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# PREFACE

Rotifers are microscopic organisms which inhabitat every type of aquatic environment. Their importance in aquatic systems varies from providing food for predators, such as fish, to maintaining viable decomposition communities in waste treatment facilities.

Using pure compounds, the potential use of rotifers to assess toxicity of water pollutants was investigated. The purpose of this research was to determine the sensitivity of these organisms to combinations of pollutant stress.

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# ABSTRACT

This research assesses the toxicity of interactions of selected inorganic compounds to the rotifer *Philodina acuticornis.* Bioassays were conducted in a synthetic dilution medium under static conditions. Effective concentrations of the compounds that produced rotifer mortality were determined by recording, after 24 and 48 hours, the number of dead or withdrawn animals in triplicate samples.

Results indicate a range of rotifer response to these compounds, from supra-additive (synergistic) to antagonistic. The interaction of chromium and chlorine was supra-additive. Additive interactions were observed for mixtures of chromium and fluoride, copper and chromium, and copper and chlorine; the interaction of copper and chromium fluctuated along the line of additivity. Combinations of iron and chlorine, iron and fluoride, zinc and fluoride, and chlorine and fluoride produced antagonistic responses. No interactions were observed for mixtures of zinc and copper and copper and fluoride. The interaction of zinc and chlorine was unique: the effect was infra-additive below a 50:50 mixture and antagonistic above this mixture. Zinc was most toxic in high concentrations, and as the concentrations of chlorine increased, rotifer sensitivity decreased, probably because of the formation of metal complexes.

Key Words: Toxicity, Invertebrate Bioassays, Rotifers, Inorganic Compounds, Pollution, Hazard Assessment.

# INTRODUCTION

State and government regulatory agencies are concerned about industrial effluent toxicity to aquatic life. Water quality standards based on inadequate toxicological information often have been established because appropriate information did not exist. Most industries, such as Virginia's artificial fiber and electroplating industries, are required to conduct periodic chemical analyses on their effluents, and some are required to conduct fish bioassays. Aggregate chemical toxicity is difficult to extrapolate from chemical analyses, and a long time lag between sample collection and availability of results may also present a major problem. By the time data are received, the toxic effluent has moved quite a distance from the point of discharge. Fish bioassays are also relatively long, but acute toxicity data are possible within four days. However, fish bioassays are expensive in time, personnel, space, and amount of effluent and diluent required.

Furthermore, most water quality standards are derived from studies on the toxicity of single or pure compounds to fish, algae, and a variety of invertebrates. Buikema et al. [1974a and 1974b] studied the response of the rotifer *Philodina* to single compounds, but when the rotifer was tested on an effluent from a metal-plating plant, the animal was even more sensitive than was anticipated. Important components of this effluent were copper, chromium, zinc, iron, fluoride, and cyanide [Virginia Water Resources Research Center, personal communication, October 4, 1974].

Interaction studies have been conducted on fish and *Daphnia.* They have investigated the effects of metals, pesticides, detergents, phenolics, polychlorinated biphenyls (PCB's), coal conversion products, and other organic and inorganic salts [see *Additional Selected References,* p. 19]. In spite of these studies, water quality recommendations still are based on the response of the most sensitive species to single compounds [National Academy of Sciences, 1973]. It will be impossible to proceed beyond this level with confidence until a substantial data base involving known combinations of pollutants is available. The purpose of this study was to examine the interaction of selected compounds present in an effluent to which the rotifer *Philodina* was most sensitive.

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# MATERIALS AND METHODS

## I. Culturing

The rotifers *(Philodina acuticornis)* were cultured using a soft dilution water (modified from Cairns and Scheier [1957] ) prepared from glass-distilled water and American Chemical Society (A.C.S.) grade chemicals. Each of the first five chemicals *(Table 1)* was measured from a concentrated (200x) stock solution directly to the distilled water. The last three chemicals were weighed and added directly to the distilled water. The volume prepared each time was 20 liters. The dilution water was bubbled with $CO_2$ until the calcium and magnesium salts were dissolved; it was then bubbled with air at least 12 hours to raise the pH, to remove $CO_2$, and to oxygenate the water. The desired characteristics of the water were a pH of 7.5, an alkalinity of 35 ppm $CaCO_3$, and a total hardness of 40 ppm $CaCO_3$ with Ca and Mg contributing 28 ppm and 12 ppm, respectively.

For the culture medium, three grams of *Purina Trout Chow* (Ralston-Purina Co.) were blended in 200 ml of distilled water for one minute; the resulting suspension was filtered through fine bolting cloth, and one part of the filtrate was added to 19 parts of dilution water. The dilute trout chow medium was autoclaved and stored in the refrigerator for up to two weeks. Before adding the medium to the cultures, the trout chow medium was diluted 50 percent, and a minute amount of a commercial vitamin mixture was added before the mixture was aerated.

The rotifer cultures were maintained in 250-ml erlenmeyer flasks (containing 150 ml of culture) or in 19-ml test tubes (containing 16 ml of culture). One-third of the culture was removed twice a week and replaced with fresh, sterile trout chow medium. The rotifer cultures contained bacteria, protozoans, and algae on which the animals fed. Dominant protozoans included *Chilomonas, Cyathomonas,* and *Bodo.*

Stock cultures of the rotifers were maintained at 20°C(±3°C) and a 16L:8D photoperiod. Seventy-two hours prior to testing, the animals were fed and then acclimated to 20°C and a 12L:12D photoperiod. Animals cultured in the test tubes were used for the experiments.

## II. Preparation of Test Solutions and Experimental Procedure

A freshly prepared solution of each toxicant (100 ppm or greater of each toxicant) was diluted to obtain the concentrations required in all experiments. The toxicants used were zinc, copper, chromium, iron, fluoride, chlorine, and cyanide *(Table 2)*.

Tests were carried out in triplicate at each concentration for each time period. Glass petri dishes of 5-cm diameter were used as test chambers. Four ml of mixed age rotifer populations (40-100 rotifers) were placed in each dish, and one ml of a 5x concentration of the toxicant was then added. The rotifers were kept at the acclimated conditions and counted at the appropriate time period.

## III. EC-50 Studies

EC-50 was defined as that (effective) concentration at which 50 percent of the animals were affected by a toxicant in a specified time period (24, 48, and 96 hours). The criterion for "affected" was no visible internal or external motion of an animal when exposed to bright light. This criterion was selected because a withdrawn animal (even if not dead) is neither feeding nor reproducing and is thus functionally removed from the community. In the majority of cases, the animals were dead.

To determine the percent of rotifers affected at the end of the assay period, the number of affected rotifers in each sample was counted; then formalin was added to the sample and the total number in each of the samples was counted. The number of animals unaffected was determined by subtraction. The accuracy of this approach has been previously determined [Buikema et al., 1974a and 1974b].

The results for the triplicates were added together for the EC-50 determination. EC-50 values were determined by the straight line estimation method [American Public Health Association, 1973].

## IV. Studies Using Combinations of Toxicants

Using the EC-50 values obtained after 24 hours in the preliminary experiments, combination experiments were set up using proportional mixtures of toxicants. For example, 80 percent of the EC-50 of toxicant A and 20 percent of the EC-50 of toxicant B were added together. For each combination the entire range [*0% (A) + 100% (B)*] to [*100% (A) + 0% (B)*] was tested in 10 percent intervals *(Figure 1)*. EC values were determined for 24 and 48-hour periods, but the 96-hour tests were not conducted because of excessive reproduction interaction [Buikema et al., 1974a and 1974b]. In addition, combination studies with cyanide were not conducted because of the low sensitivity of the rotifer to cyanide and because the high concentrations used were dangerous to humans [Office of Safety and Health Administration, personal communication]. The number of animals affected was determined as described above, and the results for the triplicates were averaged. Additivity, infra- and supra-additivity, or antagonism (any interaction that reduces toxicity) was determined graphically *(Figure 1),* using a modification of Warren [1971] and Osterhout [1914].

