 | 77:3 | 39 | 4.9 | 12:12 | 20± 1 | 5.0 | 3.3 | – |
| 86 | 83:3 | 77 | 8.4 | 12:12 | 20± 1 | 1.2 | 0.9 | – |
| 87 | 65:22 | 122 | 8.4 | 12:12 | 20± 1 | 17.2 | 7.9 | – |
| 98 | 80:18 | 67 | 7.4 | 12:12 | 20± 1 | 2.5 | 2.0 | – |
| 34 | 24:10 | 38 | 7.8 | 12:12 | 10± 1 | 4.6 | 4.2 | 2.5 |
| 37 | 30:7 | 24 | 7.8 | 12:12 | 20± 1 | 1.1 | 0.9 | 1.3 |
| 26 | 19:7 | 19 | 7.8 | 12:12 | 30± 1 | 3.8 | 2.2 | 1.5 |

Table 3
(Continued)

| Total Hardness (in ppm $CaCO_3$) | Ca:Mg (in ppm $CaCO_3$) | Alkalinity (in ppm $CaCO_3$) | pH | Photoperiod L:D (in hrs) | Temperature °C | $EC_{50}$ (in ppm zinc++) 24 hr | 48 hr | 96hr |
|---|---|---|---|---|---|---|---|---|
| 6 | 0:6 | 32 | 7.8 | 12:12 | 20± 1 | – | 3.9 | – |
| 26 | 19:7 | 121 | 8.2 | 12:12 | 20± 1 | – | 3.3 | – |
| 47 | 39:8 | 59 | 7.8 | 12:12 | 20± 1 | – | 3.3 | – |
| 81 | 53:28 | 54 | 7.8 | 12:12 | 20± 1 | – | 5.9 | – |
| 88 | 9:79 | 34 | 7.4 | 12:12 | 20± 1 | – | 2.5 | – |
| 104 | 94:10 | 37 | 7.8 | 12:12 | 20± 1 | – | 4.6 | – |
| 107 | 27:80 | 110 | 8.0 | 12:12 | 20± 1 | – | 5.7 | – |
| 117 | 102:15 | 106 | 8.1 | 12:12 | 20± 1 | – | 6.8 | – |
| 0(basic) | 0:0 | 16 | 8.2 | 0:24 | 15± 2 | 1.0 | 0.5 | – |
| 0 | 0:0 | 97 | 8.5 | 0:24 | 15± 2 | 0.6 | 0.2 | – |
| 92 | 0:92 | 96 | 8.3 | 0:24 | 15± 2 | 12.2 | 7.4 | – |
| 92 | 92:0 | 91 | 8.1 | 0:24 | 15± 2 | 5.2 | 4.2 | – |
| 100 | 0:100 | 14 | 7.6 | 0:24 | 15± 2 | 6.1 | 3.9 | – |
| 100 | 100:0 | 14 | 7.5 | 0:24 | 15± 2 | 2.1 | 1.3 | – |

Similar comparisons can be made to data for fish and the reader is referred to the analyses of Doudoroff and Katz (1953), Jones (1964) and Schweiger (1957).

c. Cadmium

Cadmium sulfate was more toxic than cadmium chloride (Table 2; Figures 1 and 2). After 24 hours, there was an effect of hardness on cadmium sulfate toxicity but this disappeared by 48 and 96 hours. For cadmium chloride (24 hr) and cadmium sulfate (24 and 48 hr) tested in soft water, the data deviate markedly from a straight line (Figures 1 and 2). This was also true for cadmium sulfate (48 hr) tested in hard water (Figure 2). From our observations, these deviations were due in part to stimulated reproduction and hatching of young rotifers. The dip in the 48 hr graph for cadmium sulfate in soft water (Figure 2) exemplifies the rapid reproduction and hatching by the rotifer and the high sensitivity of young rotifers to the toxicant. By 96 hours, the data approximate a straight line (Figures 1 and 2). *Philodina* was more sensitive to cadmium after 96 hours than were the bluegill and fathead minnow (Pickering and Henderson, 1965) and the mayfly *Ephemerella subvaria* (Warnick and Bell, 1969) and less sensitive than *Daphnia magna* (Anderson, 1948).

d. Cobalt

Cobalt chloride was not very toxic in the ranges studied (Table 2; Figure 3) and only an approximate $EC_{50}$ value of 27.8 can be determined after 24 hours. *Philodina* was less sensitive to cobalt than *Daphnia magna* (Anderson, 1944, 1948), and the mayfly *Ephemerella subvaria* (Warnick and Bell, 1969).

These data suggest that reproduction may not be affected by cobalt.

e. Copper

Cupric sulfate was quite toxic after 48 hours (Table 2 and Figure 4) and there was a definite effect of hardness after 96 hours. *Philodina* was slightly less sensitive to cupric sulfate than *Daphnia magna* (Anderson, 1944). When compared to data for copper sulfate, *Philodina* was less sensitive than the fathead minnow when tested in soft water and more sensitive when tested in hard water (Palmer and Maloney, 1956; Tarzwell and Henderson, 1960; Pickering and Henderson, 1965). Similar trends were obtained for the bluegill but the data were variable (Pickering and Henderson, 1965; Tarzwell and Henderson, 1960; Cope, 1965 and 1966). *Philodina* may be equally or less sensitive than the snails *Physa heterostropha* and *Gyralus circumstinatus*

(Wurtz and Bridges, 1961; Wurtz, 1962), the oligochaete, *Limnodeilus hoffmeisteri* (Wurtz and Bridges, 1961), the mayfly *Ephemerella subvaria* (Warnick and Bell, 1969) and more sensitive than the amphipod, *Gammarus lacustris* (Nebeker and Gaufin, 1964) and the stonefly, *Acroneuria lycorias* (Warnick and Bell, 1969). Studies on the snail, *Physa heterostropha*, suggested minor effects of water hardness (Wurtz, 1962). Toxicity in *Philodina*, the fathead minnow, and the bluegill was affected by hardness (this study; Tarzwell and Henderson, 1960; Pickering and Henderson, 1965). This lack of a hardness interaction for *Physa* may be an artifact because of convergence of data points in low concentrations of copper. In Figure 4 there was a pattern of covergence of data lines at low concentrations irrespective of water hardness or bioassay time.

f. Lead

The toxicity of lead chloride was affected by hardness and there was a slight decline in the $EC_{50}$ values after 24 hours (Table 2; Figure 5). The rotifers were seemingly less sensitive to lead than were *Daphnia magna* (Anderson, 1948), the fathead minnow (Tarzwell and Henderson, 1960; Pickering and Henderson, 1965), the bluegill, goldfish and guppy (Pickering and Henderson, 1965) and possibly the tubificid worms (Whitley, 1968). These apparent differences in sensitivity may not be real. When added to both soft and hard water, the lead immediately precipitated out in large particles presumably as lead carbonate). Most of the precipitate particles were too large to be ingested by the rotifer. The mortality of the rotifer probably was due to the ingestion of very small lead carbonate particles which were in suspension for a period of time. The lead probably became soluble by the low pH of the gut (Kwaskikpwi, 1963). Once the particles settled out of suspension, there was no further ingestion. This may explain why there were no marked differences after 24, 48, and 96 hours. However, fish are sensitive to particulate materials and particulate zinc is more toxic than soluble zinc in the bluegill (Mount, 1966) and the same may be true for particulate lead. These differences in the mode of action of particulate lead may explain the seemingly wide variation among organisms in toxicity.

g. Mercury

Mercuric chloride was increasingly toxic with time and the toxicity decreased as hardness increased (Table 2; Figure 6). There are few data for comparison and *Philodina* may be less sensitive than *Daphnia magna* (Anderson, 1948) and guppy (Shaw and Grushkin, 1967) and more sensitive than the mayfly, *Ephemerella subvaria*, the stonefly, *Acroneuria lycorias*, and the caddisfly, *Hydropsyche betteni* (Warnick and Bell, 1969).

h. Nickel

*Philodina* was more sensitive to nickel as nickelous chloride and nickelous sulfate (Table 2; Figures 7 and 8) than were *Daphnia magna* (Anderson, 1948), the fathead minnow (Tarzwell and Henderson, 1960; Pickering and Henderson, 1965), bluegill, goldfish, guppy, channel catfish, and the rainbow, brown, brook, and lake trouts (Pickering and Henderson, 1965; Wilford, 1966) and the stonefly, *Acroneuria lycorias* (Warnick and Bell, 1969). *Philodina* was more sensitive to nickel as a chloride while the mayfly, *Ephemerella subvaria*, may be more sensitive to nickelous sulfate than to nickelous chloride and the same may be true for the bluegill (Pickering and Henderson, 1965; Wilford, 1966). The slopes of the data curves were variable (Figures 7 and 8), and the data obtained for nickelous sulfate demonstrates a marked pinwheel effect with convergence of the lines at three points: (1) high concentrations and 100 percent death, (2) ~7 ppm and about 50 percent death, and (3) low concentrations near 0 percent death. No explanation is proposed for this effect.

