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ACUTE AND CHRONIC TOXICITY OF HCN TO FISH AND INVERTEBRATES

by

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FOREWORD

This report represents nearly five years of study on the acute and chronic effects of cyanide on various aquatic animals, particularly fish. It should serve as a reference for both administrators charged with setting permissible levels of cyanide in United States surface waters and researchers planning to conduct related studies.

Portions of the results herein have been published in several different scientific journals. Many tables, figures and detailed comments had to be editorially omitted from these journal articles to conserve space and reduce publication costs. This report contains all pertinent data, including previously published findings, as well as some new information never before published.

Some preliminary studies on the photodecomposition of iron-cyanides, which causes the release of HCN, also were started during this project. These results will be published later in a separate EPA Ecological Report.

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DEDICATION

During the preparation of this report the senior investigator, Dr. Lloyd L. Smith, Jr., died on June 17, 1978. His dedication and enthusiasm will be missed.

ABSTRACT

The acute toxicity of HCN was determined at various temperatures and dissolved oxygen concentrations for different life history stages of seven species of fish and two species of invertebrates. Eggs were the most tolerant stage while fry and juvenile fish, the least tolerant stages, had similar sensitivities. Temperature and dissolved oxygen concentration (DO) also have an effect on acute toxicity; juvenile fish generally became more sensitive with decreasing temperature and DO. In contrast to juvenile fish, invertebrates were less sensitive to HCN with decreasing temperature. One species of invertebrate had about the same sensitivity to HCN as juvenile fish, while the other species was considerably more tolerant of HCN.

Long-term tests conducted with fathead minnows demonstrated that the concentrations of HCN having no adverse effect on egg production was between 12.9 and 19.6 μg/l. Chronic tests with brook trout demonstrated that on the basis of spawning success the maximum acceptable toxicant concentration (MATC) was between 5.7 and 11.2 μg/l HCN. When compared with the mean number of eggs spawned in the less productive of two controls, a 42.0 percent reduction was observed at the latter concentration. Long-term tests with bluegills showed that no spawning occurred at HCN concentrations as low as 5.2 μg/l. The survival of bluegill fry was drastically reduced at 19.4 μg/l HCN in 57-day tests which began with eggs.

Chronic experiments conducted with *Asellus* and *Gammarus* demonstrated that the highest concentration of HCN having no adverse effect was between 29 and 40 μg/l for *Asellus* and between 16 and 21 μg/l for *Gammarus*. When these two invertebrate species were exposed to HCN simultaneously, the *Gammarus* almost eliminated the *Asellus* from the controls and the lowest treatment level. However, there was also a downward shift in the no-effect/effect concentration for *Gammarus* to between 4 and 9 μg/l. The values for *Asellus* remained approximately the same as when tested alone.

The presence of sublethal concentrations of HCN caused increased predation by green sunfish on fathead minnows, but it was not determined whether fatheads were easier prey or the green sunfish had greater appetites.

In acute tests where the HCN concentration is increasing with exposure time, it was determined that the amount of mortality can be roughly predicted from the relationship between exposure area (change in HCN concentration with time) and median survival time for three types of time-concentration exposure curves. Experiments conducted with intermittent and sublethal diurnal exposure regimes demonstrated that adverse effects on early growth of the fathead minnow are lessened as the exposure period is reduced.

The lethal and sublethal effects of binary toxicant mixtures on fish were evaluated according to existing models of multiple toxicity. Using the toxic unit approach, it was determined that the Zn-HCN and ammonia-HCN mixtures were more acutely toxic and the Cr-HCN mixture less toxic than what would be predicted from simply additive interaction. The sublethal joint action of toxicant mixtures on early fish growth was not predictable from existing multiple toxicity models, and in general, there was no joint interaction between toxicants when compared with the observed responses elicited by individual toxicants.

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CONTENTS

Foreword..... iii
Dedication..... iv
Abstract..... v
List of Figures..... ix
List of Tables..... x
Acknowledgments..... xiv

Sections

I. Conclusions..... 1
II. Recommendations..... 2
III. Introduction..... 4
IV. Acute Toxicity..... 6

Experimental Design..... 6
Test Animals..... 6
Water Supply..... 7
Laboratory Acclimation..... 7
Test Apparatus..... 9
Test Procedures..... 10
Statistical Analysis..... 12
Results..... 12
Fathead Minnow..... 12
Bluegill..... 14
Yellow Perch..... 14
Brook Trout..... 14
Other Fish Species..... 24
Effect of Temperature..... 24
Effect of pH..... 26
Invertebrates..... 31
Summary..... 31

V. Chronic Toxicity..... 33

Fathead Minnow..... 33
Experimental Design..... 33
Results..... 35
First Generation..... 35
Survival..... 35
Growth..... 35
Reproduction..... 35

Contents - Continued

F_1 Generation..... 37
Survival..... 37
Growth..... 37
Summary..... 41
Brook Trout..... 42
Experimental Design..... 42
Results..... 43
Adults..... 43
Growth and Survival..... 43
Reproduction..... 43
Embryos, Fry and Juveniles..... 44
Growth..... 44
Survival..... 47
Summary..... 47
Bluegill..... 49
Experimental Design..... 49
Results..... 51
Survival..... 51
Growth..... 55
Reproduction..... 55
Second Generation Hatching and Survival..... 55
Summary..... 55
Invertebrate Populations..... 59
Variability Between Two Species..... 59
Experimental Design..... 61
Results..... 61
Summary..... 66
Response to Cyanide..... 66
Experimental Design..... 66
Results..... 68
Summary..... 72

VI. Miscellaneous Tests..... 77

Predator-Prey Relations..... 77
Experimental Design..... 77
Results..... 78
Exposure to Increasing Cyanide Concentrations..... 81
Experimental Design..... 81
Results..... 84
Intermittent Sublethal Exposure Tests..... 86
Experimental Design..... 86
Results..... 87
Binary Mixtures with HCN..... 92
Experimental Design..... 92
Results..... 95
Summary.....100

VII. References.....110
VIII. Publications.....114

FIGURES

Number		Page
1	Relationship between test pH and 96-hr LC50 cyanide concentrations for the fathead minnow at 20°C	30
2	Length-frequency diagrams of all *Asellus* in each test chamber	62
3	Length-frequency diagrams of all *Gammarus* in each test chamber	63
4	Length-frequency graphs of each test concentration for chronic test--*Gammarus* alone	69
5	Length-frequency graphs of each test concentration for chronic test--*Asellus* alone (2)	70
6	Length-frequency graphs of each test concentration for chronic test--*Asellus* and *Gammarus* together	71
7	Representative curves for increasing HCN lethal exposure tests normalized to time and concentration	83
8	Diurnal fluctuation of HCN levels in test chambers as a function of their maximum concentrations	88

TABLES

Number		Page
1	Analysis of laboratory well water	8
2	Number of organisms per test chamber	10
3	Approximate initial size (mm total length) of fry, juveniles and adults for toxicity tests	11
4	Acute toxicity of HCN to fathead minnow eggs expressed as 96-hr LC50 and median lethal concentrations at hatching	13
5	Acute toxicity of HCN to fathead minnow fry expressed as 96-hr LC50 and median lethal threshold concentrations	15
6	Acute toxicity of HCN to fathead minnow juveniles expressed as 96-hr LC50 and median lethal threshold concentrations	16
7	Acute toxicity of HCN to bluegill eggs and fry expressed as 96-hr LC50 and median lethal threshold or hatching concentrations	17
8	Acute toxicity of HCN to bluegill juveniles expressed as 96-hr LC50 and median lethal threshold concentrations	18
9	Acute toxicity of HCN to yellow perch eggs and fry expressed as 96-hr LC50 and median lethal threshold or hatching concentrations	19
10	Acute toxicity of HCN to yellow perch juveniles expressed as 96-hr LC50 and median lethal threshold concentrations	20
11	Acute toxicity of HCN to brook trout eggs and sac fry expressed as 96-hr LC50 and median lethal threshold or hatching concentrations	21
12	Acute toxicity of HCN to brook trout swim-up fry expressed as 96-hr LC50 and median lethal threshold concentrations	22
13	Acute toxicity of HCN to brook trout juveniles expressed as 96-hr LC50 and median lethal threshold concentrations	23