## V. Determination of Amount of Toxicant Present

Heavy metals were analyzed with an atomic absorption spectrophotometer [American Public Health Association, 1973]. Fluoride concentrations were determined using an Orion fluoride-ion electrode. Chlorine was measured amperometrically using a Wallace and Tiernan titrator. Cyanide concentrations, as amount of HCN present, were calculated from introduced amounts [Broderius, 1973].

# RESULTS AND DISCUSSION

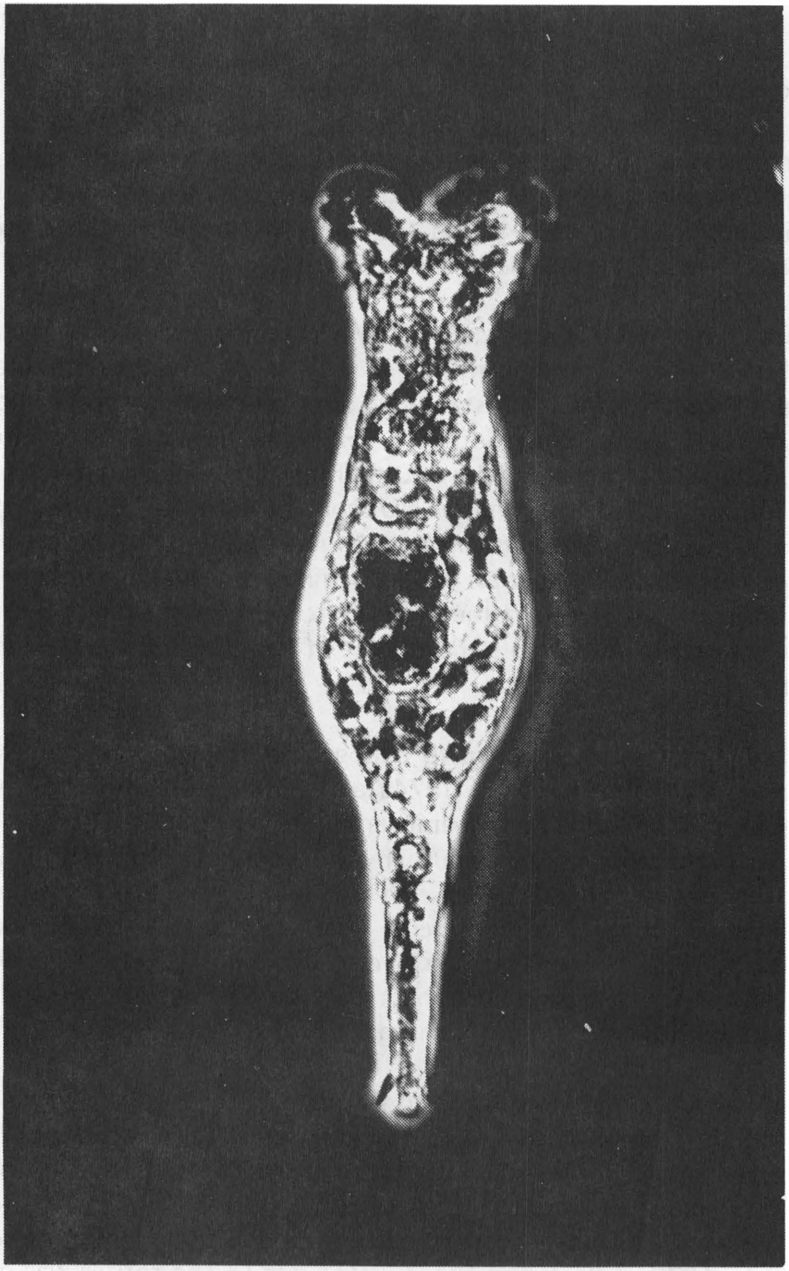
## I. EC-50 Studies

Studies showed that the water characteristics were within the guidelines established previously, except for cyanide experiments [see *Culturing,* p. 5; Buikema et al., 1974a and 1974b]. Concentrated HCl was used to adjust the pH in these experiments.

In preliminary tests, the percent survival over time was used to determine EC-50 values for each toxicant *(Table 3).* After 24, 48, and 96 hours, the order of toxicants, from most toxic to least toxic, was: chlorine > copper >> zinc >> iron > chromium >>> HCN >> fluoride. Results for the metals compare favorably with results obtained earlier by Buikema et al. [1974a and 1974b] for *Philodina acuticornis* and Jones [1937 and 1940] for the planarian *Polycelis nigra* and the amphipod *Gammarus pulex.* Jones's order of toxicity was: copper > zinc > chromium. Biesinger and Christensen [1972] found the order of toxicity of the heavy metals to *Daphnia magna* to be the same as that of *Polycelis nigra* and *Philodina acuticornis.*

*Philodina* was more sensitive to copper and zinc than was previously reported by Buikema et al. [1974a and 1974b]. A comparison of the responses of *Philodina* and other invertebrates to copper, zinc, and chromium has been discussed previously by Buikema et al. [1974a and 1974b]. The rotifer was more sensitive than fish to chromium and zinc but less sensitive to copper. Aquatic insects [Warnick and Bell, 1969] and fish [Ebeling, 1928] were more sensitive to iron than was *Philodina,* which was probably as sensitive to iron as *Daphnia magna* [Dowden and Bennet, 1965; Biesinger and Christensen, 1972].

Fluorides are much more toxic to fish than to the rotifer [Angelovic et al., 1961]. After 48 hours *Philodina* was as sensitive to chlorine as *Daphnia pulex;* but *Daphnia magna,* the riverine snail *Nitocris,* and the ogligochaete *Aeolosoma* were more tolerant to chlorine than was the rotifer [Cairns et al., 1977]. Similarly, fish are more tolerant than the rotifer [Zillich, 1972; Arthur and Eaton, 1971, etc.]. The rotifer was one of the most tolerant organisms to cyanide with the possible exception of the oligochaete *Aeolosoma* [Cairns et al., 1977]. Fish were at least 70 times more sensitive to cyanide than was the rotifer [Jones, 1964; Doudoroff et al., 1966, and Broderius, 1973].



Philodina acuticornis

(~600x)

## II. Combination Studies

Combination studies were conducted for 24 and 48 hours to minimize the effects of reproduction. Many methods have been proposed to analyze graphically the interaction of two compounds. Sprague [1970] used the concept of toxic units, while Warren [1971] proposed plotting the solution combination versus the reciprocal of the $TL_m$. Marking and Dawson [1975] proposed an equation for describing quantitatively the additive toxicity of chemicals and determining the significance to their additive toxicity index. We have elected to use a modification of the method of Warren [1971] and Osterhout [1914] , and the data are presented as percent mortality versus solution combinations *(Figures 2 - 12)* rather than the reciprocal of the $TL_m$ or EC value versus solution combinations.

A hypothetical graph *(Figure 1)* demonstrates the possible kinds of interactions that may occur between two toxicants (modified from Warren, [1971] ). The base of the pyramid represents a "strictly" additive interaction where the percent survival remains at 50 percent irrespective of the proportions of equally toxic solutions, i.e., [*80% (A) + 20% (B)*] is as toxic as [*40% (A) + 60% (B)*], etc. No interaction or independent action occurs when a 50:50 combination is half as toxic as either solution tested separately. In this case, each toxicant is being proportionately diluted by the other and no interactions occur. The apex of the pyramid represents 75 percent in a 50:50 mixture. The sides of the pyramid represent the survival expected if there is no interaction between the chemicals as they are proportionately diluted. Data above the pyramid indicate that survival is greater than expected if there is no interaction (i.e., greater than 75 percent in a 50:50 mixture). These data suggest that the interaction between toxicants was antagonistic.