i. Chromium

An increase in water hardness increases the toxicity of chromium after 24 and 48 hours and the effect of hardness was reversed after 96 hours (Table 2; Figure 9). Chromium was more toxic in soft water to the fathead minnow and the bluegill (Pickering and Henderson, 1965). In general, the rotifer *Philodina* probably was more sensitive to chromium than the mosquito fish, *Gambusia affinis*, Wallen, Greer, and Lasater, 1957), the bluegill (Cairns, 1957; Cairns and Scheier, 1959a; Pickering and Henderson, 1965; Dowden and Bennett, 1965), the goldfish (Dowden and Bennett, 1965), and the zebra fish, *Brachydanio rerio*, (Cairns, Scheier and Loos, 1965). Among the invertebrates, the caddisfly, *Hydropsyche* and the mayfly *Stenonema* may be more sensitive than this rotifer (Roback, 1965) and *Daphnia magna* may be equally sensitive (Dowden and Bennett, 1965). Variability does exist among the insects if chromium is in a different form (Warnick and Bell, 1969). The data curves vary in slope, especially after 24 hours and 48 hours in soft water. These variations in the curves may reflect the mechanism of poisoning by destruction of the gut tissue. This effect has been observed for the largemouth bass, *Micropterus salmonoides* (Fromm and Schiffman, 1958).

j. Silver

Silver toxicity has not been studied very much. These data suggest that silver toxicity varies between 24, 48, and 96 hours (Table 2; Figure 10) and toxicity was the least after 48 hours. *Daphnia magna* (Anderson, 1948) were

quite sensitive to silver (nitrate). The guppy, tadpole of *Bufo valliceps* (Shaw and Gruskin, 1967) and the stickelback (Jones, 1939b) are probably more sensitive.

k. Zinc

Zinc toxicity was similar after 96 hours regardless if it was introduced as chloride or sulfate (Table 2; Figures 11 and 12). Initially, the sulfate salt was more toxic than the chloride. Increased hardness decreased the toxicity of zinc. (See section below – Factors affecting toxicity – for a detailed discussion of factors affecting zinc toxicity). In comparing the 96 hr $EC_{50}$ values for zinc chloride and zinc sulfate, *Philodina* was more sensitive than the bluegill (Cairns, 1957; Cairns and Scheier, 1957 and 1968; Patrick, Cairns and Scheier, 1968; Pickering and Henderson, 1965), goldfish (Pickering and Henderson, 1965), zebra fish, *Brachydanio rerio*, (Cairns, Scheier and Loos, 1965), rainbow trout (Herbert and Shurberg, 1964), perch, *Perca fluviatillis*, roach, *Rutilus rutilis*, gudgeon, *Gobio gobio*, and common bream, *Abramis brama* (Ball, 1967a).

Data for the fathead minnow suggest that *Philodina* was more sensitive in soft water (Pickering and Henderson, 1965). The guppy (Pickering and Henderson, 1965) and the stickleback (Jones, 1939b) may be slightly more sensitive. In comparing the data for other invertebrates, *Daphnia magna* may be more sensitive (Anderson, 1948) but the midge, *Tendipes decorus*, the isopod, *Asellus communis*, the dragonfly, *Argia sp*, the oligochaete, *Limnodrilus hoffmeisteri*, (Wurtz and Bridges, 1961), unidentified tubificid worms (Whitely, 1968), the stonefly, *Acroneuria lycorias*, the mayfly, *Ephemerella subvaria*, the caddisfly, *Hydropsyche betteni* (Warnick and Bell, 1969), and possibly the snail, *Physa heterostropha* (Wurtz and Bridges, 1961; Cairns and Scheier, 1958) are less sensitive.

Hardness affected the toxicity of zinc to the snail, *P. heterostropha*, and so did temperature (Cairns and Scheier, 1958). Hardness effects also were demonstrated for the bluegill and fathead minnow (Pickering and Henderson, 1965).

The toxicity data for zinc exhibit plateaus at the lower concentrations (Figures 11 and 12). These plateaus are most distinct for zinc sulfate and they occur in soft and hard water. These plateaus may be the result of reproduction but they could reflect an age effect on toxicity.

l. Ammonium Chloride

*Philodina* was quite resistant to ammonium chloride (Table 2; Figure 13). Resistance to ammonium chloride was greater than was reported for goldfish (Dowden and Bennett, 1965), mosquito fish, *Gambusia affinis* (Wallen, Greer and Lasater, 1957), brown trout (Herbert and Shurberg, 1964), *Daphnia magna* (Dowden and Bennett, 1965; Anderson, 1948), and the eggs of the snail, *Lymnaea sp*, (Dowden and Bennett, 1965). The data for the bluegill were variable and range from a 96 hr $TL_m$ of 7.7 ppm (Cairns and Scheier, 1959b) to 725 ppm (Dowden and Bennett, 1965). The toxicity curves were quite linear.

m. Phenol

The toxicity of phenol varied between 24, 48, and 96 hours (Table 2; Figure 14). Again this rotifer was extremely tolerant. Most of the literature for fish report 96 hr $TL_m$'s below 50 ppm (Pickering and Henderson, 1965; Trama, 1955; Wallen, Greer and Lasater, 1957). Of the invertebrates tested the snail, *Physa heterostropha*, had a 96 hr $TL_m$ of 94 ppm. Forty-eight hr $TL_m$'s for the caddisfly (*Hydropsyche*), the mayfly (*Stenonema*) (Roback, 1965), and *Daphnia magna* were less than 30 ppm (Dowden and Bennett, 1965).

There are some plateaus in the data curves (Figure 14) and again these represent the effect of reproduction or age.

n. Probit Analysis

One of the principal methods for determining $EC_{50}$ values in bioassay research is the probit analysis (Finney, 1969; Dixon, 1970). To date this method has been used successfully for fish and certain invertebrates where it is easy to control age or size of the test organisms.

The data presented above are for populations of rotifers where the only control was to use populations in a log growth phase. There was no control of age or size of the test organisms. In comparing $EC_{50}$ values read from the actual data and computed from the probit analysis (Table 2), there are some discrepancies in values. Further, when one examines the variations in the slopes of the data curves (Figures 1 through 14), we see that the effects of reproduction, age, and possibly other factors modify the results. Because of the variation in data, it may be that the probit analysis is not a satisfactory bioassay analysis when studying populations of organisms (Dr. F. Calhoun, pers. comm.).

## IV. Physiological and Behavioral Effects

Because *Philodina* lack gills, produce no noticeable mucus, and can withstand low oxygen tensions, a physiological cause of death other than anoxia should be considered. *Philodina* feed by setting up currents with the ciliated corona (Hyman, 1951). It is likely that the site of penetration for the heavy metals is through the gut wall rather than through the tougher lorica. When exposed to zinc, this rotifer withdrew its corona and ceased feeding. Despite this avoidance behavior, the exposed rotifers did not recover when subsequently transferred to clean water. This suggests that either zinc pentration may occur through the lorica or that the damage done was extensive enough to make recovery unlikely despite the avoidance behavior. Allee and Rosenthal (1949) observed that the rotifer, *Philodina roseola*, contracted when it ingested colloidal silver, but did recover when transferred to clean water. This apparent difference in results can possibly be explained by a difference in the chemical behavior of the two metals in the gut. For example, the zinc possibly was more soluble under the acid conditions of the gut compared with the silver.

In both the cadmium bioassay and in the chromium bioassay, there was a marked difference between the 24 and 96 hour $EC_{50}$ values (Table 2). This observation coupled with the observation that the larger bluegill was less sensitive to these compounds at 96 hours compared to *Philodina*, suggests that the toxicity of these compounds may depend upon their penetration into the organism. This was further supported by the observation of Ball (1967b) that although cadmium was very toxic to rainbow trout (*Salmo gairdnerii*, Richardson) in seven days ($LC_{50}$ = 0.008 to 0.010 cadmium), it still takes 30 ppm to kill 50 percent of the fish in one day. Because the speed and extent of toxicity depends on the surface:volume ratio, it appears that the principal site for action for acute chromium and cadmium poisoning may be internal.

Hexavalent chromium compounds are different toxicologically and chemically from the other heavy metals (Doudoroff and Katz, 1953). One property of dichromate compounds that should be mentioned when discussing their toxicity is their strong oxidizing power. Because of this property, $K_2Cr_2O_7$ could denature biological material by oxidizing it.