Tables - continued

Number		Page
14	Acute toxicity of HCN to rainbow trout, largemouth bass and black crappie juveniles expressed as 96-hr LC50 concentrations	24
15	Acute toxicity of HCN to wild stock fathead minnow juveniles at 5°C intervals from 5° to 30°C expressed as 96-hr LC50 and median lethal threshold concentrations	25
16	Acute toxicity of HCN to brook trout juveniles at temperatures from 4° to 18°C expressed as 96-hr LC50 and median lethal threshold concentrations	27
17	Mean test conditions for composite free cyanide bioassays	28
18	Biological assay by the log-probit analysis method of fathead minnow cyanide bioassays at 20°C grouped according to test pH	29
19	Acute toxicity of HCN to *Asellus communis* and *Gammarus pseudolimnaeus* adults expressed as 96-hr LC50 and median lethal threshold concentrations	32
20	Survival and weight of first-generation fathead minnows after 28, 56, and 84 days of exposure to hydrogen cyanide	36
21	Egg production, hatchability, and terminal weights for first-generation fathead minnows exposed to various concentrations of hydrogen cyanide	38
22	Survival, length, and weight of F_1 fathead minnows after 28 and 56 days of exposure to hydrogen cyanide	40
23	Egg production of adult brook trout exposed to HCN for 144 days prior to start of spawning	44
24	Growth of embryo and juvenile brook trout exposed to various levels of cyanide	45
25	Effect of HCN on the survival of brook trout eggs to hatching and from hatching to 90-day-old juveniles	48
26	Percentage reduction in mean number of eggs spawned by female brook trout exposed to HCN	49
27	Survival and fecundity of adult bluegills after 289 days, Test 4	52

Tables - continued

Number		Page
28	Percentage survival of bluegills from fertilized egg to the 57-day juvenile stage in various HCN concentrations for Test 1	53
29	Percentage survival of bluegills from fertilized egg to the 57-day juvenile stage in various HCN concentrations for Test 3	54
30	Mean lengths (cm) and mean weights (g) of adult bluegills exposed to HCN for 289 days, Test 4	56
31	Terminal mean lengths and weights of bluegill juveniles at various HCN concentrations in Tests 1 and 3	57
32	Percentage survival, length, and weight of bluegill juveniles in Test 2 (mean initial weight = 0.66 g)	58
33	Test conditions in *Gammarus* and *Asellus* experiments	60
34	Population parameters in *Asellus* and *Gammarus* tests	64
35	Means for the population indices of five replications	65
36	Test conditions for simultaneous *Gammarus* and *Asellus* bioassays in HCN	67
37	Biological indices in the chronic test series (1) on *Asellus* (both tests included)	68
38	Biological indices in chronic test series (2) on *Asellus* (both tests included)	73
39	Biological indices in the chronic test series on *Gammarus* (both tests included)	74
40	Biological indices in the chronic series with *Asellus* (A) and *Gammarus* (G) together (both tests included)	75
41	Comparisons of the no-effect/effect concentrations (μg/l) of hydrogen cyanide for *Asellus* and *Gammarus*	76
42	Mean HCN concentrations in each experimental tank of each test and the overall means for each tank at designated nominal concentrations	78
43	Prey consumption (grams of prey consumed per gram of predator present) for each test tank and the overall means for each nominal HCN concentration	79

Tables - continued

Number		Page
44	Prey consumed for each test tank as a percentage of the control values and the overall means for each nominal HCN concentration	80
45	Diluter and aquaria conditions as related to water turnover times	82
46	Maximum HCN concentration, median survival time (MST) and exposure area derived from the time-concentration curves for the increasing HCN lethal exposure tests	85
47	Mean areas, standard deviations, and coefficients of variation for the combined tests from Table 46	86
48	Growth of fathead minnows in 30 days when exposed to continuous or intermittent HCN concentrations	89
49	Linear regression analysis of the toxicant dose response curves expressing terminal mean dry weight in mg (Y) and percentage terminal mean dry weight normalized to controls (Y) as a function of log toxicant concentration in μg/l (X)	91
50	Mean test conditions and description of test fish for acute toxicity bioassays of individual toxicants and binary mixtures	96
51	Dose-response parameters, lethal concentrations and additive indices for acute toxicity bioassays of individual toxicants and binary mixtures	97
52	Growth of fathead minnows in 30 days when exposed to individual or mixture solutions of hexavalent chromium and HCN	101
53	Growth of fathead minnows in 30 days when exposed to individual or mixture solutions of zinc and HCN	103
54	Growth of rainbow trout in 30 days when exposed to individual or mixture solutions of ammonia and HCN	105
55	Linear regression analysis of the toxicant dose response curves expressing terminal mean dry weight in mg (Y) and percentage terminal mean dry weight normalized to controls (Y) as a function of log toxicant concentration in mg/l (X)	107

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SECTION I

CONCLUSIONS

The results of experimental studies on the acute and chronic toxicity of cyanide to aquatic organisms have demonstrated that HCN is toxic to fish and invertebrates in low concentrations which are frequently found in polluted waters. Lethal tests have shown that the susceptibility of fish to HCN is dependent on their life history stage. Eggs are the most tolerant while fry and juveniles, the least tolerant stages, have similar sensitivities. Acute toxicity was demonstrated to vary among species, sources of fish, and with environmental factors such as temperature, dissolved oxygen, and pH. The toxicity of HCN to all species of juvenile fish which were tested increased as temperature decreased, yet the opposite was observed for an invertebrate species. Decreased DO levels also increased the toxicity of HCN, but pH levels within the range 6.8 to 8.3 had little effect.

In view of the uncertainty of predicting long-term adverse effects from results of acute toxicity tests, the results of chronic exposures provide a more reliable estimate of acceptable levels of HCN. On the basis of our tests with representative freshwater organisms, it was demonstrated that egg production (reproductive potential) and spawning success are reduced at low HCN levels. From these experiments, it can be concluded that to insure cyanide does not seriously impair the success of most freshwater organisms a continuous and constant concentration of HCN should not exceed 5 μg/l. This level should protect most aquatic populations during the most sensitive period of population maintenance and can also be tolerated by all other life history stages under reasonably adverse environmental conditions. If effects on reproduction and fry survival are not considered, our results suggest that concentrations up to about 25 μg/l HCN can be tolerated without marked adverse effects on natural populations of the species which were studied.

SECTION II

RECOMMENDATIONS

The acute toxicity to aquatic organisms of most simple and metallocyanide complexes has been demonstrated in the scientific literature and from work conducted during this investigation to be almost entirely due to the presence of HCN as derived from ionization, dissociation, and photodecomposition of cyanide containing compounds. Therefore, the analytically determined HCN concentration in cyanide polluted waters is the most reliable chemical measurement of the actual cyanide toxicity to aquatic organisms.

The relationship between total cyanide and HCN in natural waters has not been well defined, but it is known to vary markedly with receiving water conditions, the type of cyanide compounds present, the presence of other chemical compounds, and the degree of exposure to natural light. The total cyanide concentration in some waters may consist almost entirely of free cyanide, or may contain cyanides which may readily photodecompose or dissociate to yield HCN. It must therefore be assumed that a standard total cyanide measurement is indicative of the free cyanide concentration unless it can be demonstrated otherwise. However, under most circumstances it would be expected that the concentration of total cyanide will be greater than that of HCN. Since total cyanide concentrations in most cases will not be toxicologically meaningful, such a measurement would not be representative of the actual cyanide toxicity but would most likely be a liberal estimate. Therefore, a simple but accurate method which measures free cyanide or HCN directly in the micrograms per liter range is needed and should be used by regulatory agencies in their implementation of receiving water cyanide standards that reflect sound water quality criteria.

Determination of maximum acceptable toxicant concentrations of HCN should be based on the most sensitive life history stage of an aquatic organism when exposure is continuous throughout its lifetime. Any impairment of long-term survival, growth rate, fecundity, or spawning behavior must be considered detrimental to population maintenance unless ecological studies show that some degree of impairment of these vital functions can be sustained on a continuous basis without degradation of the aquatic population.

The effect of intermittent discharges of HCN at concentrations higher than acceptable acute or chronic levels has not yet been fully determined. However, the effect of low temperatures on acutely toxic levels of HCN to fish and the extreme sensitivity of some life history stages demand caution in assuming that temporary or seasonal deviations from demonstrated no-effect levels of HCN can be safely tolerated by fish populations. Further testing is needed to deter-

mine the long-term sensitivity of fish to HCN at low temperatures. Until this is accomplished, it is recommended that a concentration no greater than 5 μg/l HCN be considered a safe limit for the protection of freshwater fish and invertebrates.

SECTION III

INTRODUCTION

Compounds containing the cyanide group are present in many effluents, including those from iron and steel processing plants, petroleum refineries, and metal plating plants, and constitute a hazard to aquatic ecosystems in certain waste receiving waters. These compounds usually exist in aqueous solutions as free cyanide (i.e., CN^- ion and molecular HCN) or as metallocyanide complexes. The toxicity of different forms of cyanide varies markedly. Certain cyanide complexes have relatively low toxicity, although they may photodecompose and dissociate into the highly toxic HCN. Wuhrmann and Woker (1948), Bridges (1958), and Doudoroff *et al.* (1966) have concluded that HCN is the principal toxic form of cyanide. However, Broderius *et al.* (1977) have demonstrated that even though molecular HCN is more toxic than the CN^- ion, the anion does contribute to the total toxicity in proportion to its concentration. In aqueous solution the cyanide radical of simple alkali cyanides such as NaCN hydrolyzes to form free cyanide. The molecular (un-ionized) component predominates at pH values found in most natural waters with less than 6 percent of free cyanide occurring in the ionic form below pH 8 at 25°C. As the pH of the aqueous simple cyanide solution is increased, the percentage of free cyanide present as the CN^- ion is increased to satisfy the equilibrium reaction $HCN \rightleftarrows H^+ + CN^-$.