Data that occur within the pyramid are considered infra-additive. By definition, infra-additive interactions are less than "strictly" additive, while supra-additive interactions are more than "strictly" additive. Supra-additive interactions also are known as "synergistic interactions," but Warren [1971] and others do not recommend the use of "synergism" because of its ambiguity. The pyramid depicted in *Figures 2 - 12* approximates the level of "strict additivity" (50 percent survival irrespective of proportions of equally toxic solutions) and "no interaction" (75 percent survival in a 50:50 mixture, etc.).

The results of the combination study are summarized in *Table 4* and depicted in *Figures 2-12.* Part of the fluctuations of the values are the result of testing mixed-age, reproducing populations of rotifers [Buikema et al., 1974a and 1974b]. The interaction of chromium and chlorine was the only truly supra-additive interaction observed *(Figure 2).* Antagonistic responses were observed for iron and chlorine *(Figure 3),* zinc and fluoride *(Figure 4),* iron and fluoride *(Figure 5),* and chlorine and fluoride *(Figure 6).* All other combinations varied from additive to no interaction. The results also varied after 24 and 48 hours for some mixtures such as chromium and fluoride *(Figure 7),* and chromium and copper *(Figure 8).* Additionally, some mixtures varied from one type of interaction to another type of interaction depending on the concentrations of toxicants studied. A good example of this was the interaction of zinc and chlorine *(Figure 9).*

Two combinations of metals were studied. After 24 hours, copper and chromium *(Figure 8)* appeared to be strictly additive even though the valued fluctuated around the line of additivity. The effect may be slightly infra-additive when the proportion of copper and chromium is 50:50. After 48 hours, the response varied from infra-additive when chromium was most concentrated, to supra-additive when copper was most concentrated. The shift to the left in infra-additivity after 48 hours may be in part the result of rotifer reproduction because the young are more sensitive than the adults and because chromium sensitivity increases with time *(Table 3).* The low EC value (30 percent) for 100 percent copper was not expected and also may have been due to reproduction of the rotifers and excessive kill of the young. Similar reproductive interactions were observed by Buikema et al. [1974a and 1974b].

The study of zinc and copper *(Figure 10)* basically showed no interaction between the two metals. This effect may be expected when the effects of the metals are independent [Warren, 1971]. This no-interaction effect is different than the response obtained by Cairns and Scheier [1968], Doudoroff [1952], Wilbur [1969], and LaRoche [1972]; but similar to those obtained by Lloyd [1961], Sprague and Ramsey [1965], and Eisler and Gardner [1973].

The anionic interactions, chlorine and fluorine *(Figure 6),* apparently were antagonistic, although this antagonism may be an artifact. The chlorine was added as calcium hypochlorite. Because calcium and fluoride combine readily to form an insoluble precipitate, much of the fluoride effectively was removed from solution. In the range from 40 percent chlorine and 60 percent fluoride to 90 percent chlorine and 10 percent fluoride, the percent survival exceeded 80 percent *(Figure 6)*. Only when the solution contained 100 percent of the chlorine concentration did the mortality reach 50 percent. Consequently, part of the "antagonism" was really a decrease in the effective concentration of fluoride while the chlorine levels were not sufficiently toxic to provide a 50 percent mortality.

Of the metal-chlorine mixtures *(Figures 2,3,9,* and *11)*, the results varied. Chromium and chlorine *(Figure 2)* were supra-additive, and the effect was most apparent as chlorine concentration increased. The chromium interaction was different than when it was combined with copper *(Figure 8)*. Copper and chlorine *(Figure 11)* were basically additive. In this case, metal complexes probably were not formed, and all the mixtures were as toxic as either solution tested alone. Iron and chlorine *(Figure 3)* were antagonistic probably because of the formation of metal complexes. Zinc and chlorine *(Figure 9)* were infra-additive below a 50:50 mixture and antagonistic above that mixture. In this instance zinc was probably most toxic when in high concentration, but as the chlorine concentration increased, rotifer sensitivity decreased as metal complexes probably were formed.

Of the four metal-fluoride combinations, the results for iron and zinc indicated that the interactions were antagonistic *(Figures 4* and *5)*. This antagonism was probably due to metal complexes which were formed. Brown et al. [1969] suggested that when zinc predominated a mixture of zinc, phenol, and ammonia, antagonistic effects were demonstrated in trout bioassays. Conversely, preexposure to zinc enhanced the sensitivity of rainbow trout to alkylbenzene sulfonates (ABS) [Brown et al., 1968]. Chromium and fluoride *(Figure 7)* generally were additive during the test period. The effect of copper and fluoride *(Figure 12)* appeared to be slightly antagonistic, but in all probability there was no interaction. The National Academy of Sciences [1973] discussed the idea that "the toxicity of a mixture of pollutants may be estimated by expressing the actual concentration of each toxicant as a proportion of its lethal threshold concentration (usually equal to the 96-hr LC-50) and adding the resulting numbers for all toxicants. If the total is 1.0 or greater, the mixture will be lethal." The basic premise for this system is that lethal actions are additive.

In this research on *Philodina*, the proportions of each combination studied always equaled 1.0. If the lethal actions were strictly additive, we would predict 50 percent mortality in all situations. Of the 11 combinations studied, only two, chromium plus fluoride and copper plus chlorine, were additive. The nine remaining results were not simple additive interactions. Some of the antagonistic responses, e.g., iron and fluoride, probably were due to the formation of chemical complexes. Similarly, Doudoroff et al. [1966] demonstrated that the form of metal-cyanide complexes affected their toxicity to fish. When metal complexes are formed, we could expect a deviation from an additive interaction. For chromium and chlorine and zinc and chlorine, other interactions occurred which were not strictly additive.

It is proposed that different modes of action had an effect on the results obtained. It appears that the additive interaction of toxicant mixtures demonstrated for fish [National Academy of Sciences, 1973] does not always occur for the rotifer *Philodina.* Physiological differences between rotifers and fish may account for some of the variation in their responses to toxic mixtures.

# SUMMARY

Interaction studies of selected inorganic pollutants to the rotifer *Philodina acuticornis* indicated that:

1. The interaction of chromium and chlorine was supra-additive.

2. The interactions of the following mixtures were additive: chromium and fluoride, copper and chromium, and copper and chromium. The interactions of copper and chromium varied but were probably additive.

3. The interactions of the following mixtures were antagonistic: iron and chlorine, iron and fluoride, zinc and fluoride, and chlorine and fluoride.

4. The interaction of zinc and chlorine was infra-additive when the proportion of zinc was the greatest and antagonistic as the proportion of chlorine increased.