## V. Factors Affecting Toxicity, Especially Zinc

a. Hardness

The results obtained with *Philodina* agreed with the literature. The higher the hardness, the less toxic the zinc (Table 3 and Figure 15). In Figure 15, only

the "b" value for 48 hours was significant at the 95 percent confidence level. It appeared that there may be a slightly greater effect at 24 hours compared with 48 hours.

b. Alkalinity

Alkalinity in the absence of hardness had little effect on toxicity. This was evidenced by the low $EC_{50}$ values in those test treatments where the alkalinity came from $NaHCO_3$, rather than from $MgCO_3$ or $CaCO_3$ (Table 3 and Figure 16). In Figure 16, although the "b" values were both positive, they were not significant at the 95 percent confidence level.

Alkalinity appeared to have a greater effect at 24 hours compared to 48 hours (Figure 16). It has been demonstrated using vital stains that sections of the gut of *Philodina* are acidic, pH 3.0 to 5.0 (Kwasikpui, 1963). The greater death at 48 hours could be explained by the ingestion of $ZnCO_3$ by the rotifers. As the rotifers feed, they may ingest suspended zinc salts which became soluble when they reached the more acidic regions of the digestive tract.

c. Hardness Combined with Alkalinity

In the rotifer bioassays, the most marked antagonism was observed when both high hardness and high alkalinity were present (Table 3). Note that the highest point in Figure 17 was from a treatment which had both calcium and magnesium in an all carbonate water.

Although neither the "b" value for the effect of hardness at 24 hours (Figure 15) nor the "b" values for the effect of alkalinity (Figure 16) were significant at the 95 percent confidence level, both the 24-hour and 48-hour regression lines for the effect of hardness plus alkalinity on the toxicity of zinc were significant (Figure 17). The wide scatter in Figure 17 is probably a reflection of the varied chemical composition of the dilution waters.

Since toxicity goes down as the sum of hardness and alkalinity goes up (Figure 17), it should be safer to add zinc effluent to a body of water that is hard and high in carbonates. It may also be advisable to mix sewage containing zinc with this type of water before introducing it into a biological treatment facility. Because preexposure to hard or alkaline water did not increase zinc antagonism or control survival in these experiments, preconditioning of the rotifers with this type of water does not appear necessary.

d. Calcium:Magnesium Ratio

For *Philodina* it appears that calcium, by itself, may be a better antagonist to zinic toxicity at a low hardness (total hardness 60 to 89 ppm) and magnesium a stronger antagonist at a higher hardness (total hardness 90 to 162 ppm) (Table 3 and Figure 18). Still, at the lower hardness range the greatest zinc antagonism was at a calcium:magnesium ratio of 7:3 (Figure 18). Reid (1961) reports that natural bodies of water of about this total hardness would have a calcium:magnesium ratio of 5:3. Possibly the absence of one of these two ions puts the animal under a slight stress making it more sensitive to zinc. At the higher hardnesses this stress could be compensated for by the greater zinc antagonizing effect of magnesium by itself. The change from calcium antagonism to magnesium antagonism with the increase in hardness is possibly an indication that calcium and magnesium have different physiological effects which are most apparent at different hardnesses. Possibly, the two hardness ions compete. Heilbrunn (1952) reports that magnesium acts like calcium on protoplasm, but that calcium is much stronger. Thus calcium could antagonize when present in only small amounts; whereas magnesium, although a greater zinc antagonist, must be present in greater amounts to displace the calcium and effect this antagonism.

The calcium:magnesium ratio of the body fluids changes seasonally in animals that hibernate. The percentage of magnesium rises in the winter, as has been shown for both vertebrates and invertebrates (Heilbrunn, 1952; Prosser, 1973). Although seasonal changes in the calcium:magnesium ratio have not been studied for rotifers, *Philodina* will go into a diapause state when exposed to adverse conditions such as drought or cold. It has been shown for crabs that the less active forms, such as *Maja*, have a high magnesium level in the body fluids compared with the more active genera, such as *Carcinus* (Prosser, 1973). An anesthetized state can be produced artificially by the injection of mammals with, or the submergence of some invertebrates in, a magnesium solution (See the review by Heilbrunn, 1952; Prosser, 1973). Possibly a high external magnesium concentration causes a decrease in the rotifer's metabolism. This metabolic drop could protect the rotifer if it decreased the influx of the heavy metal into the rotifer or just put less stress on the rotifer.

e. Temperature

The effect of temperature is summarized in Table 3. In soft water at 10 C the toxicity of zinc was less than at 20 and 30 C after 96 hours. The initial effect after 24 hours suggests that zinc was more toxic at 20 C. The toxicity of zinc was seemingly lower at 30 C after 24 hours and the difference between 20 and 30 C becomes negligible after 96 hours.

Similar effects of low temperature on decreased zinc toxicity have been observed in the snail, *Physa heterostropha*, (Cairns and Scheier, 1958; Wurtz, 1962), bluegill (Cairns et al., 1972), and rainbow trout (Lloyd, 1960). The initial discrepancy at 30 C between the rotifer and other organisms reflects in part the rapid reproduction rate of the rotifer and the age structure of the rotifer population.

f. Life Cycle Stage

Qualitative observations of the sensitivity of the rotifer eggs and newly hatched rotifers made in the rotifer experiments suggest that this rotifer may differ in its sensitivity to some of these metals at different stages of its life cycle. This was evidenced by the observation that eggs which had been exposed to a concentration of zinc or cadmium that had killed all the hatched rotifers in 24 hours when removed and placed in clean water hatched. This suggests that the eggs may have a relatively impermeable membrane which could withstand short pulses of zinc and cadmium pollution in the field. It was observed that young rotifers were more sensitive than the adults.

g. Body Size and Population Size

Possibly, the observation that the young rotifers were more sensitive to cadmium than the adults and that the rotifers were more sensitive than the bluegill can be explained by an effect of body size on toxicity.

Although Allee and Rosenthal (1949) showed an effect of grouping of the rotifer, *Philodina roseola*, on survival when exposed to copper sulfate, there was no apparent effect of sample size on toxicity in these rotifer bioassays.

# SUMMARY

1. The rotifer, *Philodina acuticornis*, feasibly can be used as a bioassay organism for the assessment of water pollution, especially the heavy metals.

2. When comparing the 96 hr $EC_{50}$ values (as ppm of metal) obtained in soft water, the sensitivity from the most toxic to the least toxic was: cadmium, mercury and copper, zinc, silver, nickel (chloride), chromium, nickel (sulfate), lead, and cobalt.

3. When comparing the 96 hr $EC_{50}$ values (as ppm of metal) obtained in hard water, the sensitivity from the most toxic to the least toxic was: cadmium, copper, mercury, chromium, and lead. The $EC_{50}$ data suggest that zinc will follow mercury in toxicity when tested in hard water.

4. When comparing the toxicity of the chloride and sulfate salts of cadmium, nickel and zinc in soft water, cadmium sulfate and zinc sulfate were more toxic than the chloride salt. Nickelous chloride was more toxic than nickelous sulfate.

5. The results suggest that this rotifer may be more sensitive than the bluegill to the salts of cadmium, copper, nickel, zinc, and chromium. The rotifer may be equally or less sensitive to lead. Data for cobalt, silver, and mercury were not available.

6. The toxicity of zinc decreases as water hardness and alkalinity increases. The effect of water hardness and alkalinity is most marked if these are considered together.

7. The data suggest that calcium was the principal antagonist of zinc at water hardness less than 90 ppm and that magnesium was at hardness greater than 90 ppm.

8. After 96 hours at 10 C zinc toxicity was less than at 20 and 30 C. There were no differences between 20 and 30 C.

9. *Philodina* was extremely tolerant of ammonium chloride and phenol.

10. Rotifer eggs were very resistant to toxicants and young were more sensitive than adults.

11. There was no apparent effect of population size on toxicity.

13. The probit analysis may not be appropriate for populations of organisms which vary in age and size.

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# FIGURES

Figure 1

The Toxicity of Cadmium (as Chloride) to *Philodina* in Soft Water

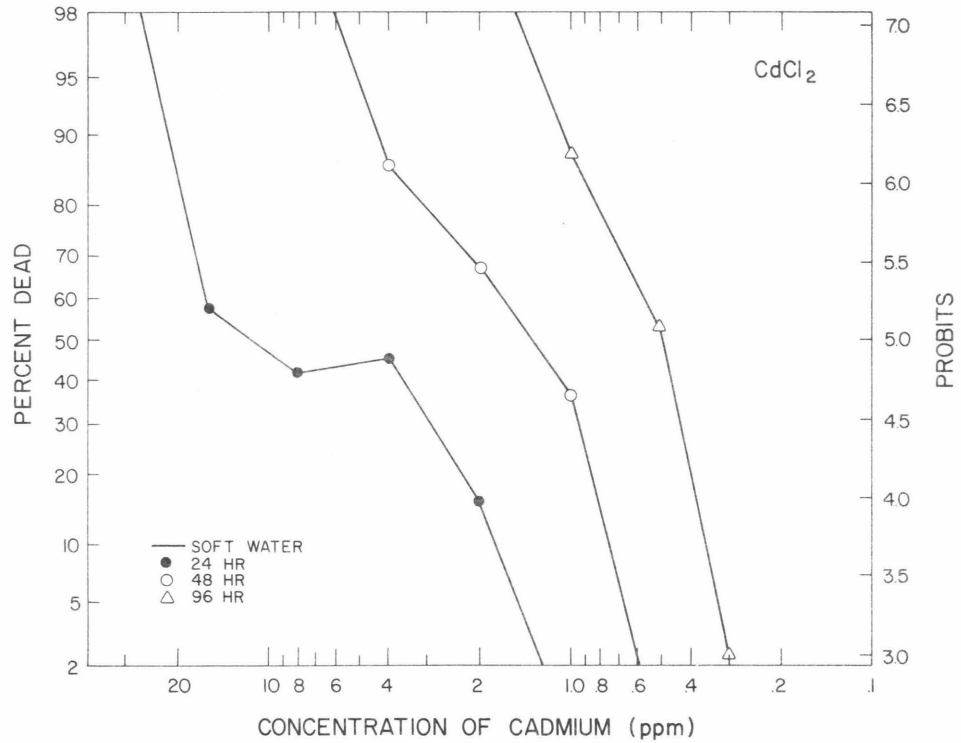


Figure 2

The Toxicity of Cadmium (as Sulfate) to *Philodina* as a Function of Water Hardness