The literature on the toxicity to fish of various cyanides has been recently reviewed by Doudoroff (1976). The results vary widely among species and among experimenters. Much of the data is difficult to assess because the exact character of the test solutions, wastes, test conditions, test organisms, etc. were often not clearly defined. Further, variations in the sensitivity of organisms during different life history stages have not been well examined. In view of the lack of comparative toxicity tests with fish in various life history stages, and the absence of comprehensive chronic tests to determine sublethal effects, the present study was undertaken using seven species of fish and two species of invertebrates. The fish which were used in toxicity tests were the fathead minnow, *Pimephales promelas* Rafinesque; bluegill, *Lepomis macrochirus* Rafinesque; yellow perch, *Perca flavescens* (Mitchill); brook trout, *Salvelinus fontinalis* (Mitchill); rainbow trout, *Salmo gairdneri* Richardson; black crappie, *Pomoxis nigromaculatus* (LeSueur), and largemouth bass, *Micropterus salmoides* (Lacépède). The invertebrates *Asellus communis* Say* and *Gammarus pseudolimnaeus* Bousfield were also studied.

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*Keyed out to *Asellus militaris* Hay in Pennak (1953) but to *Asellus communis* Say in Williams (1970).

Acute toxicity tests were conducted using all of the species listed above, during various life history stages. The chronic toxicity of cyanide to fathead minnows, bluegills, brook trout, *Asellus*, and *Gammarus* was also investigated. In addition, a series of toxicity tests was conducted at different pH values, using fathead minnows, to determine the relationship of hydrogen ion concentration and cyanide form on the toxicity of free cyanide. Tests were conducted to determine the effect of temperature on the acute toxicity of HCN to fathead minnows and brook trout. The lethal and sublethal effects of binary mixtures of HCN and hexavalent chromium, zinc, or ammonia were evaluated. The response of fathead minnows to continuously increasing and fluctuating sublethal levels of HCN was also studied.

Iron-cyanides are relatively nontoxic, but when exposed to sunlight they photodecompose to release HCN. Preliminary studies of this phenomenon were begun during this project, but the results will be incorporated in a more detailed study to appear in a future EPA report.

SECTION IV

ACUTE TOXICITY

Acute toxicity tests were designed to determine the 96-hr median lethal toxicant concentration (LC50) of HCN and the median lethal threshold concentration (LTC) (Sprague 1969) at various temperatures and dissolved oxygen concentrations. These tests were conducted with eggs, fry, and juveniles of four species of fish, juveniles of three other fish species, and two species of invertebrates.

EXPERIMENTAL DESIGN

Test Animals

For brevity in this paper, when referring to the various specific phases in the life history of a fish as defined by Balon (1975), the less specific common terms will be used. Toxicity tests using fish eggs encompassed both the cleavage phase and the embryo phase in all cases. For brook trout the term sac fry refers to the eleutheroembryo phase, swim-up fry to the alevin phase and juvenile to the smolt phase. For the other species, fry refers to the eleutheroembryo phase and juvenile to the juvenile phase. Sexually mature individuals were used for experiments with *Asellus* and *Gammarus*.

Brook trout and rainbow trout were procured as recently hardened eggs, or as sac fry within 24 hr after hatch. They were provided by state hatcheries (Lanesboro and French River, Minnesota, and Osceola, Wisconsin) where both the parents and the offspring were maintained at about 10°C. Trout eggs were incubated in the laboratory in total darkness. Fathead minnows which were used in toxicity tests were reared in the laboratory at 25°C from parents obtained as eggs from the Environmental Research Laboratory at Duluth, Minnesota. Bluegills were collected from several local lakes. Eggs and fry were obtained in the laboratory by artificially spawning adult bluegills which had been collected the same day at temperatures between 24° and 28°C. The water temperature measured during collection was maintained in the laboratory during spawning. The male fish was killed with a blow to the head. The testes were dissected out, rinsed with water, and placed in a cheesecloth bag. The female was wiped dry, and the eggs were stripped into a wetted Petri dish. Sperm was immediately squeezed from the bag and dripped onto the cluster of eggs. Water was then added as the dish was moved to gently swirl the eggs and sperm. After 1 min a light suspension of bentonite clay was added to prevent clumping of the eggs or their adherence to the dish. Juvenile bluegills (13-28 mm total length) were collected about 2 weeks before being used in toxicity tests, when

lake temperatures were between 13° and 28°C. Yellow perch eggs were obtained from adults which had naturally spawned in large holding tanks in the laboratory. Eggs of yellow perch which had been spawned naturally and were determined to be less than 24 hr old were also collected from several northern Minnesota lakes having water temperatures between 9° and 16°C. Some of the eggs were hatched in the laboratory, and the fry were used in toxicity tests. Juvenile perch were collected from Lower Red Lake, Minnesota about 2 weeks before testing, when their total length was 48-62 mm and lake temperatures were 13° to 17°C. Largemouth bass (48-56 mm total length) and black crappies (41-44 mm total length) were collected from Round Lake, adjacent to Lake Phalen in Ramsey County, at water temperatures between 21° and 25°C. *Asellus* were collected from Rainy Lake, near Ranier, Minnesota, and *Gammarus* from a small stream running into the St. Croix River near Marine on St. Croix, Minnesota.

Water Supply

The laboratory well water came from the Jordan sandstone underlying the Minneapolis-St. Paul area and was delivered through black iron pipes to an epoxy-coated storage tank. From there it passed through a catalytic iron removal filter and to the testing apparatus through PVC pipes. Table 1 lists the chemical characteristics of the water at the point of delivery to the test apparatus.

Laboratory Acclimation

The temperature of the water used for acclimation was controlled within a range of ±0.1°C by electric immersion heaters. Incoming well water was aerated by its passing over a series of baffles. Water having a specific oxygen concentration was obtained by siphoning it from a point along the series of baffles. All acclimation chambers were constructed of glass and silicone sealant.

Prophylactic treatments for disease control were administered as soon as juvenile fish were brought into the laboratory. Neomycin and tetracycline were used at a concentration of 20 mg/l. Fish were treated for 4 hr each day for three consecutive days.

The type of food and frequency of feeding varied with the size and development phase of the fish and species being acclimated. The various foods used were (1) finely mashed, hard-boiled egg yolk, (2) "Glencoe" dry pellets,* (3) live, recently hatched brine shrimp,† and (4) frozen, mature brine shrimp.‡ All foods were supplied in quantities slightly in excess of what the fish would eat in 5 min. *Asellus* and *Gammarus* fed upon small, dead fish and dried leaves of deciduous trees which had been soaked in flowing water for at least 30 days just prior to use as food.

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* Glencoe Mills, Glencoe, Minnesota

† San Francisco Bay Brand, Metaframe Inc.

‡ Ibid.

TABLE 1. ANALYSIS OF LABORATORY WELL WATER*

Item	Concentration (mg/l)
Total hardness as $CaCO_3$	225
Alkalinity as $CaCO_3$	230
Calcium as $CaCO_3$	140
Magnesium as $CaCO_3$	85
Iron	0.02
Chloride	0.64
Sulfate	<5
Sulfide	0.0
Fluoride	0.16
Total phosphorus	0.021
Sodium	4.8
Potassium	1.6
Copper	0.0004
Manganese	0.0287
Zinc	0.0044
Cobalt, nickel	<0.0005
Cadmium, mercury	<0.0001
Ammonia nitrogen	<0.20
Organic nitrogen	<0.30
Specific conductivity (μmhos/cm at 25°C)	430

* Water taken from well head before aeration and heating; pH 7.5

The most rapid rate of change in temperature from that of collection to the desired test temperature was 10°C/hr for eggs and fry, and 2°C/hr for juvenile fish. The maximum change in oxygen concentration after collection of fish eggs and fry was 4 mg/l, and occurred in less than 1 min. Juvenile fish were subjected to a maximum change in oxygen concentration of 1 mg/l/hr. Fish eggs and fry were transferred to the test chamber immediately upon reaching the desired test conditions of temperature and oxygen concentration. Juvenile fish were held for 7 days under the desired conditions before being placed in the test chambers. Holding temperatures for *Asellus* and *Gammarus* were adjusted to test temperatures at a rate of 1°C/hr and were then maintained for a minimum of 7 days before testing.

Test Apparatus

Each test apparatus was supplied with water at the desired temperature and oxygen concentration from a separate supply tank. Temperature was controlled within a range of ±0.1°C by a thermostat in conjunction with a solenoid valve, which allowed hot water to pass through a stainless steel heat exchange coil. Oxygen concentrations were adjusted and maintained by regulating the amount of aeration in the head tank. The flow rate of air for aeration was adjusted with a needle valve and monitored with a flow meter.