5. No interactions were observed for mixtures of zinc and copper and copper and fluoride.

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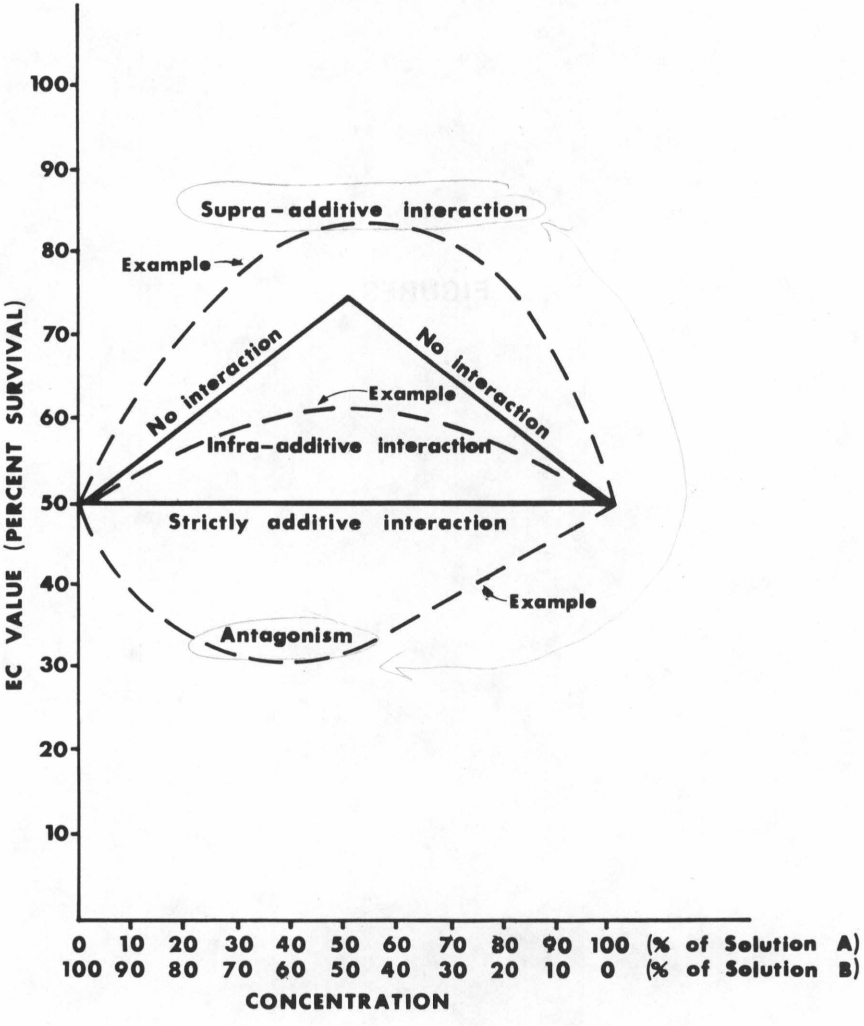
# FIGURES

## FIGURE 1
Possible Interactions Between Two Hypothetical Toxicants



## FIGURE 2

Interaction Effects of Chromium and Chlorine to the Rotifer *Philodina acuticornis*

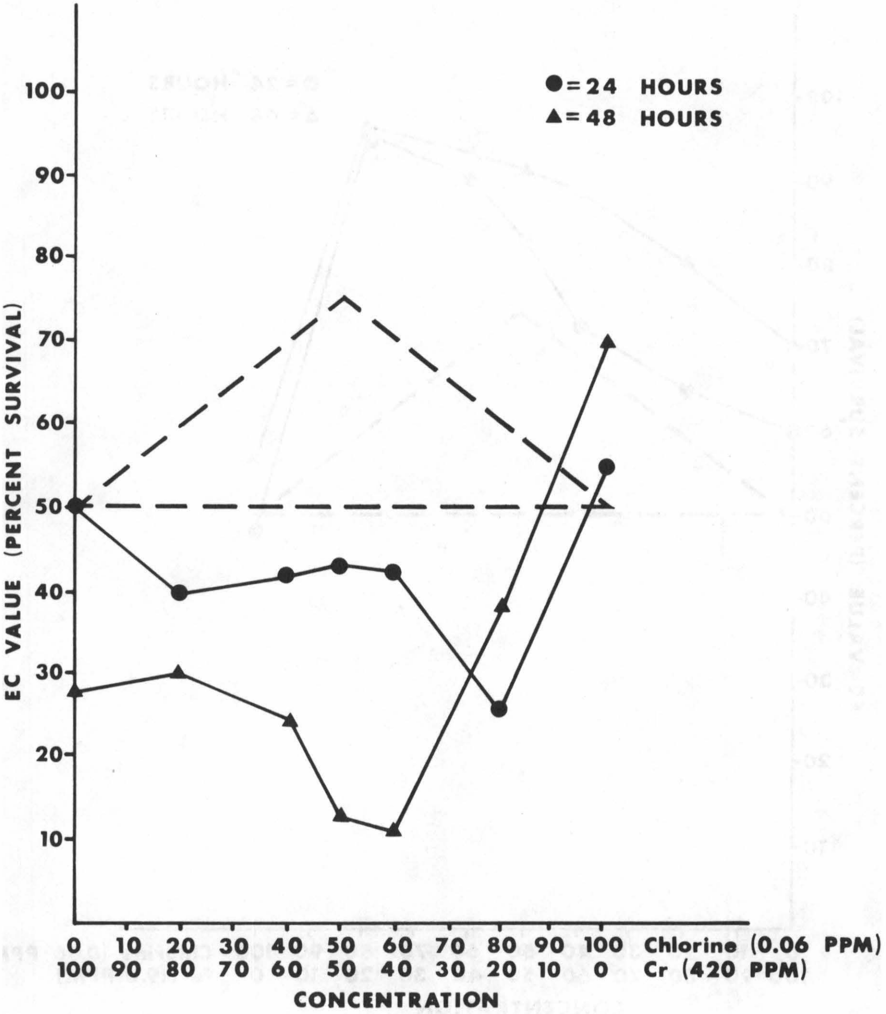
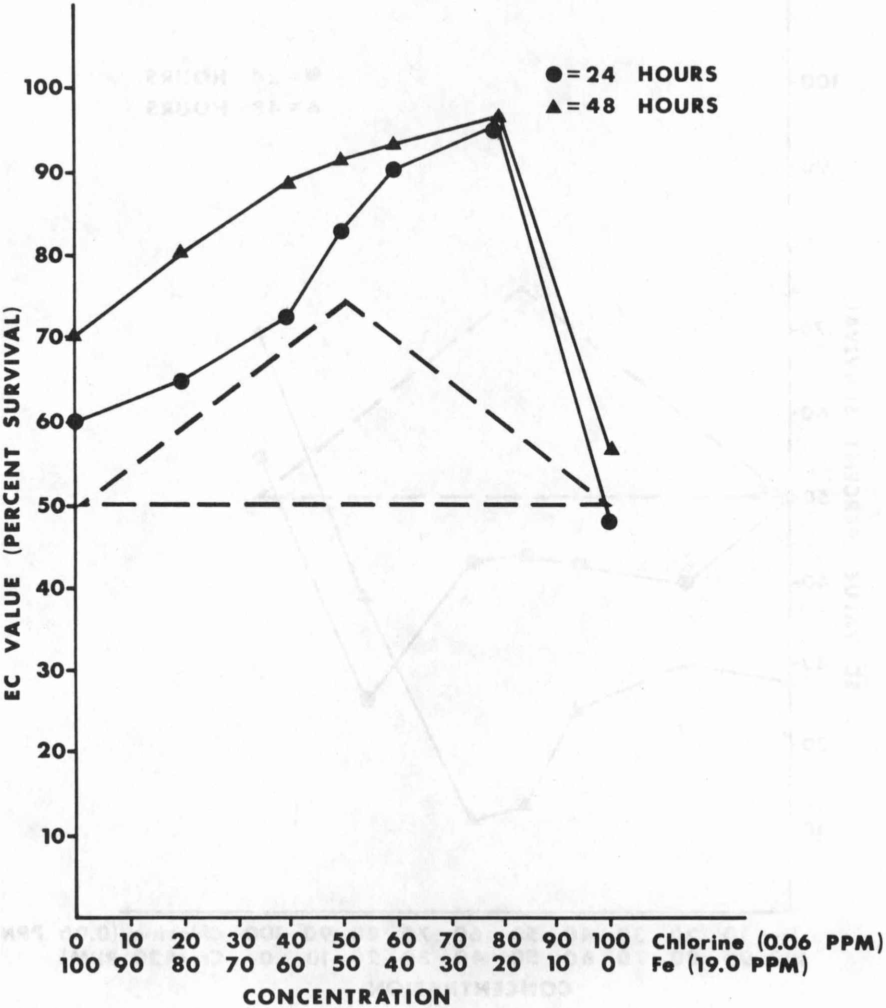