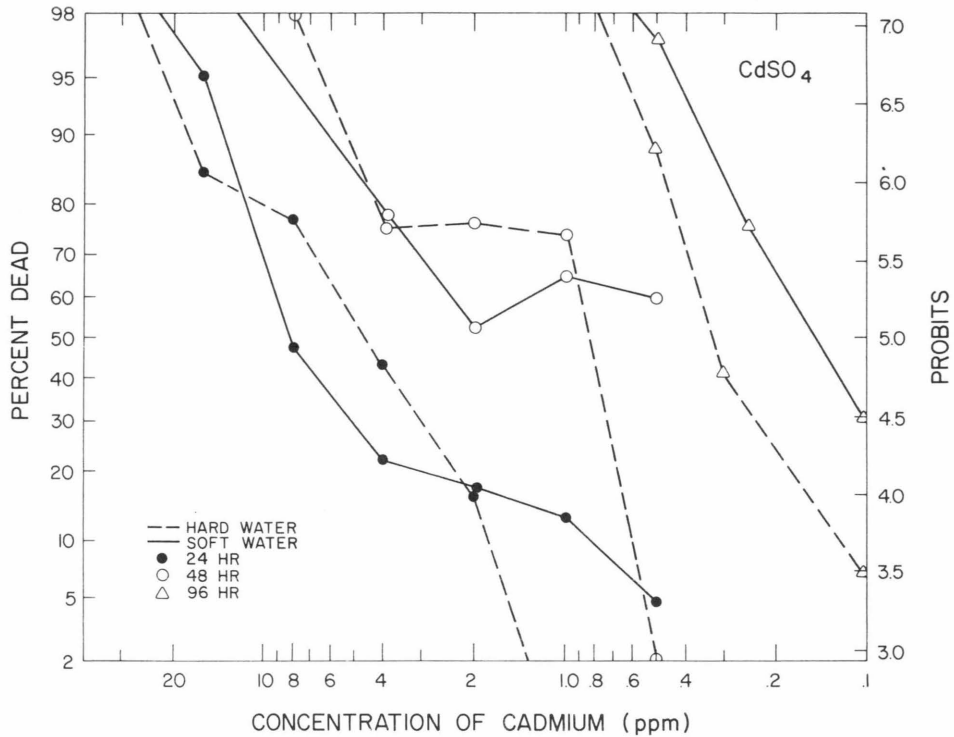


Figure 3

The Toxicity of Cobalt (as Chloride) to *Philodina* in Soft Water

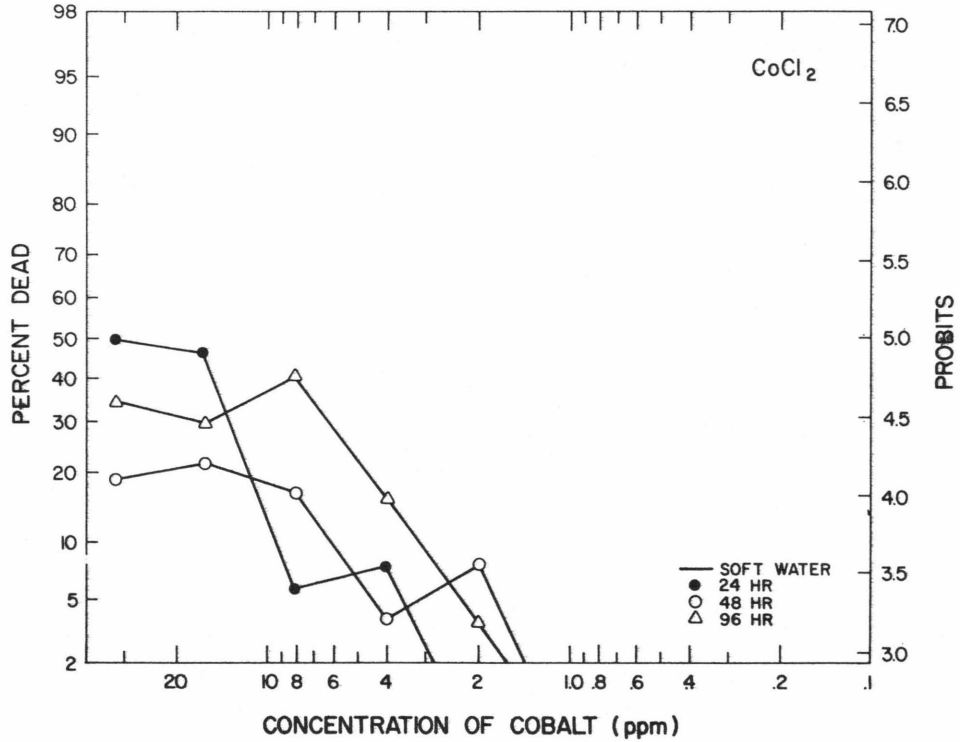


Figure 4

The Toxicity of Copper (as Sulfate) to *Philodina* as a Function of Water Hardness