Toxicant water was delivered to each test chamber by a diluter modified from that of Brungs and Mount (1970). The "C" cells were eliminated and "W" cells dispensed a volume of 1.3 l. Water from the last "W" cell was used to activate the water flow control bucket and was then wasted to the drain. Movement of the water flow control bucket tripped a microswitch which in turn activated a solenoid valve in the water supply line. The contents of each "W" chamber flowed to a separate chemical metering apparatus (CMA) (Mount and Warner, 1965). Each CMA dispensed a different volume (0.5 to 5.0 ml) of sodium cyanide solution, so that each test chamber contained a different cyanide concentration. The sodium cyanide stock solution was maintained at a pH of about 11.0 using sodium hydroxide. When the stock solution was mixed with 1.3 l of test water at a pH of about 7.8, the resulting test solution was within a few hundredths of the original test water pH. The dispensing volume of each CMA was adjusted by moving a flexible plastic sleeve to increase or decrease the length of the pipet bulb. The flow of water to the diluter was adjusted so that 1.3 l of water was introduced into each test chamber every 3 min. There were 12 diluters of the type described above, each supplying 1 control and 4 different treatment levels.

The test chambers used for experiments with invertebrates, juvenile fish, and the swim-up fry of brook trout were constructed of glass the silicone sealant. The tanks measured 50 x 25 x 20 cm high with an outlet 16 cm high, and contained a volume of 20 l. For experiments with fish eggs and fry, except for brook trout swim-up fry, a vertical, cylindrical, screen-bottomed, acrylic plastic chamber was attached to the inside of the outlet tube of the 20-l chamber described above. The eggs or fry rested on the top side of the horizontal screen. Water leaving the 20-l chamber passed upward through the screen (452-μ opening, nylon filament cloth), past the eggs or fry and out the chamber outlet. The cylinders measured 5.6 cm in diameter and 7.5 cm high. The screened outlet was 2.3 cm from the bottom. To prevent surface stagnation in

the cylinders, two 0.9-cm screened inlet ports were made at a height of 3 cm and opposite the outlet hole. A series of twelve 0.3-cm holes were drilled in the tube connecting the screened cylinder to the 20-l chamber outlet tube. By varying the number of holes that were open, the flow rate of water past the eggs could be regulated. The flow rate was adjusted to a level which would slowly move the eggs without lifting them off the screen.

The test chambers were lighted by two 40-W "Vita-lite"* fluorescent tubes 80 cm above the surface of the water. Light intensity at the water surface was 590-840 lux. Timers were used to provide a constant 12 hr of light and 12 hr of darkness per day. In experiments with eggs, black bakelite covers on top of the cylindrical chambers excluded all direct light from the eggs.

Test Procedures

The number of organisms placed in each test chamber depended on the species and the developmental phase being tested (Table 2). Brook trout and bluegill eggs required no special treatment prior to exposure. Fathead minnow eggs, obtained from natural spawnings of a laboratory-reared stock, were removed from the underside of a cement-asbestos tile according to the method described by Gast and Brungs (1973). To facilitate the handling of perch eggs, sections which had the approximate number of eggs desired were cut from a strand. These sections were then placed in flowing, aerated water for 1 hr. The viable eggs in each section were then counted. The eggs were less than 24 hr old when toxicity tests were started. The sizes of fry, juveniles and adults when toxicity tests began are listed in Table 3. Eggs and fry were exposed to cyanide immediately when placed in the test chambers. Juvenile fish were held in the test chambers for 3 days before the initial introduction of cyanide. *Asellus* and *Gammarus* were placed in the test chambers 2 hr before cyanide was introduced.

TABLE 2. NUMBER OF ORGANISMS PER TEST CHAMBER

Species	Eggs	Fry		Juveniles	Adults
		Sac	Swim-up		
Brook trout	25	10	10	10	-
Fathead minnow	25-50	25		10	-
Bluegill	25	10-50		10-20	-
Yellow perch	20-70	25		10-20	-
Largemouth bass, black crappie, rainbow trout	-	-	-	10	-
Asellus	-	-	-	-	10
Gammarus	-	-	-	-	20

*Luxor Lighting Products, New York

TABLE 3. APPROXIMATE INITIAL SIZE (MM TOTAL LENGTH) OF FRY, JUVENILES AND ADULTS FOR TOXICITY TESTS

Species	Fry		Juveniles	Adults
	Sac	Swim-up		
Brook trout	9-10	14-16	40-68	-
Fathead minnow	5-6		26-45	-
Bluegill	4		13-28	-
Yellow perch	4		48-62	-
Largemouth bass	-		48-56	-
Black crappie	-		41-44	-
Rainbow trout	-		49-58	-
Asellus				9
Gammarus				11

Determinations of free cyanide, temperature, dissolved oxygen, pH, and total alkalinity were made on samples taken from the center of the water mass of each test chamber. All variables except total alkalinity (determined once per test) were measured daily. Free cyanide concentrations were determined by the Epstein colorimetric method (American Public Health Association *et al.* 1971). Calculated HCN concentrations were based on corresponding pH and temperature measurements using dissociation constants of molecular HCN defined by Izatt *et al.* (1962). Oxygen was determined by means of an oxygen specific electrode, standardized by titration. The method of total alkalinity determination was that of Dobie and Moyle (1962).

During the cyanide exposure, brook trout swim-up fry and all juveniles were fed twice per day in amounts slightly in excess of what they would eat. Brook trout and fatheads were fed Glencoe dry pellets, and the other species were fed mature, frozen brine shrimp. *Asellus* and *Gammarus* could feed on presoaked leaves placed in the tank for both food and cover.

Observations on the mortality of test organisms were made daily. Eggs were determined to be dead when the egg contents began to turn an opaque white. Fry mortality was based on the lack of a heart beat. Juvenile and adult mortality was assumed when there were no opercular or swimming movements and no response to a physical stimulus. Dead and surviving fish and invertebrates from all tests, including fish fry which hatched during egg experiments, were measured. The survivors of experiments with juvenile fish were also weighed.

Statistical Analysis

Concentration-percentage mortality data were analyzed with a logarithmic-probability (log-probit) program (Dixon 1973). Upper and lower 95 percent confidence limits on the 96-hr median lethal toxicant concentration (LC50) and the median lethal threshold concentration (LTC) were calculated from the equations

$$\log LC50 \pm 1.96(1/b)(N'/2)^{-\frac{1}{2}} \text{ for homogeneous data or}$$

$$\log LC50 \pm t_{.05(K-2)}(1/b)(\chi^2/D-2)^{\frac{1}{2}}(N'/2)^{-\frac{1}{2}} \text{ for heterogeneous data.}$$

The symbol, N', refers to the number of organisms in treatments having expected mortality rates between 16 to 84 percent. The reciprocal of the log-probit line's slope (1/b) is equivalent to the standard deviation of the logarithm of the population's tolerance frequency distribution ($\hat{\sigma}$) or the logarithm of Litchfield and Wilcoxon's (1949) slope function, S. The symbol, K, refers to the number of treatment levels excluding controls, and χ to the $(Chi)^2$ value calculated for goodness of fit of the regression line to the data points. If this value is greater than the tabulated $(Chi)^2$ with K-2 degrees of freedom, the data are significantly heterogeneous. The formulas given above were derived from Litchfield and Wilcoxon (1949) and Finney (1971). The median lethal threshold concentrations were determined at the end of a time interval when no mortality in any test treatment had occurred for at least 48 hr. When the data were such that analysis by computer was not possible, the dose-response line was graphically fitted to the data points on semilog paper (APHA, 1975).

RESULTS

The acute toxicity of HCN to fish depended on their stage of development. Eggs were the most tolerant, and newly hatched fry and juveniles were the most sensitive. The 96-hr LC50's and LTC's, their 95 percent confidence limits, and log-probit regression analysis of the concentration-percent mortality curves for each exposure period are detailed in Tables 4 through 15.

Fathead Minnow

Tests using the fathead minnow during different life history stages were conducted at about 15° to 25°C and in dissolved oxygen concentrations (DO) from about 4 to 7 mg/l. Delayed embryonic development at low temperature and low DO resulted in no apparent difference in tolerance with decreasing DO but an increase in tolerance with decreasing temperature for 96-hr egg tests (Table 4). Median lethal HCN concentrations at hatching were markedly reduced for tests at 25°C and when the DO was less than 5.52 mg/l. At similar DO levels it appears that a reduction in temperature from 25.0° to 15.2°C resulted in greater sensitivity at hatching. The time required for eggs to hatch at 25°, 20°, and 15°C was 6, 10, and 17 days, respectively.