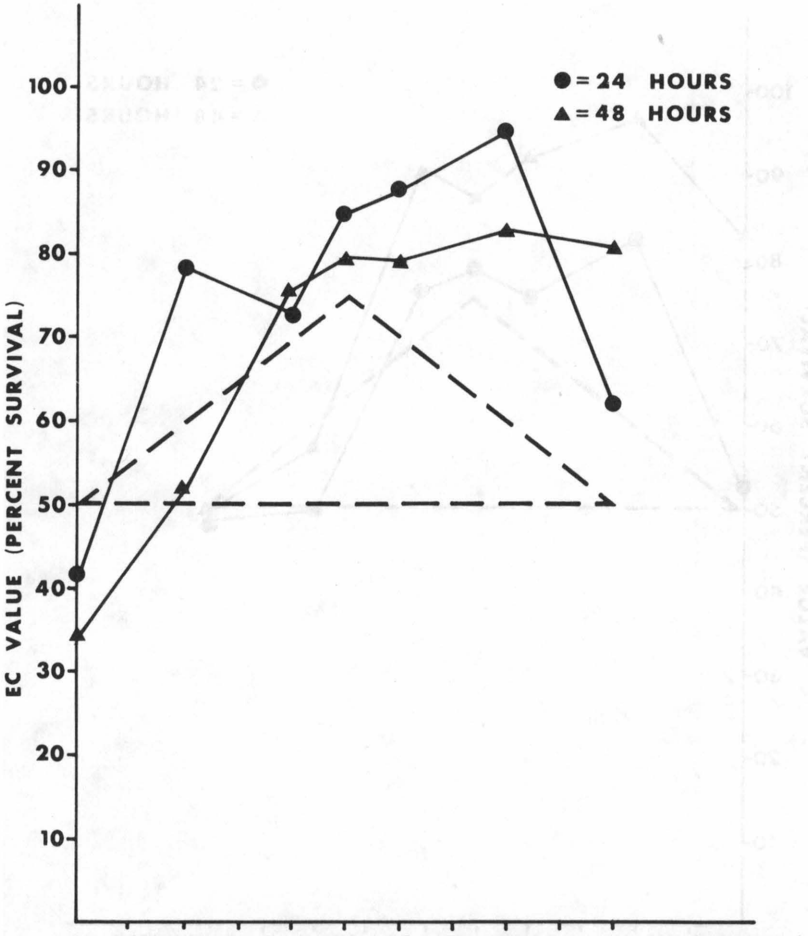
FIGURE 3

Interaction Effects of Iron and Chlorine to the Rotifer *Philodina acuticornis*



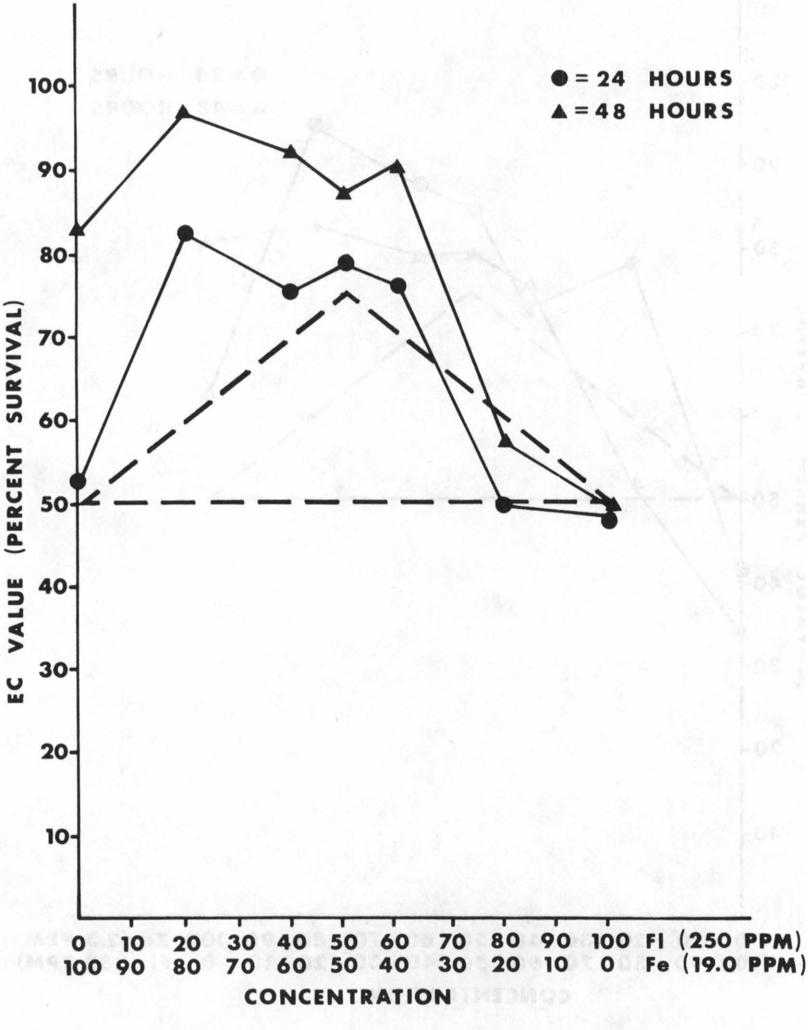
## FIGURE 4

Interaction Effects of Zinc and Fluoride to the Rotifer *Philodina acuticornis*



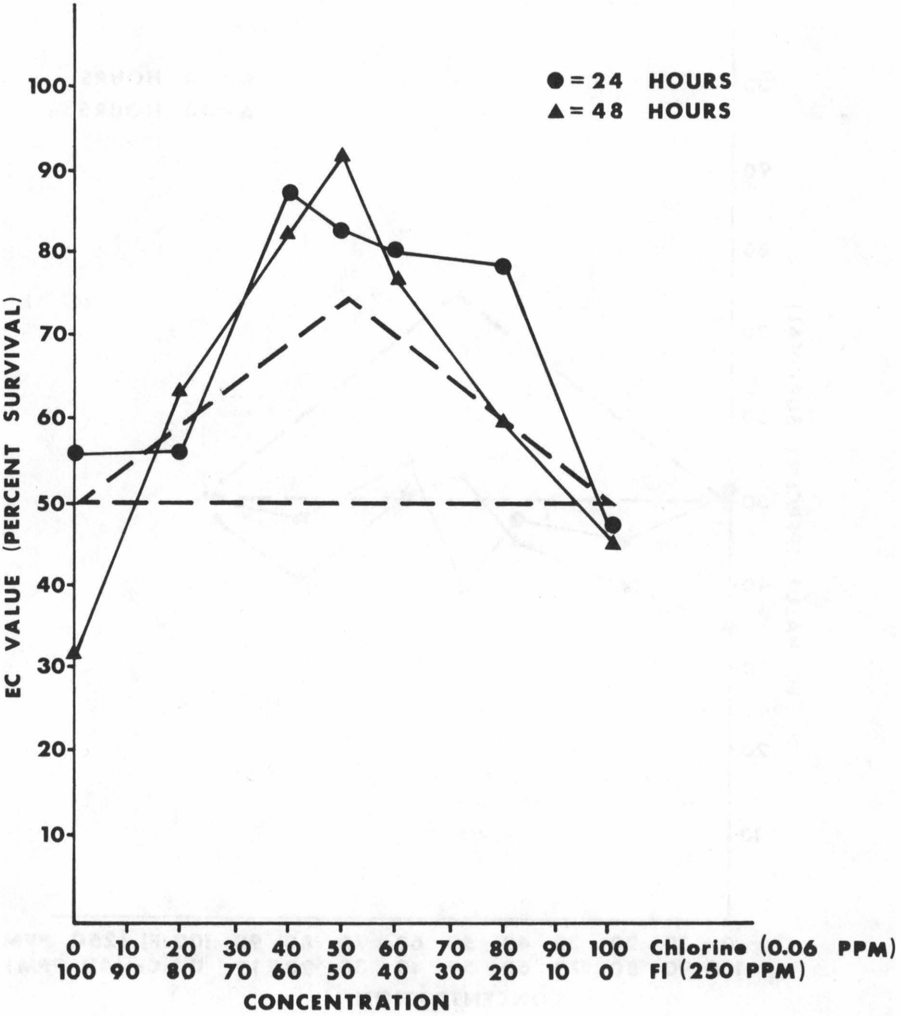
# FIGURE 5

## Interaction Effects of Iron and Fluoride to the Rotifer *Philodina acuticornis*



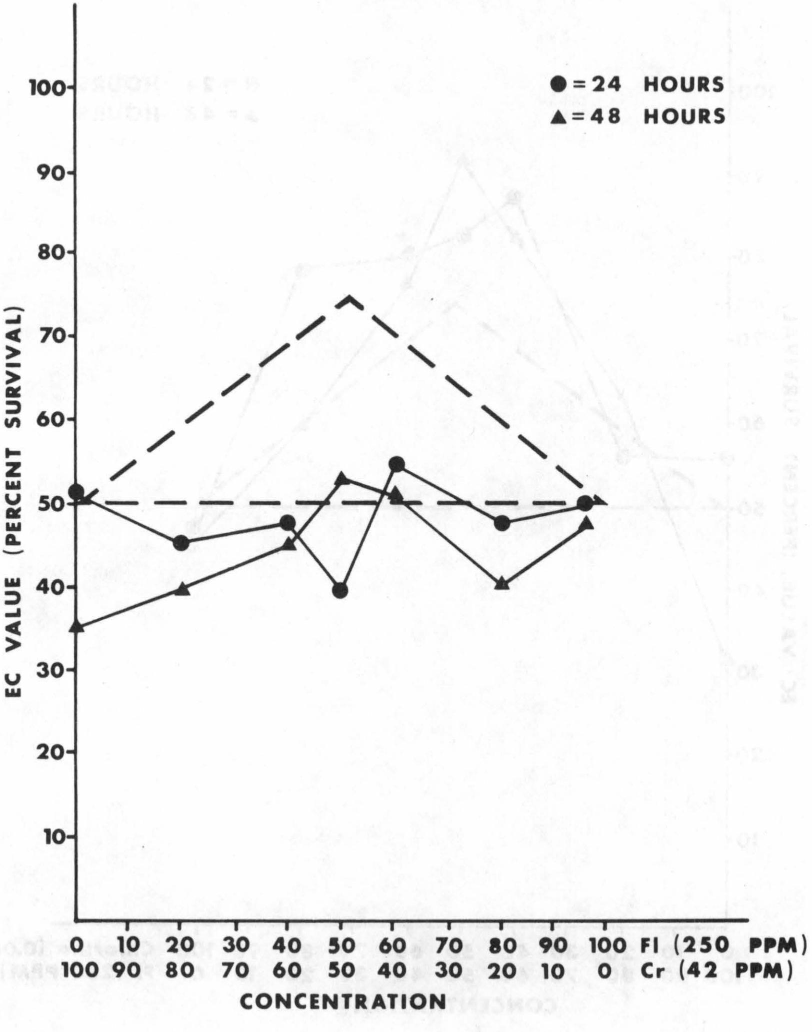
## FIGURE 6

Interaction Effects of Chlorine and Fluoride to the Rotifer *Philodina acuticornis*