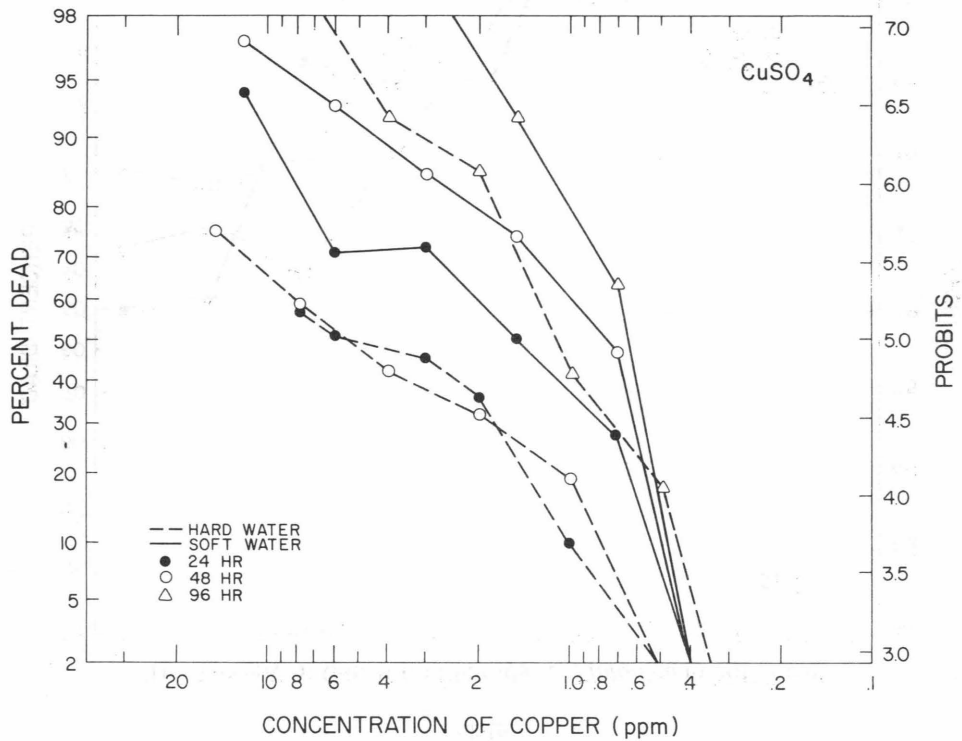


Figure 5

The Toxicity of Lead (as Chloride) to *Philodina* in Soft Water

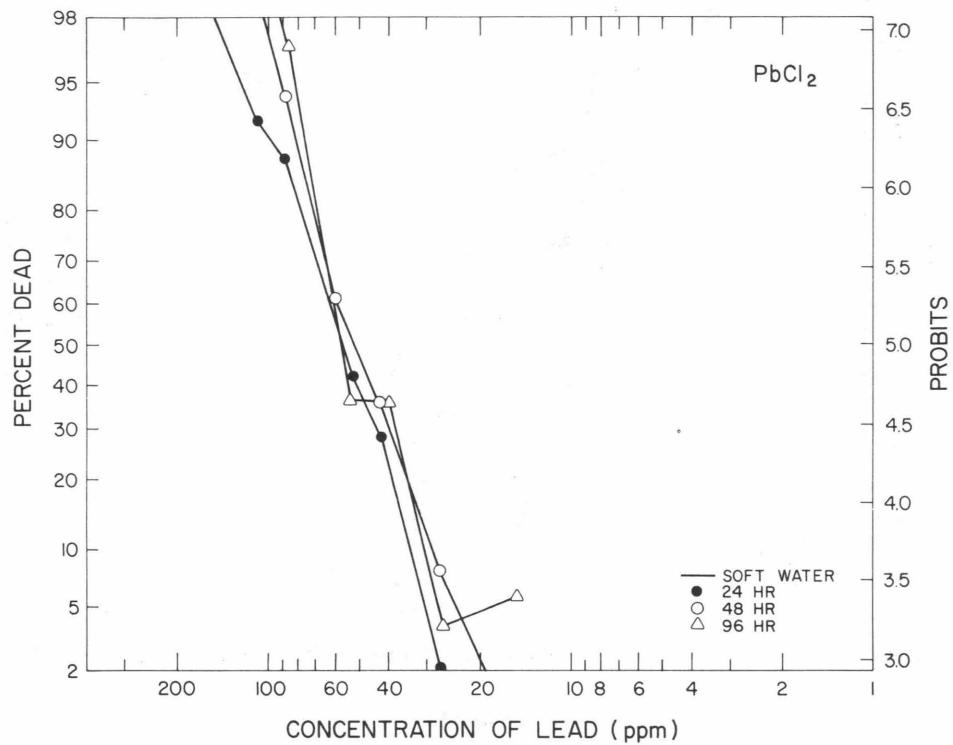


Figure 6

The Toxicity of Mercury (as Chloride) to *Philodina* as a Function of Water Hardness

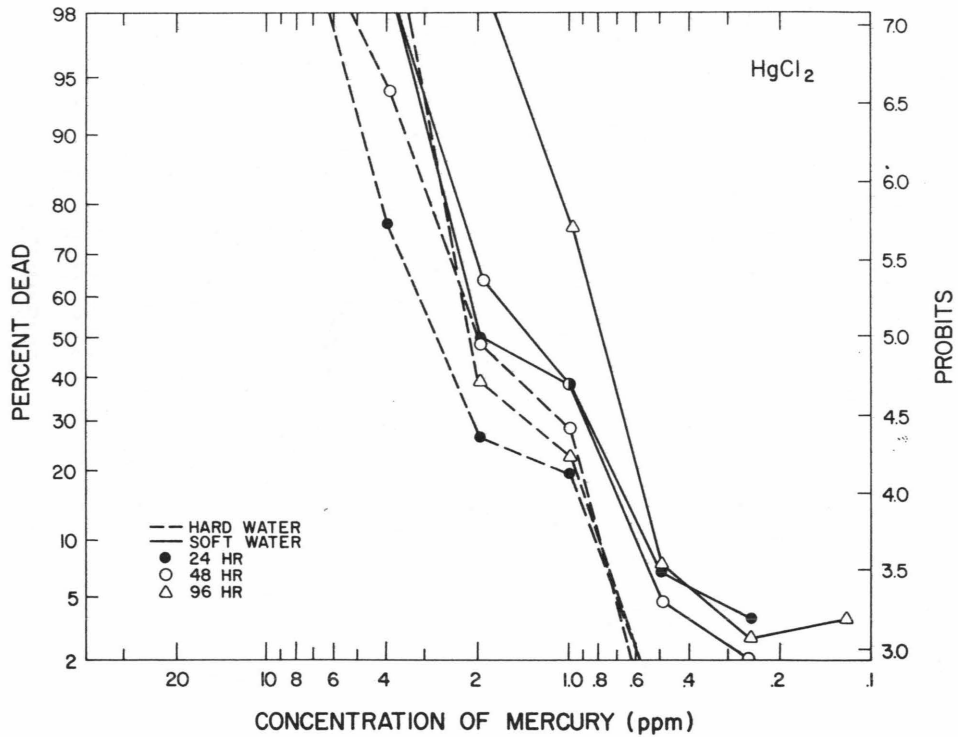


Figure 7

The Toxicity of Nickel (as Chloride) to *Philodina* in Soft Water

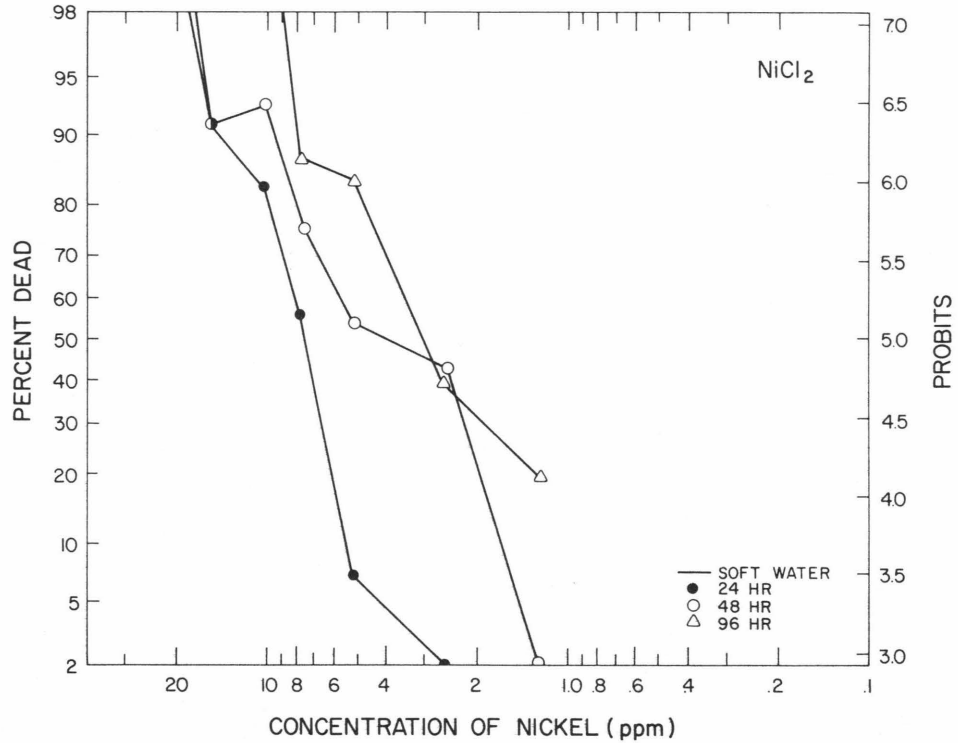


Figure 8

The Toxicity of Nickel (as Sulfate) to *Philodina* in Soft Water

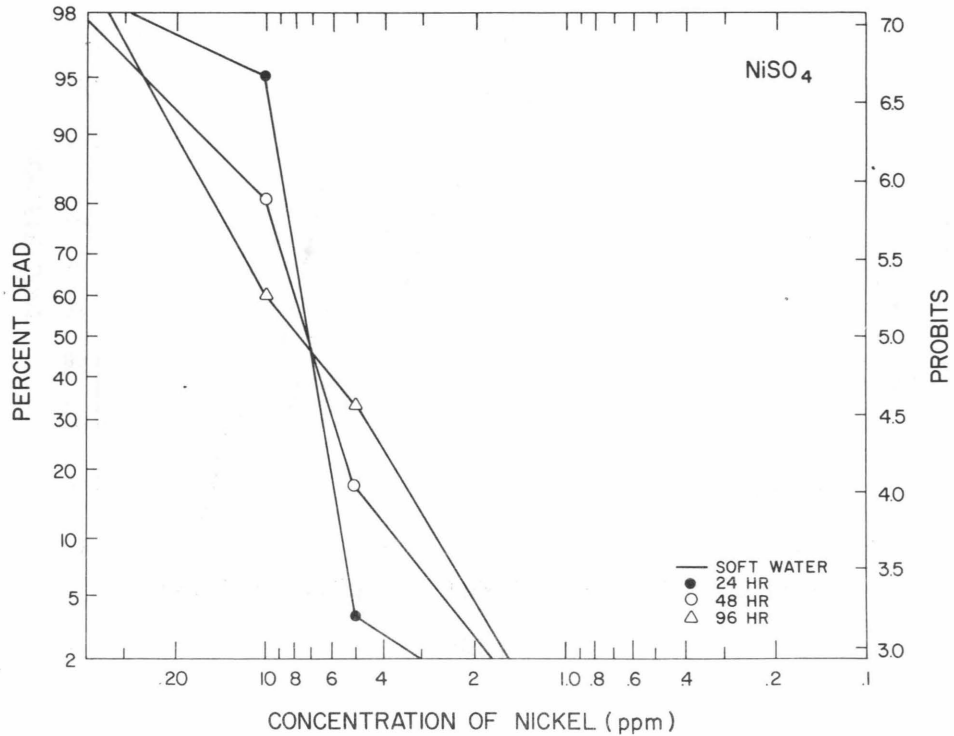


Figure 9

The Toxicity of Chromium (as Potassium Dichromate) to *Philodina* as a Function of Water Hardness