In experiments with fry and juvenile fish, there was little difference between the 96-hr LC50 and the median lethal threshold concentration. There was no clear trend in sensitivity to cyanide associated with temperature in fry experiments, but in tests at 25°C and 3.77 mg/l DO, the fry were obviously

TABLE 4. ACUTE TOXICITY OF HCN TO FATHEAD MINNOW EGGS EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL CONCENTRATIONS AT HATCHING

Mean test conditions			No. of tests	Log-probit regression analysis*			96-hr LC50		Hatch LC50	
°C	DO mg/l	pH		Treat-ments†	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
15.2	6.36	7.86	2	8	- 5.033	3.940	352	274-453	-	--
				5	-15.508	9.765	-	--	126	90.9-174
20.0	6.13	7.88	11	40	2.175	1.159	273	162-463	-	--
				36	- 2.063	3.411	-	--	118	97.3-142
24.9	3.51	7.72	2	5	- 5.358	4.494	202	130-314	-	--
				8	- 8.411	6.491	-	--	116	86.6-157
24.8	4.46	7.95	2	6	- 8.488	6.475	121	77.3-190	-	--
				6	-13.291	8.909	-	--	113	83.0-154
25.0	5.52	7.90	3	9	- 6.262	4.973	184	115-293	-	--
				10	- 5.288	4.563	-	--	180	122-266
25.0	6.34	8.00	5	12	- 4.612	4.194	196	140-274	-	--
				10	-14.863	8.992	-	--	162	135-193
24.9	7.25	7.99	1	-	--	--	202	--	187	--

*A probit of 4.0, 5.0, and 6.0 corresponds to 16, 50, and 84 percent mortality, respectively, when $\hat{Y}_i$ is the maximum likelihood probit value of percent mortality and X_i is log HCN concentration in µg/l for the regression equation $\hat{Y}_i = a + b(\log X_i)$.

†Number of HCN concentrations in the regression analysis.

more sensitive (Table 5). The sensitivity of juvenile fathead minnows to cyanide was unrelated to temperature or DO (Table 6).

Bluegill

The sensitivity to cyanide of bluegill eggs which had been incubated at about 25°C and in DO's ranging from 6.90 to 3.39 mg/l was not affected at the time of hatching by differences in DO (Table 7). At cyanide concentrations equivalent to the hatching LC50, essentially all the newly hatched sac fry were premature and deformed in appearance. Fry were considerably more sensitive to cyanide than eggs, and at 25°C, the 7-day LTC values for fry decreased with a decrease in DO.

In tests using juveniles, conducted at 8.4° to 24.9°C and relatively constant DO levels, the LTC values increased progressively from 61.7 to 120 µg/l HCN with increase in temperature (Table 8). However, only a slight reduction in sensitivity to cyanide with decreasing DO was observed for juveniles tested at DO's ranging from 6.90 to 3.48 mg/l and 25°C.

Yellow Perch

Results from yellow perch egg and fry experiments conducted at temperatures from about 10° to 18°C and DO's from about 4 to 7 mg/l were so variable that specified median lethal concentrations cannot be considered definitive (Table 9). For tests with juveniles at relatively constant DO levels, LTC values increased progressively from 87.6 to 101 µg/l HCN as test temperatures increased from 15.0° to 21.0°C (Table 10). An increase in sensitivity with decreasing DO was observed for juveniles tested at 7.10 to 3.56 mg/l DO and 21°C; LTC values decreased from 107 to 75.0 µg/l HCN over the range of DO's. There was little difference between the 96-hr LC50 and the LTC values for juvenile perch.

Brook Trout

In brook trout egg tests conducted at temperatures from about 7° to 13°C and DO's from about 4 to 8 mg/l, the 96-hr LC50 values were all greater than 212 µg/l HCN. The median lethal concentrations at the time of hatching for tests conducted at about 6 mg/l DO varied from 98.0 to 155 µg/l HCN but did not appear to be correlated with temperature (Table 11).

Tests using sac fry at 10.0°C and 3.50 to 7.96 mg/l DO demonstrated that dissolved oxygen concentrations markedly influenced HCN toxicity. The 96-hr LC50 and LTC at 3.50 mg/l DO were 108 and less than 71 µg/l HCN while at 7.96 mg/l DO they were 518 and 207 µg/l HCN, respectively. At the intermediate level of 6.03 mg/l DO the 96-hr LC50 and LTC values were 350 and 169 µg/l HCN, respectively (Table 11).

The response to DO of swim-up brook trout fry was similar to that of sac fry; their sensitivity to cyanide did not appear to be correlated to test temperature. The LTC values at 3.90, 6.04, and 8.02 mg/l DO were 53.9, 83.4, and 103 µg/l HCN, respectively (Table 12). There was little difference between the 96-hr and LTC values.

TABLE 5. ACUTE TOXICITY OF HCN TO FATHEAD MINNOW FRY EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC	
°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
15.0	6.38	7.86	6	13	-12.743	8.507	122	104-143	-	--
				15	-13.376	9.143	-	--	102	92.8-113
20.0	6.14	7.89	6	21	- 6.419	5.721	99.1	88.9-111	-	--
				13	- 6.145	5.621	-	--	96.1	83.6-110
24.6	3.77	7.84	3	10	- 9.989	7.840	81.6	71.2-93.6	81.6	71.2-93.6
24.7	5.14	7.96	2	8	- 8.170	6.471	108	90.3-130	108	90.3-130
24.9	6.17	8.02	9	28	- 7.971	6.314	113	96.5-133	113	96.5-133

TABLE 6. ACUTE TOXICITY OF HCN TO FATHEAD MINNOW JUVENILES EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC	
°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
15.0	6.07	7.86	4	15	-27.167	15.455	121	116-125	-	--
				15	-28.565	16.176	-	--	119	115-123
20.0	3.58	7.70	4	11	- 8.512	6.417	128	109-149	-	--
				11	- 9.924	7.146	-	--	123	105-143
19.8	4.68	7.80	2	4	-24.538	15.415	82.4	76.4-88.9	82.4	76.4-88.9
20.0	5.20	7.78	4	8	-21.086	12.446	125	117-133	-	--
				9	-19.292	11.616	-	--	123	116-132
20.0	6.07	7.91	4	13	-20.485	11.933	137	122-153	137	122-153
20.0	7.13	7.90	4	9	-28.818	15.978	131	124-138	131	124-138
24.8	3.58	7.75	2	7	-18.494	11.592	106	87.9-129	106	87.9-129
25.0	5.08	7.83	2	7	-23.502	13.722	119	111-129	119	111-129
25.1	6.13	7.98	8	28	-20.032	11.868	129	124-133	129	124-133
25.2	7.04	7.96	2	6	-27.069	15.415	120	113-128	120	113-128

TABLE 7. ACUTE TOXICITY OF HCN TO BLUEGILL EGGS AND FRY EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD OR HATCHING CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC or hatch LC50	
°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
Eggs										
25.2	3.39	7.70	2	6	- 1.349	2.236	-	--	690	461-1033
25.0	4.99	7.79	2	6	- 4.902	3.630	-	--	535	240-1192
25.1	6.09	7.92	2	8	-12.494	6.157	-	--	693	572-841
25.0	6.90	7.90	2	6	- 3.802	3.185	-	--	580	343-980
Fry										
20.0	5.99	7.89	6	11	- 2.618	2.973	365	188-709	-	--
				11	- 8.388	5.791	-	--	205	156-270
24.9	3.59	7.72	3	4	-40.381	22.256	-	--	109	99.9-120
24.9	5.08	7.80	4	10	- 2.756	3.279	232	147-366	-	--
				7	-18.630	10.872	-	--	149	117.189
24.9	6.01	7.93	8	22	-13.580	7.611	276	241-316	-	--
				28	-18.117	9.883	-	--	218	193-247
24.8	6.81	7.90	4	9	- 8.222	5.433	271	200-368	-	--
				5	-11.820	7.355	-	--	194	110-340

TABLE 8. ACUTE TOXICITY OF HCN TO BLUEGILL JUVENILES EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC	
°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
8.4	6.08	7.80	2	-	--	--	83.0	--	-	--
				5	-40.261	25.283	-	--	61.7	59.3-64.2
9.7	8.35	7.94	1	-	--	--	<92.0	--	-	--
15.0	6.07	7.83	3	6	-44.847	25.692	87.1	81.0-93.8	87.1	81.0-93.8
15.1	7.03	7.92	2	3	-15.982	11.190	75.0	66.0-85.2	-	--
17.8	7.97	8.12	1	-	--	--	99.0	--	-	--
20.0	6.06	7.86	6	10	-31.160	17.797	108	103-112	-	--
				9	-32.839	18.609	-	--	108	104-112
25.1	3.48	7.71	6	12	-16.173	10.593	99.7	86.0-116	99.7	86.0-116
25.0	5.05	7.78	6	15	-21.671	13.003	113	55.8-227	113	55.8-227
24.9	6.17	7.92	5	16	-19.485	11.766	120	109-133	120	109-133
24.9	6.90	7.86	6	12	-30.955	17.160	125	115-135	125	115-135

TABLE 9. ACUTE TOXICITY OF HCN TO YELLOW PERCH EGGS AND FRY EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD OR HATCHING CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC or hatch LC50	
°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
Eggs										
14.0	3.36	7.66	2	8	-11.968	6.900	288	270-307	-	--
				3	-14.050	9.006	-	--	130	--
14.1	4.96	7.76	2	6	-14.491	8.849	>329	--	159	131-194
14.0	6.04	7.87	2	6	- 5.099	4.813	>317	--	125	92.7-170
14.1	7.2	7.90	2	8	- 0.072	2.340	>389	--	147	70.0-308
18.0	6.17	7.90	2	-	--	--	>276	--	<109	--
Fry										
10.0	7.04	7.90	2	-	--	--	>357	--	<170	--
14.0	3.84	7.70	2	8	-10.431	6.249	295	256-339	-	--
14.0	5.32	7.72	2	8	- 1.910	2.734	337	202-561	-	--
14.0	7.44	7.87	4	-	--	--	>395	--	<179	--
18.1	7.00	7.94	2	-	--	--	>338	--	<165	--