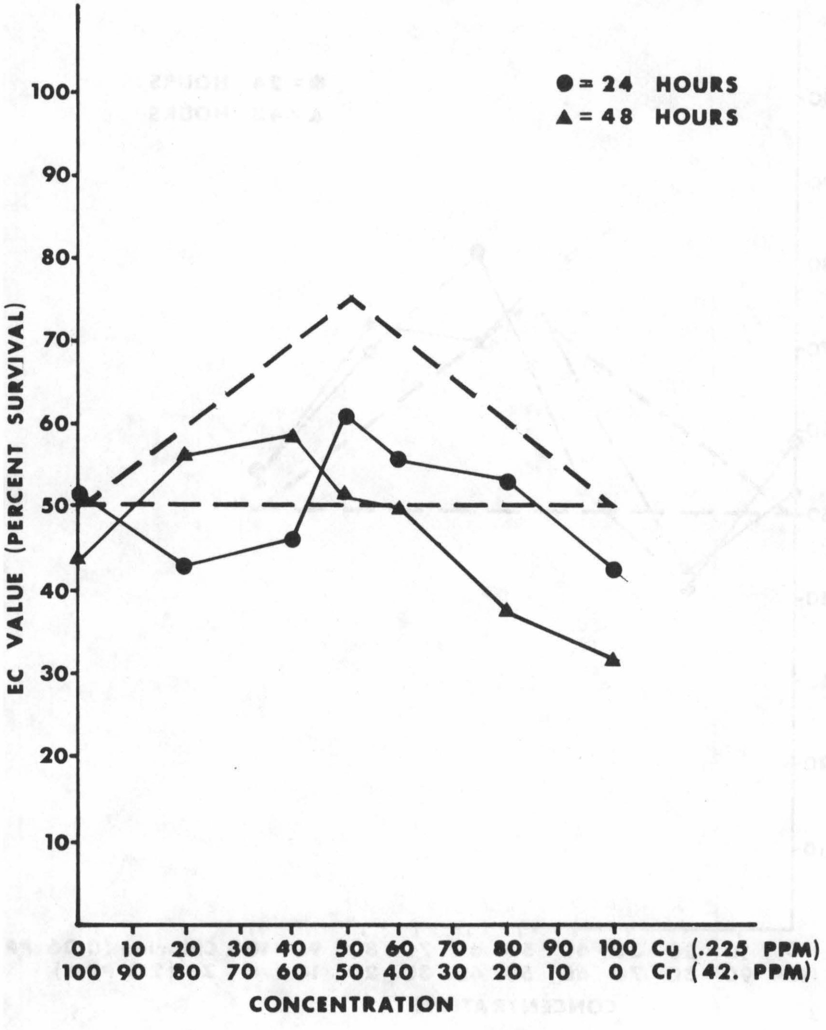
# FIGURE 7

## Interaction Effects of Chromium and Fluoride to the Rotifer *Philodina acuticornis*



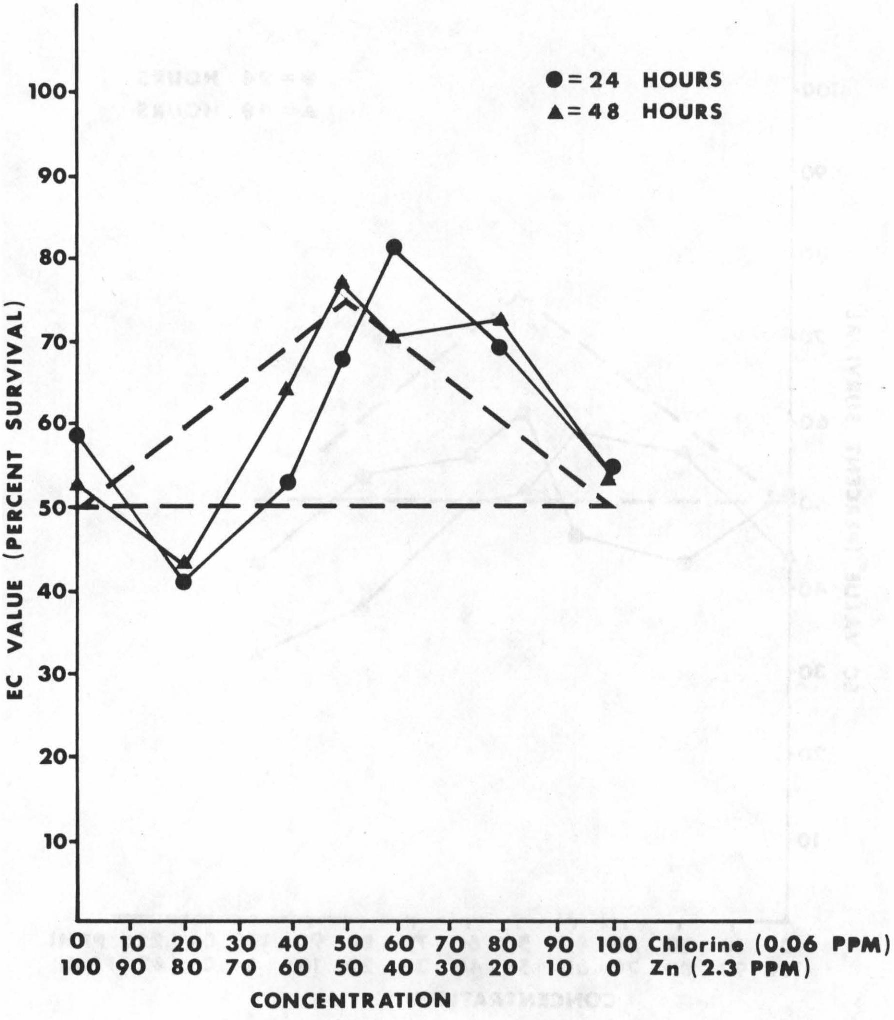
## FIGURE 8

## Interaction Effects of Chromium and Copper to the Rotifer *Philodina acuticornis*



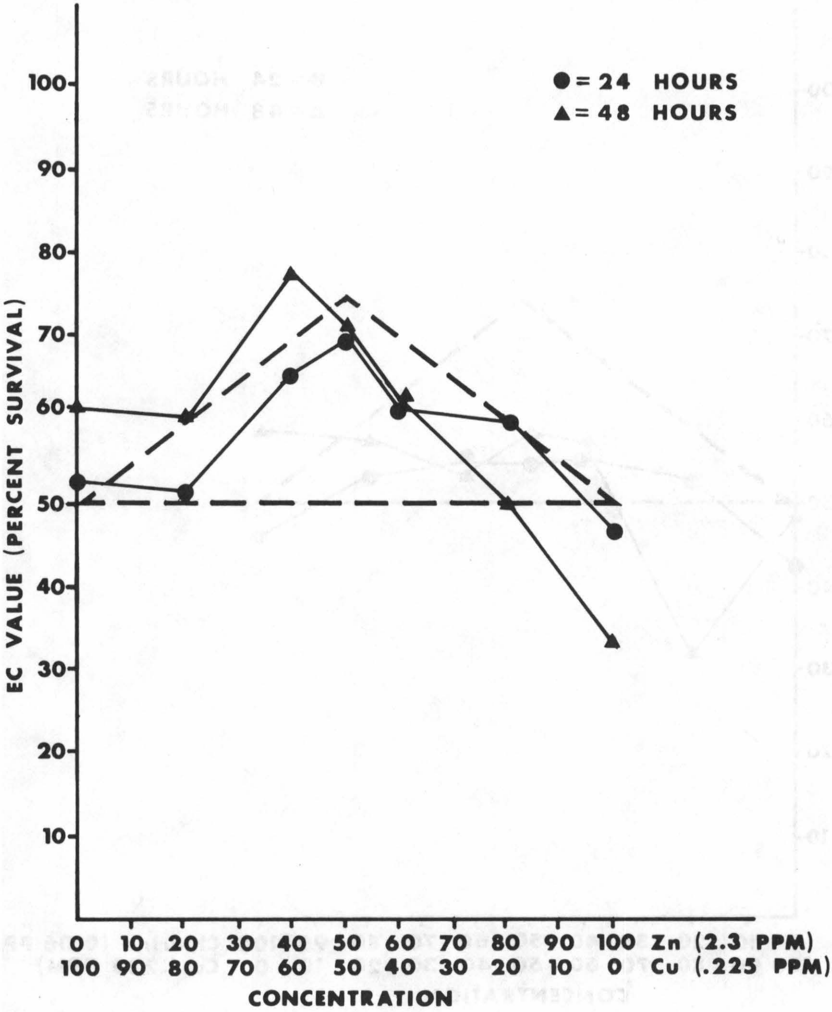
# FIGURE 9

## Interaction Effects of Zinc and Chlorine to the Rotifer *Philodina acuticornis*



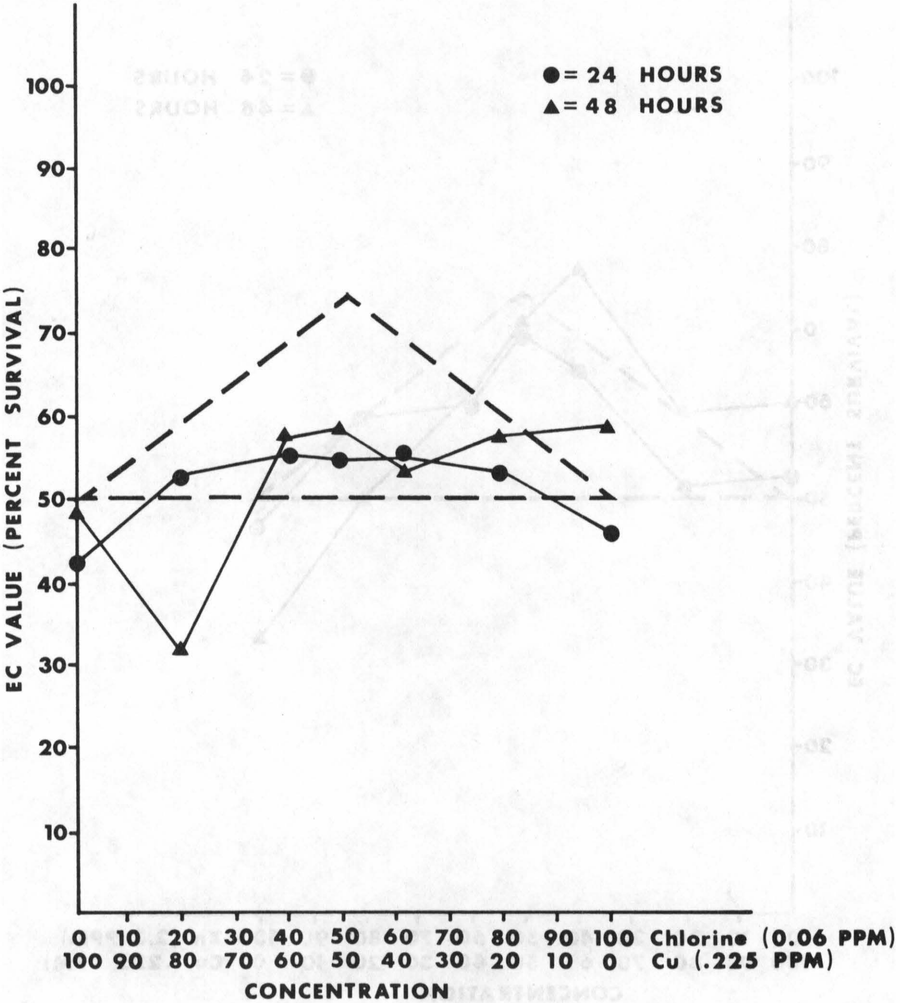
# FIGURE 10

## Interaction Effects of Zinc and Copper to the Rotifer *Philodina acuticornis*



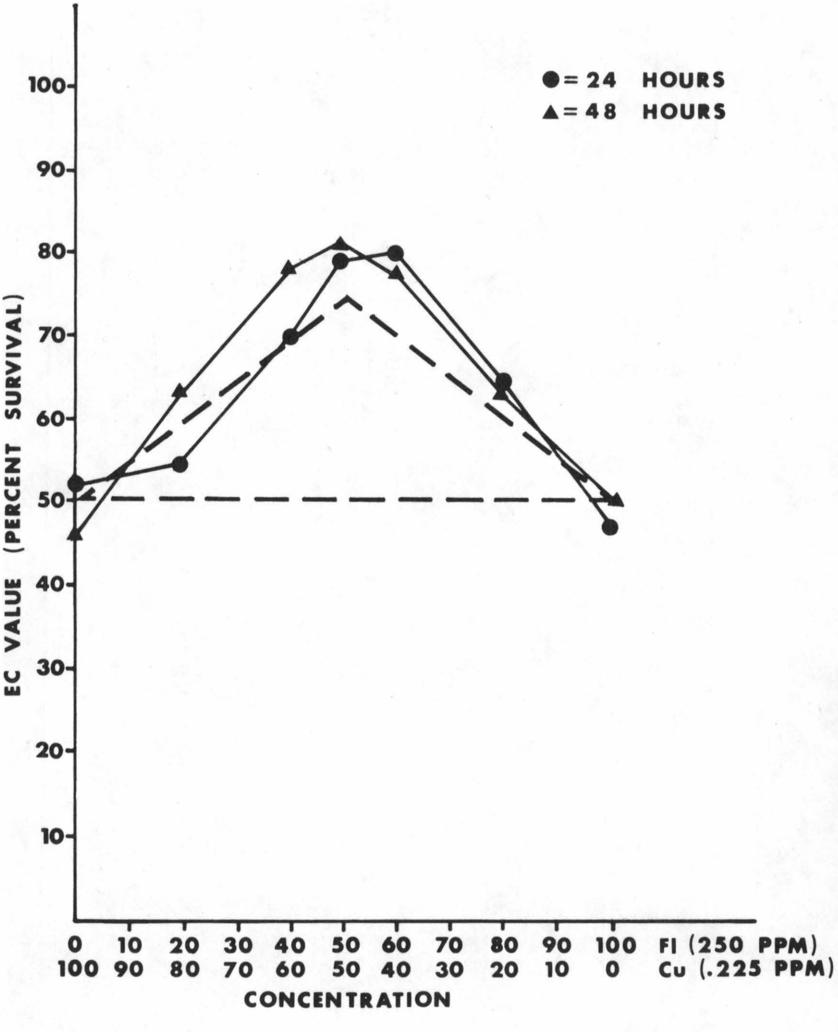
# FIGURE 11

Interaction Effects of Copper and Chlorine to the Rotifer *Philodina acuticornis*



## FIGURE 12

Interaction Effects of Copper and Fluoride to the Rotifer *Philodina acuticornis*



# TABLES

## TABLE 1

## Chemical Composition of the Soft Dilution Water Used for Culturing and Bioassays

| Compound | Concentration (g/l) |
|---|---|
| $NaHCO_3$ | $2.30 \times 10^{-2}$ |
| $NH_4NO_3$ | $3.60 \times 10^{-3}$ |
| KCl | $1.46 \times 10^{-2}$ |
| $H_2SiO_3$ | $1.00 \times 10^{-3}$ |
| Fe citrate | $2.00 \times 10^{-5}$ |
| $CaCO_3$ | $2.00 \times 10^{-2}$ |
| $CaSO_4$ | $3.44 \times 10^{-2}$ |