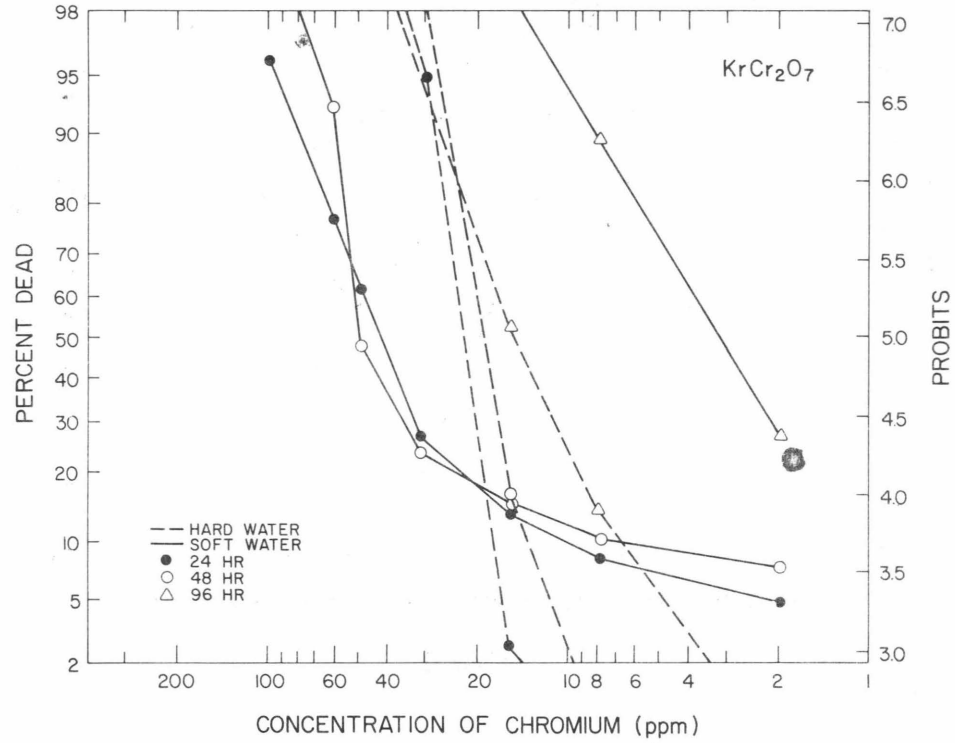


Figure 10

The Toxicity of Silver (as Nitrate) to *Philodina* in Soft Water

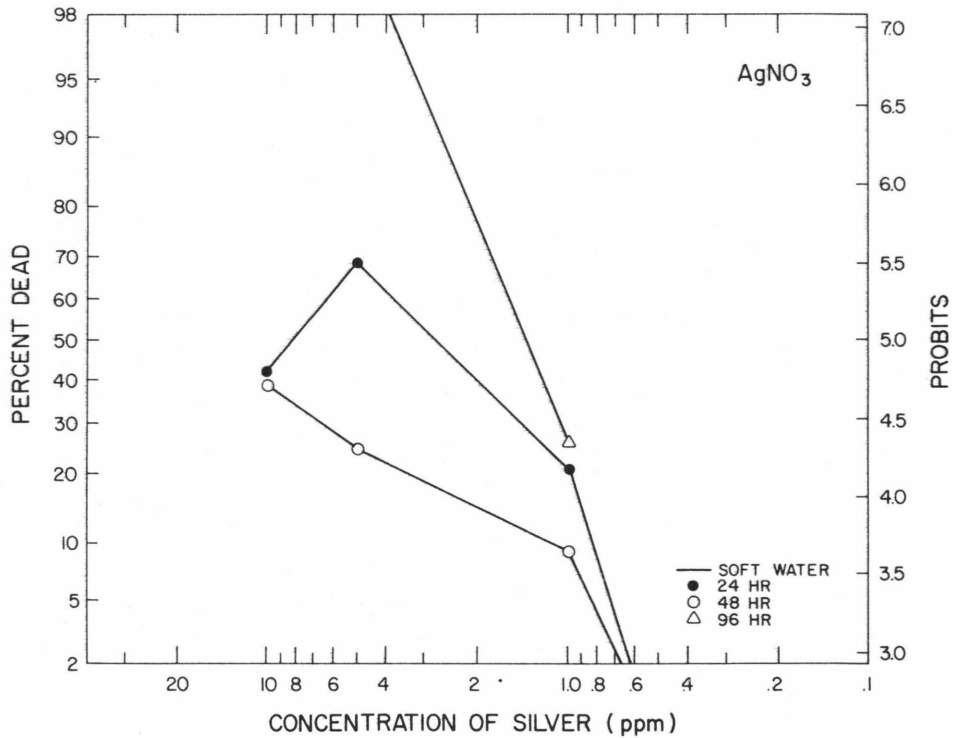


Figure 11

The Toxicity of Zinc (as Chloride) to *Philodina* in Soft Water

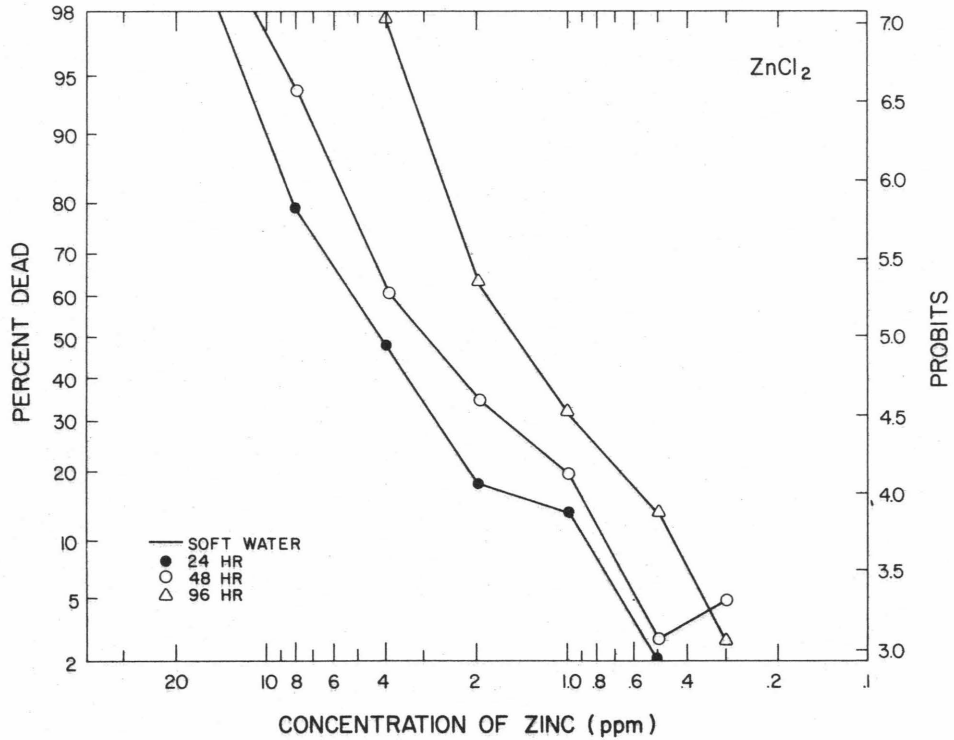


Figure 12

The Toxicity of Zinc (as Sulfate) to *Philodina* as a Function of Water Hardness

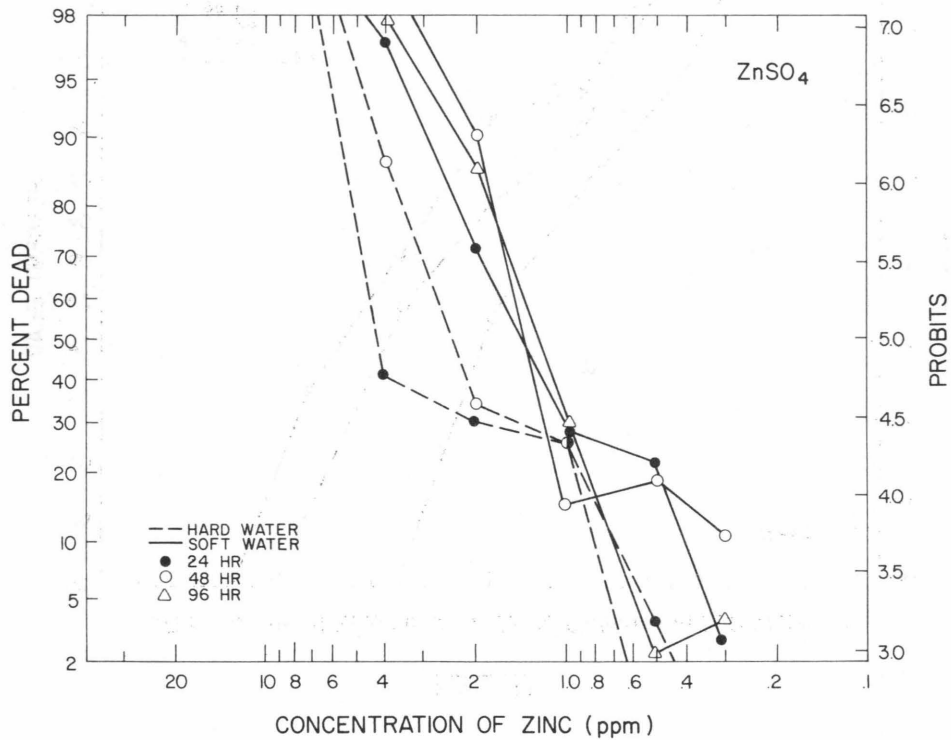


Figure 13