TABLE 10. ACUTE TOXICITY OF HCN TO YELLOW PERCH JUVENILES EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC	
°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
15.0	6.10	7.82	6	20	-17.790	11.650	90.4	85.8-95.2	-	--
				20	-21.367	12.575	-	--	87.6	83.4-92.0
18.0	6.05	7.83	6	22	-28.964	17.215	94.0	91.8-96.2	-	--
				22	-30.573	18.062	-	--	93.2	91.0-95.5
21.4	3.56	7.69	5	13	-19.935	13.266	75.8	69.8-82.2	-	--
				11	-21.323	14.037	-	--	75.0	68.7-82.0
21.0	5.09	7.74	5	13	-19.206	12.214	95.9	88.8-104	-	--
				13	-19.711	12.538	-	--	93.5	86.7-101
21.0	6.13	7.82	6	20	-31.329	18.082	102	98.7-106	-	--
				21	-30.843	17.868	-	--	101	97.6-105
21.1	7.10	7.82	6	17	-19.768	12.172	108	102-115	-	--
				18	-19.512	12.069	-	--	107	101-114

TABLE 11. ACUTE TOXICITY OF HCN TO BROOK TROUT EGGS AND SAC FRY EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD OR HATCHING CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC or hatch LC50	
°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
Eggs										
7.1	5.96	7.80	2	6	-2.743	3.735	>212	--	118	63.0-222
10.0	3.64	7.71	2	-	--	--	>232	--	<98	--
10.0	5.96	7.79	3	10	-1.225	2.842	>228	--	155	93.4-257
10.1	8.13	7.88	3	-	--	--	>242	--	-	--
13.0	5.98	7.77	1	4	-3.695	4.367	>232	--	98.0	43.9-219
Sac fry										
10.0	3.50	7.68	2	4	-2.114	3.502	108	77.1-150	<71	--
10.0	6.03	7.78	4	11	-9.989	5.891	350	290-423	-	--
				6	-7.149	5.453	-	--	169	106-270
10.0	7.96	7.84	2	6	-5.939	4.030	518	403-665	-	--
				7	-3.095	3.496	-	--	207	107-401
13.0	6.00	7.78	2	7	-3.478	3.518	257	193-342	-	--
				8	-8.993	6.327	-	--	163	143-185

TABLE 12. ACUTE TOXICITY OF HCN TO BROOK TROUT SWIM-UP FRY EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC	
°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
7.0	6.04	7.83	6	14	- 7.954	6.695	86.0	71.4-104	-	--
				20	- 9.524	7.582	-	--	82.3	70.5-96.2
10.0	3.90	7.73	6	10	-10.709	8.996	55.8	46.8-66.5	-	--
				10	-12.962	10.372	-	--	53.9	46.8-62.1
10.0	6.04	7.80	14	37	- 8.898	7.137	88.6	82.8-94.7	-	--
				39	- 7.793	6.659	-	--	83.4	77.2-90.0
10.0	8.02	7.85	6	14	-19.180	11.944	106	100-112	-	--
				15	-11.872	8.377	-	--	103	92.1-116
13.0	6.04	7.79	8	19	-17.914	11.663	92.2	79.8-106	-	--
				21	-12.874	9.195	-	--	87.9	78.2-98.7

TABLE 13. ACUTE TOXICITY OF HCN TO BROOK TROUT JUVENILES EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC	
°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
6.9	6.01	7.84	4	8	-41.017	24.507	75.4	71.2-80.0	-	--
				7	-39.851	24.000	-	--	73.9	62.0-88.0
6.8	9.26	8.06	2	6	-50.061	28.585	84.4	77.2-92.2	-	--
				6	-47.866	27.486	-	--	83.8	75.9-92.6
10.0	4.02	7.74	6	10	-31.995	19.613	76.9	71.5-82.8	-	--
				10	-21.303	14.290	-	--	69.3	62.6-76.7
10.0	6.00	7.82	6	14	-59.000	32.883	88.4	86.0-90.8	-	--
				9	-46.346	26.698	-	--	83.8	81.0-86.7
10.0	8.06	7.90	6	11	-19.910	12.634	93.7	85.1-103	-	--
				10	-36.891	21.504	-	--	88.7	85.1-92.5
10.0	8.82	8.08	2	5	-35.896	20.435	100	95.5-105	-	--
				5	-53.042	29.153	-	--	97.9	93.2-103
13.0	6.04	7.84	4	8	-42.817	23.986	98.5	94.9-102	-	--
				8	-28.569	16.916	-	--	96.5	91.9-101
13.1	8.40	8.08	1	-	--	--	112	--	112	--

Toxicity tests with juvenile brook trout demonstrated little difference between the 96-hr LC50 and LTC values. A positive correlation between resistance to HCN and either temperature or dissolved oxygen was noted (Table 13). When the test temperature was 6.9°, 10.0°, and 13°C the LTC values, at DO levels of about 6 mg/l, were 73.9, 83.8 and 96.5 µg/l HCN, respectively. When DO levels were 4.02, 6.00, 8.06, and 8.82 mg/l, the LTC values at 10.0°C were 69.3, 83.8, 88.7, and 97.9 µg/l, respectively.

Other Fish Species

Tests conducted with juvenile black crappies and largemouth bass indicate that their sensitivity to HCN was similar to that of the other warm-water species used in toxicity tests (Table 14). Juvenile rainbow trout were the most sensitive of all species tested (Table 14). The difference in median lethal concentration between wild stock fathead minnows (Table 15), the most resistant species tested, and rainbow trout was approximately threefold. The eggs of all species tested were the most tolerant life history stage. Fry and juveniles, the least resistant stages, had similar sensitivities.

Slopes of the concentration-mortality curves were similar for all species but were not the same for all life history stages. In general, the slope for a specific species is smallest for egg bioassays and then increases with successive developmental phases to the value determined for juveniles.

TABLE 14. ACUTE TOXICITY OF HCN TO RAINBOW TROUT, LARGEMOUTH BASS AND BLACK CRAPPIE JUVENILES EXPRESSED AS 96-HR LC50 CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50	
°C	DO mg/l	pH		Treatments	a	b	µg/l	95 percent confidence limits
Rainbow trout								
10.1	8.82	8.04	6	15	-35.492	23.039	57.2	55.7-58.7
Largemouth bass								
25.0	5.94	7.88	2	4	-46.487	25.676	101	95.7-107
Black crappie								
25.0	5.74	7.90	1	3	-17.808	11.376	101	84.7-121

Effect of Temperature

A number of tests with different species indicated a marked positive correlation between tolerance to HCN and temperature rather than a negative one as might be anticipated. An attempt was made to evaluate the acute toxicity of HCN to wild fathead minnows subjected naturally to different temperatures at

TABLE 15. ACUTE TOXICITY OF HCN TO WILD STOCK FATHEAD MINNOW JUVENILES AT 5°C INTERVALS FROM 5° TO 30°C EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC	
°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
5.0	6.07	7.87	2	6	-12.903	9.128	>167	--	91.5	52.0-161
10.0	6.09	7.82	2	7	-15.532	9.259	165	140-194	-	--
				5	-44.809	23.374	-	--	135	129-141
15.0	6.02	7.88	2	8	-17.799	9.995	191	178-205	-	--
				8	-20.673	11.331	-	--	184	173-196
20.0	6.16	8.02	2	8	-31.483	16.282	174	158-192	174	158-192
25.0	5.82	7.99	2	6	-32.804	17.160	160	140-182	160	140-182
30.0	5.66	8.15	2	6	-21.242	11.957	157	126-194	157	126-194

different times of the year in a local lake. Fish were tested at 5°C intervals from 30°C in August to 5°C in December 1975. For each test, a group of fish was collected when the lake temperature was close to the desired test temperature. All tests were at a dissolved oxygen concentration of about 6.0 mg/l (Table 15). A slight increase in tolerance to HCN was observed with a decrease in test temperature from 30° to 20°C. The corresponding 96-hr LC50 values, which were equal to the respective LTC's, increased from 157 to 174 µg/l HCN. The maximum tolerance to HCN was observed in October and at 15°C with the 96-hr LC50 and LTC being 191 and 184 µg/l HCN, respectively. As the test temperature decreased in the fall, threshold tolerance also markedly decreased so at 5°C the 96-hr LC50 was greater than 167 µg/l, but the LTC reached after 35 days of exposure was 91.5 µg/l. The increase in sensitivity of field stock fathead minnows at temperatures above 15°C, a relationship not observed for laboratory reared individuals, was probably a result of environmental stresses imposed on the wild fish during the hot summer months.