| $MgSO_4 \cdot 7H_2O$ | $2.46 \times 10^{-2}$ |

See text for preparation; modified from Cairns and Scheier [1957].

## TABLE 2

## Toxicant Salt and Concentrations Used For Initial Bioassay Studies

| Toxicant | mg of cpd/l for 100 ppm Solution of Toxicant | Concentrations Used (ppm of Toxicant) |
|---|---|---|
| Zinc as $ZnSO_4 \cdot 7H_2O$ | 440.0 | 3.0; 2.5; 2.0; 1.5; 1.0; 0 |
| Copper as $CuSO_4 \cdot 5H_2O$ | 392.8 | 2.0; 1.0; 0.5; 0.25; 0 |
| Chromium as $K_2Cr_2O_7$ | 282.8 | 75; 50; 25; 8; 4; 2; 1; 0 |
| Iron as $FeSO_4 \cdot 7H_2O$ | 497.8 | 50; 25; 10; 5; 1; 0 |
| Fluoride as NaF | 221.0 | 960; 750; 500; 247.5; 100; 0 |
| Chlorine as $Ca(OCl)_2 \cdot 2H_2O$ | 252.3 | 0.087; 0.066; 0.034; 0.012; 0 |
| Cyanide as KCN | 250.3 | 190.0; 180.5; 166.3; 152.0; 142.5; 137.8; 19.0; 9.5; 4.8; 0 |

# TABLE 3

## EC-50 Values for Toxicants Studied
## (in ppm of Toxicant)

| | Time (hr) | | |
|---|---|---|---|
| **Toxicant** | **24** | **48** | **96*** |
| Zinc | 2.40 | 2.30 | 1.70 |
| Copper | 0.22 | 0.14 | 0.16 |
| Chromium | 47.50 | 41.50 | 21.00 |
| Iron | 19.10 | 11.30 | 14.80 |
| Fluoride | 272.00 | 158.00 | 212.00 |
| Chlorine | 0.08 | 0.07 | 0.07 |
| HCN† | 142.5 - 237.5 | 142.5 - 237.5 | 19.0 - 142.5 |