The Toxicity of Ammonium Chloride to *Philodina* in Soft Water

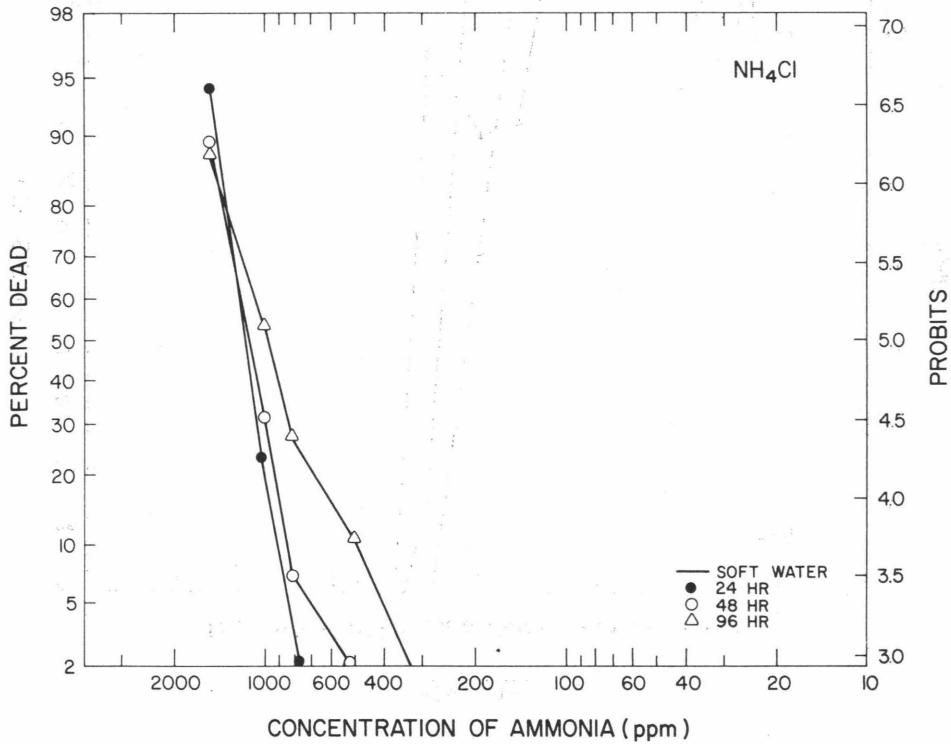


Figure 14

The Toxicity of Phenol to *Philodina* in Soft Water

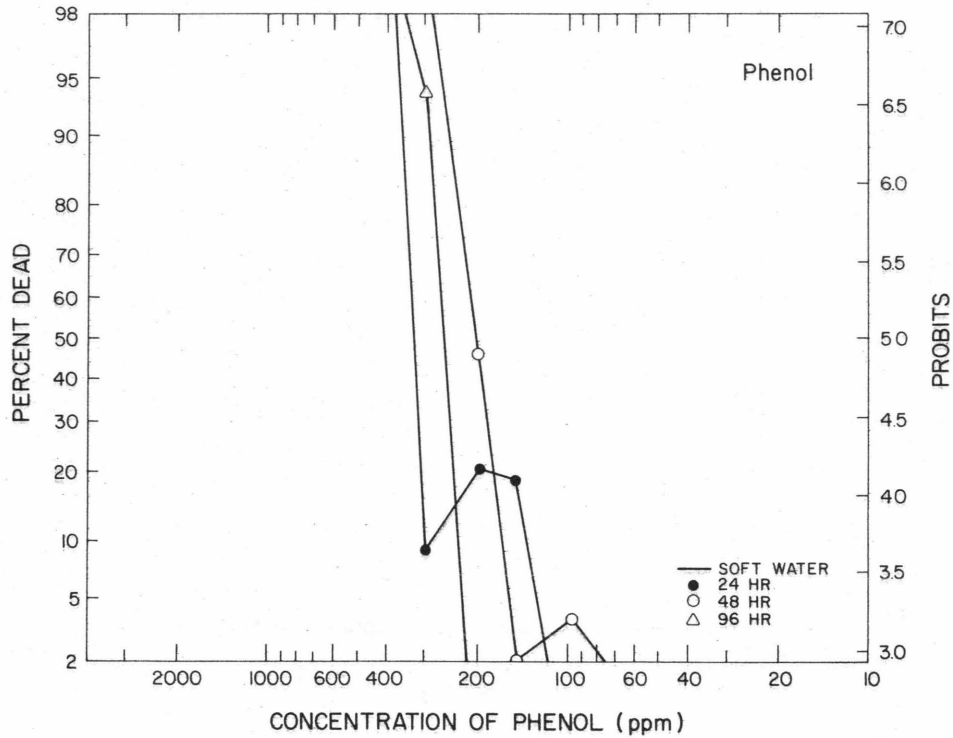


Figure 15

The Effect of Water Hardness on the Toxicity of Zinc Sulfate to *Philodina*

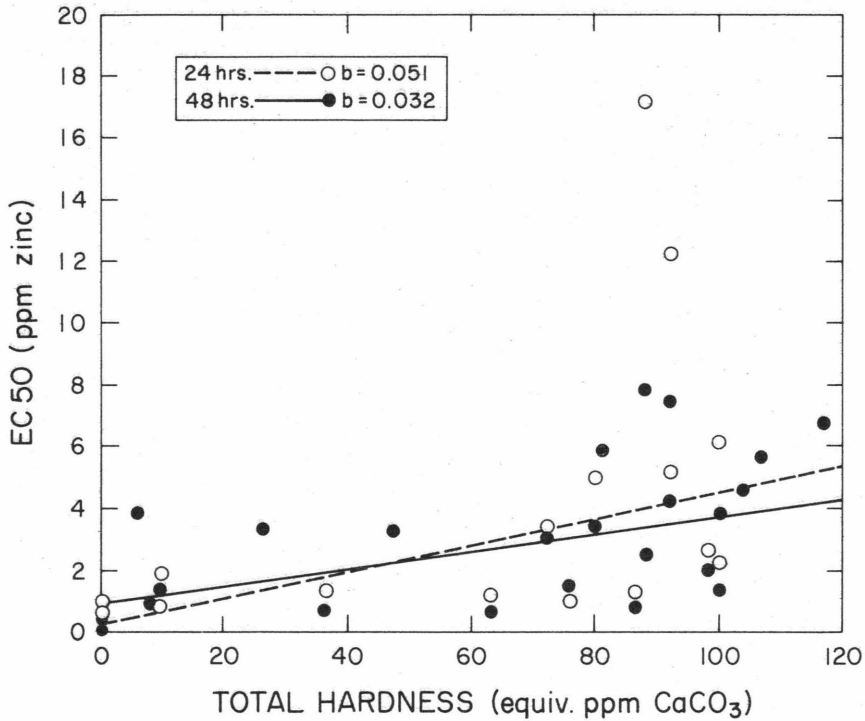


Figure 16

The Effect of Water Alkalinity on the Toxicity of Zinc Sulfate to *Philodina*

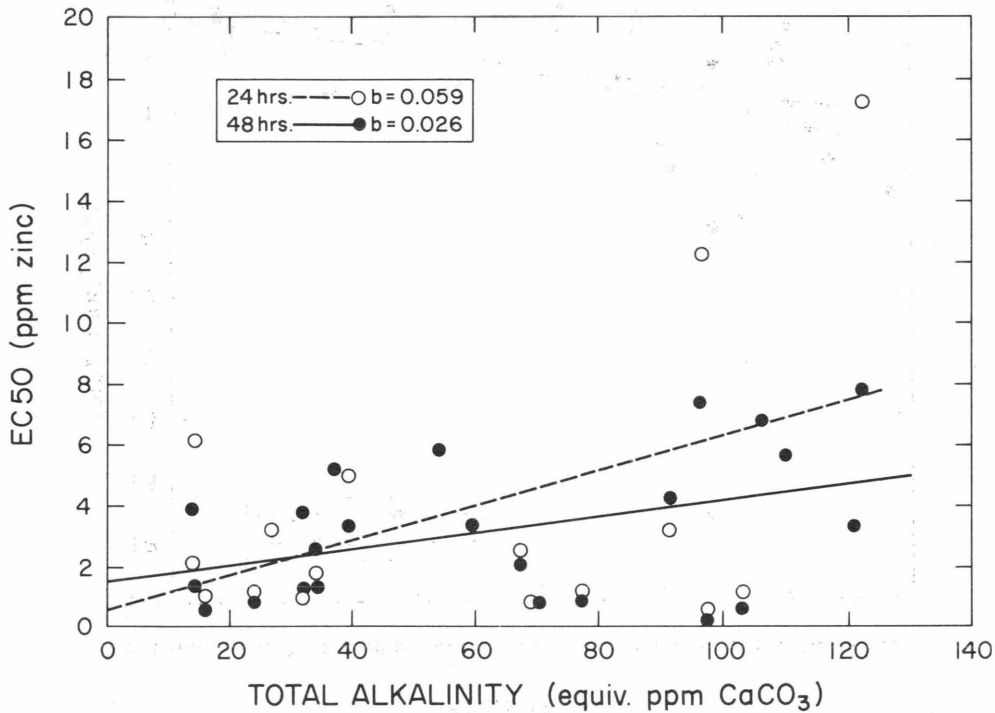