In a series of tests run during a 2-mo period with brook trout from a single hatchery lot, the positive relationship between temperature from 4.0° to 18°C and tolerance to HCN was more apparent than it had been for fathead minnows. The 96-hr LC50 varied from 53.0 µg/l at 4.0°C to 143.0 µg/l at 18°C and the LTC from 51.8 to 138.0 µg/l, respectively (Table 16). The tolerance increased at a relatively uniform rate with temperature increases to 12°C, but between 12° to 18°C the tolerance increased rapidly with successive 3°C intervals. In tests run with bluegills at dissolved oxygen concentrations of about 6.0 mg/l (Table 8), there was approximately a twofold increase in tolerance between 8.4° and 24.9°C.

Effect of pH

There are a number of fish surfaces where exchange of gases and ions between blood and water can occur, but the gill epithelium is recognized as the primary site. It is also generally accepted that ions have a lesser toxicity than more lipid-soluable, un-ionized molecules. This is because of difficulty in penetrating strongly charged membranes by the relatively large hydrated ions, and the resulting repulsion by or adsorption of ions to the charged protein surface of the membrane. The acute toxicity to fish of solutions containing free cyanide (i.e., HCN plus CN^-) is mainly attributed to the toxic action of molecular HCN, varying with the concentration of gas in solution and inversely with test pH, and not with the CN^- anions which are considerably less toxic (Doudoroff and Katz 1950; Doudoroff 1976).

Because the toxicity of weak acids and bases is known to be pH dependent, the change in tolerance of juvenile fathead minnows to free cyanide as a function of pH was investigated. The fathead minnow was selected for these tests as an experimental organism because it has a wide distribution in various chemically diverse natural waters from acid bog lakes to lakes of high pH. The minnows were reared in the laboratory under a constant photoperiod in 30-l glass aquaria receiving a continuous supply of well water at 25°C and with a pH of approximately 7.9. Six lots of fish were tested during a 15-week period. All fish were approximately 13 weeks old at the time of testing, and had a mean total length of 30.8 mm. The mean wet weight of survivors was 289 mg. The fish were fed Oregon Moist and Glencoe pellets twice daily until

TABLE 16. ACUTE TOXICITY OF HCN TO BROOK TROUT JUVENILES AT TEMPERATURES FROM 4° TO 18°C EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD CONCENTRATIONS

Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC	
°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
4.0	7.19	7.92	2	5	-28.849	19.626	53.0	49.3-57.0	-	--
				4	-32.301	21.757	-	--	51.8	48.5-55.3
6.0	7.24	7.83	2	-	--	--	61.8	--	-	--
				4	-25.903	17.403	-	--	59.7	55.0-64.8
9.0	7.30	7.88	2	-	--	--	68.3	--	-	--
				4	-31.135	19.827	-	--	66.3	61.7-71.2
12.0	7.04	7.94	2	-	--	--	72.5	--	72.0	--
15.0	7.24	8.02	2	5	-32.474	18.840	97.5	69.3-137	-	--
				5	-26.234	15.737	-	--	96.6	79.3-118
18.0	7.06	8.00	2	-	--	--	143	--	138	--

24 hr prior to exposure to the toxicant.

The 96-hr toxicity bioassays were conducted in three identical test systems, each including one control and four treatment chambers with a similar experimental design as previously described for acute toxicity tests. The flow rate through each chamber provided 99 percent replacement in about 3 hr. The pH of the test water was controlled by dispensing a sulfuric acid or sodium hydroxide solution into the water supply reservoirs. The temperature of the test water was controlled at 20°C, and the water was aerated in the head reservoirs to maintain dissolved oxygen concentrations in the test chambers at about 7.5 mg/l. Each test chamber was illuminated for 12 hr each day by a 40-W incandescent bulb placed 25 cm above the chamber. Three days before beginning a set of bioassays, 10 or 20 fish which had been acclimated to 20°C for 1 week were randomly placed into each of the 12 treatment and 3 control chambers. Sulfuric acid or sodium hydroxide was then slowly added to the head reservoirs to attain the desired pH. The fish were acclimated to the specified pH for at least 2 days before the toxicant was introduced.

The relationship between test pH and the acute toxicity of free cyanide to the fathead minnow was determined for pH values from about 6.8 to 9.3 at 20°C. A summary of the test conditions and log-probit analysis of composite tests grouped according to pH is presented in Tables 17 and 18 and Figure 1. It is apparent that the 96-hr LC50 values for free cyanide and molecular HCN were fairly constant within the pH range 6.8 to 8.3. Above this pH range to 9.3 the values diverged; free cyanide levels increased and HCN decreased.

TABLE 17. MEAN TEST CONDITIONS FOR COMPOSITE FREE CYANIDE BIOASSAYS; STANDARD DEVIATIONS IN PARENTHESES

Test pH	Temperature °C	DO, mg/l	Alkalinity mg/l $CaCO_3$ Bicarbonate	Alkalinity mg/l $CaCO_3$ Total	Free CO_2 in test solution* mg/l	Free CO_2 in test solution* mm Hg
6.830(0.028)	20.1(0.12)	7.65(0.06)	96	96	28.0	12.4
7.559(0.041)	20.0(0.02)	7.56(0.09)	193	193	11.0	4.9
8.286(0.034)	20.0(0.01)	7.66(0.06)	240	240	2.5	1.1
8.672(0.067)	20.1(0.09)	7.59(0.09)	229	244	1.0	0.44
8.974(0.068)	20.0(0.12)	7.71(0.10)	214	243	0.47	0.21
9.262(0.091)	20.2(0.09)	7.63(0.12)	206	250	0.24	0.11

* Free CO_2 evaluated by nomographic method (APHA 1975). Assuming $K = H_2CO_3/P_{CO_2}$ and log K = -1.41 at 20°C and one atmosphere, then 1 mg/l CO_2 = 0.444 mm Hg CO_2 tension (Stumm and Morgan, p. 148, 1970).

TABLE 18. BIOLOGICAL ASSAY BY THE LOG-PROBIT ANALYSIS METHOD OF FATHEAD MINNOW CYANIDE BIOASSAYS AT 20°C GROUPED ACCORDING TO TEST pH

Test pH	Log-probit regression analysis* Treat-ments	α	β	96-hr LC50 µg/l as HCN	95 Percent confidence limits for LC50, µg/l
			HCN		
6.830	6	-23.80	13.76	124	106-144
7.559	7	-23.47	13.81	115	102-130
8.286	6	-27.37	15.52	112	115-128
8.672	9	-33.46	18.88	109	105-113
8.974	17	-16.52	10.68	104	96-112
9.262	10	-28.73	17.55	83	80-87
			Free Cyanide		
6.830	6	-23.82	13.76	124	107-144
7.559	7	-23.60	13.82	117	102-135
8.286	6	-28.00	15.54	133	126-140
8.672	9	-34.59	18.64	133	128-138
8.974	17	-16.44	9.83	152	140-165
9.262	10	-28.57	15.29	157	142-173

*For equation $Y_i = \alpha + \beta (\log X_i)$ when Y_i is the maximum likelihood probit value and X_i is log cyanide concentration as µg/l HCN (Dixon 1973).