* EC-50 values are subject to error because of possible reproductive interactions [Buikema et al., 1974a and 1974b].

† Values are ranges only.

## TABLE 4

## Summary of Interactions Between Selected Chemicals

| Figure | Toxicant Combination | Time (hr) | Interaction Effect* |
|---|---|---|---|
| 2 | Chromium + Chlorine | 24 | Supra-additive |
| | | 48 | Supra-additive |
| 3 | Iron + Chlorine | 24 | Antagonistic |
| | | 48 | Antagonistic |
| 4 | Zinc + Fluoride | 24 | Antagonistic |
| | | 48 | Antagonistic |
| 5 | Iron + Fluoride | 24 | Antagonistic |
| | | 48 | Antagonistic |
| 6 | Chlorine + Fluoride | 24 | Antagonistic |
| | | 48 | Antagonistic |
| 7 | Chromium + Fluoride | 24 | Additive |
| | | 48 | Additive |
| 8 | Chromium + Copper | 24 | Additive to infra-additive |
| | | 48 | Infra- to supra-additive |
| 9 | Zinc + Chlorine | 24 | Infra-additive to antagonistic |
| | | 48 | Infra-additive to antagonistic |
| 10 | Zinc + Copper | 24 | No interaction |
| | | 48 | No interaction |
| 11 | Copper + Chlorine | 24 | Additive |
| | | 48 | Additive |
| 12 | Copper + Fluoride | 24 | No interaction to antagonistic |
| | | 48 | Antagonistic |

* Terminology based on that of Warren [1971].

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