Figure 17

The Effect of Water Alkalinity Plus Hardness
on the Toxicity of Zinc Sulfate to *Philodina*

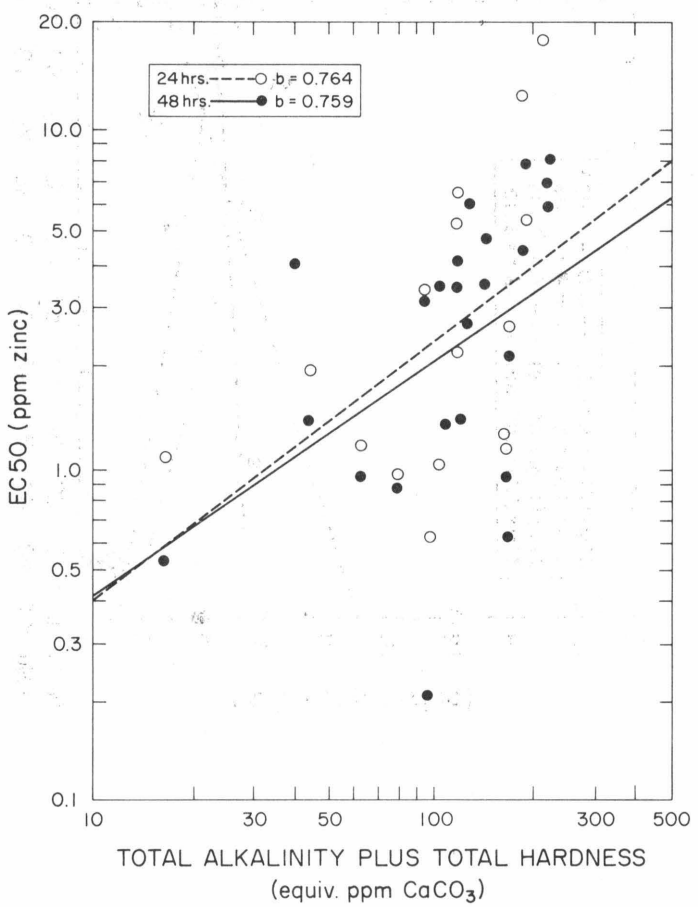
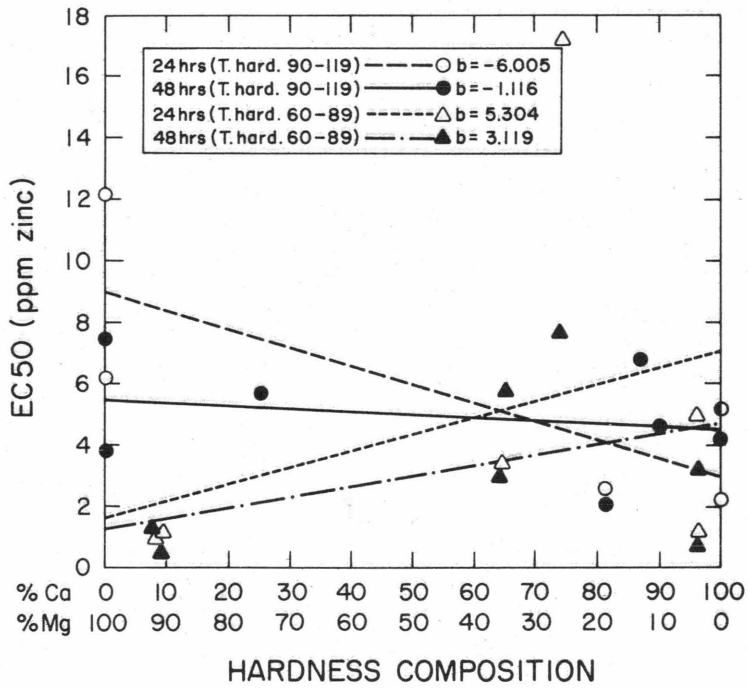


Figure 18

The Effect of the Ratio of Calcium to Magnesium on the Toxicity of Zinc Sulfate to *Philodina*



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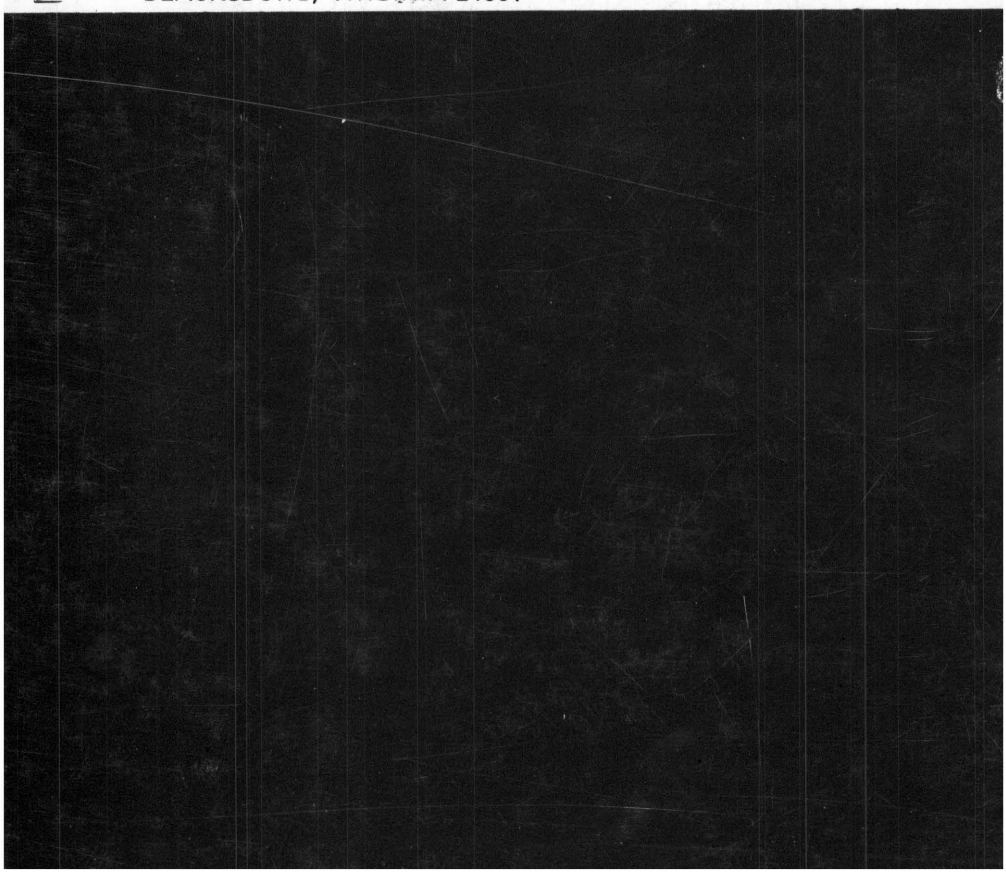
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