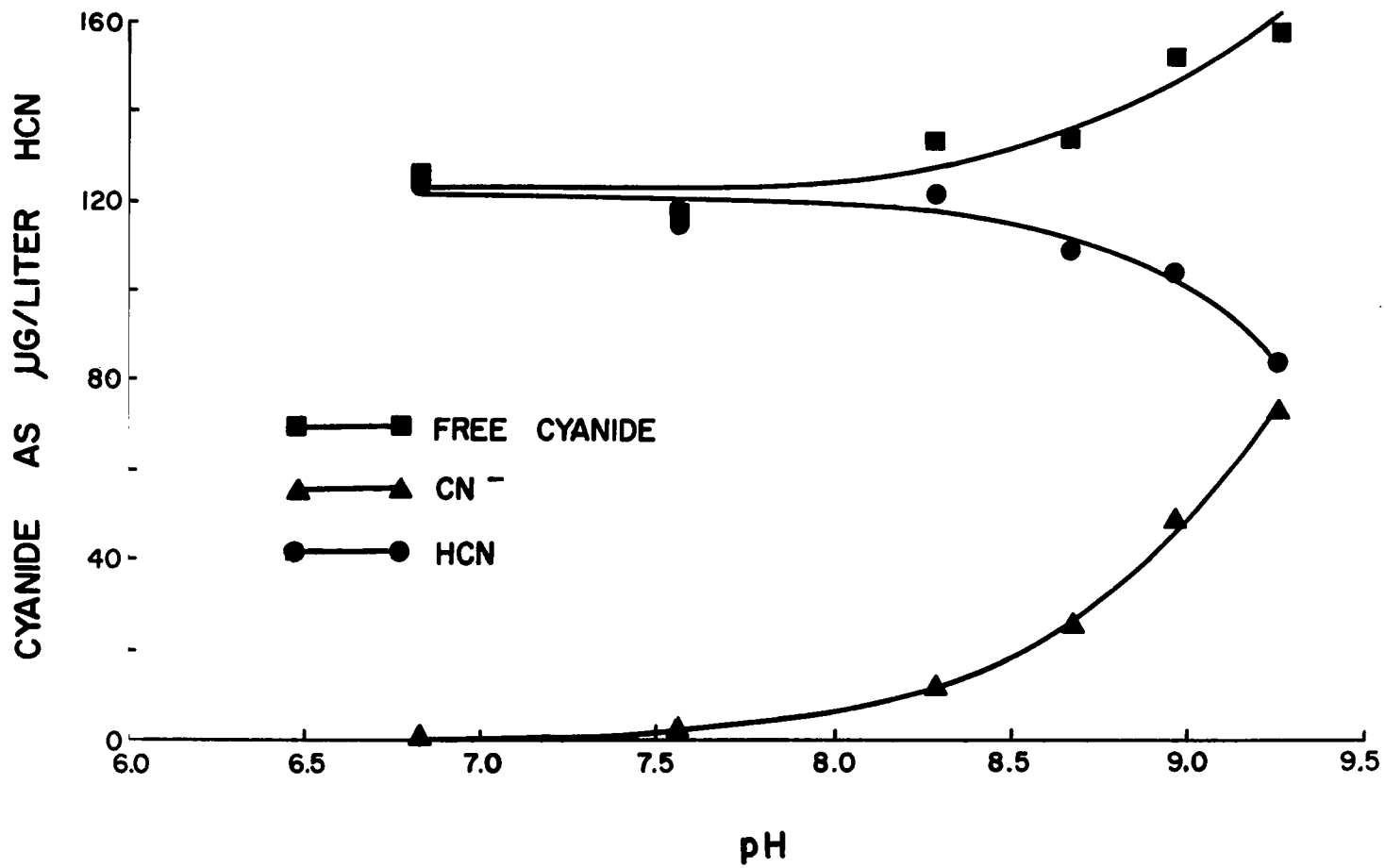

Figure 1. Relationship between test pH and 96-hr LC50 cyanide concentrations for the fathead minnow at 20°C

At pH 8.7 and below the toxicity of free cyanide ($HCN + CN^-$) is due principally to HCN but CN^- apparently contributes to the observed toxicity at higher pH values. Since pH is seldom greater than 8.7 in most natural waters, it is proposed that the CN^- ion contributes very little to the toxicity of solutions containing free cyanide (Broderius *et al.* 1977).

Invertebrates

In acute toxicity tests with *Asellus communis* Say at 18°C and DO of 7.52 mg/l the 96-hr LC50 was 2328 µg/l and LTC (at 11 days) was 1834 µg/l, both with broad confidence limits (Table 19). Tests with *Gammarus pseudolimnaeus* Bousfield at temperatures from 5.2°C to 20°C and DO of 6.70 to 7.88 mg/l, indicated that the median lethal threshold concentrations of HCN varied from 58.1 µg/l at 20°C to 183.8 µg/l at 5.2°C. The linear regression of temperature (X) versus LTC (Y) for *Gammarus* is expressed by the equation

$$Y = 225.7 - 8.117\ (X)$$

with $r^2 = 0.96$.

Summary

The 96-hr LC50 values for most species and their life history stages occurred at a slightly higher HCN concentration than when acute toxicity is measured at the median lethal threshold level. The life history stage most sensitive to HCN varies among the fish species, but it was generally observed that eggs are the most tolerant and juveniles the least tolerant. The response of fish eggs is often so variable that an acute toxic concentration of HCN cannot always be confidently assigned. The range of acutely toxic concentrations of HCN among species is not great in the juvenile stage, except that rainbow trout are appreciably more sensitive than brook trout or any of the other species which were studied. At high dissolved oxygen levels and temperatures above 9°C, the acute toxicity of HCN to juvenile fish varied from 57 µg/l for rainbow trout to 191 µg/l for wild fathead minnows. Temperature has a marked effect on acute toxicity of HCN; juvenile fish in general become more sensitive at lower temperatures. The sensitivity of wild stock fathead minnows also increased at high temperatures. Dissolved oxygen levels below 5 mg/l increased the sensitivity to cyanide of the juveniles of all species tested. The implications of lowered oxygen and lower temperatures during cold weather seasons in systems receiving cyanide wastes should be carefully evaluated in the development of standards set by regulatory agencies. No chronic tests have been conducted incorporating low temperature cycles, but the possibility of increased sensitivity and adverse effects from this factor should not be ignored.

TABLE 19. ACUTE TOXICITY OF HCN TO *ASELLUS COMMUNIS* AND *GAMMARUS PSEUDOLIMNAEUS* ADULTS EXPRESSED AS 96-HR LC50 AND MEDIAN LETHAL THRESHOLD CONCENTRATIONS

	Mean test conditions			No. of tests	Log-probit regression analysis			96-hr LC50		LTC LC50	
	°C	DO mg/l	pH		Treat-ments	a	b	µg/l	95 percent confidence limits	µg/l	95 percent confidence limits
Asellus communis	18.0	7.52	8.13	2	6	- 4.756	2.898	2328	1710-3168	--	--
					6	-10.152	4.643	--	--	1834	1513-2224
Gammarus pseudolimnaeus	5.2	7.05	7.86	2	8	- 0.654	2.497	--	--	183.8	145.5-232.1
	10.0	7.06	7.88	2	7	--*	--*	--	--	140.0*	--*
	15.0	7.03	7.91	2	8	- 5.760	4.826	169.6	142.8-201.5	--	--
					8	- 3.422	4.479	--	--	75.9	65.7-87.7
	18.0	7.60	8.00	3	11	- 2.342	3.271	175.6	133.5-231.0	--	--
					11	- 1.113	3.318	--	--	69.6	53.0-91.2
	20.0	6.70	8.14	2	8	1.149	2.002	83.9	63.7-110.6	--	--
					8	- 0.158	2.924	--	--	58.1	38.8-87.0

* LC50 calculated by graphical interpolation; computer program could not handle data

SECTION V

CHRONIC TOXICITY

Three species of fish, the fathead minnow, brook trout, and bluegill, and two invertebrates, *Asellus* and *Gammarus*, were used in experiments to determine the chronic toxicity of cyanide.

Little information is available on the chronic effects of hydrogen cyanide on fish. Neil (1957) and Broderius (1970) found that free cyanide concentrations of 10 μg/l expressed as CN impaired the swimming performance of salmonid fishes. Leduc (1966) measured the growth of juvenile cichlids (*Cichlasoma bimaculatum*) receiving unrestricted rations of tubificid worms and subjected to various levels of free cyanide for 36 days at 25°C. Fish exposed to free cyanide levels of 60 μg/l and higher, expressed as HCN, grew more slowly than control fish during the first 12 days of the experiments, but grew faster than control fish during the final 12 days so that the effect of free cyanide up to 100 μg/l, the highest treatment, on total weight gain was slight.

Leduc (1966) observed a similar pattern of growth with time in a single 24-day experiment at 16°C with juvenile coho salmon (*Oncorhyncus kisutch*) fed an unrestricted diet of earthworms. In this experiment, however, total weight gain was markedly reduced compared to controls at both the lowest (10 μg/l) and highest (80 μg/l) treatment levels but appeared to be unaffected at intermediate levels of 20 μg/l and 40 μg/l free cyanide as HCN. The author did not explain the poor growth in the lowest treatment level.

Since experimental designs were different for each species, descriptions of apparatus and design are handled separately. The test water and analytical procedures were the same as those described in the section on acute toxicity.

FATHEAD MINNOW

Experimental Design

The experiment was started in fifteen 20-l glass chambers. A cyclic flow of water was supplied to the test chambers at the rate of 1 l every 3 min. Twelve different treatment levels and three controls were randomly assigned to test chambers. A toxicant metering system [Mount and Warner (1965)] introduced sodium cyanide stock solutions to treatment chambers with each water-delivery cycle. Nominal treatment levels were from 5 to 100 μg/l HCN. Mean temperatures in test chambers varied over the experimental period from 24.8° to 25.1°C with standard deviations from 0.2° to 0.3°C. Mean pH readings ranged from 8.06 to 8.09 with standard deviations from 0.04 to 0.12, and mean

concentrations of dissolved oxygen were from 5.9 to 6.3 mg/l, with standard deviations from 0.5 to 0.7 mg/l. Each test chamber was lighted with a 40-W incandescent bulb 30 cm above the water surface. The photoperiod simulated Evansville, Indiana day length starting at the December 15 day length of 10 hr, 30 min, and was adjusted biweekly.

Over a 4-day period, 80 newly hatched fathead minnow larvae of a strain from the U.S. EPA Environmental Research Laboratory, Duluth, Minnesota, were placed in each test chamber. When all chambers contained 80 larvae, sodium cyanide was introduced into the treatment chambers. Free cyanide concentrations in each chamber were determined three times weekly thereafter.

Through the first week of exposure to HCN, the fish were fed a fine, hard-boiled egg yolk suspension three times daily. Beginning on the second week, a supplement of finely ground Glencoe granules was fed twice daily. The fish were fed newly hatched brine shrimp (*Artemia*) once daily beginning the third week, and finely chopped lettuce was added to the egg yolk suspension in the fifth week. During the first 4 weeks of the test, fish were fed an excess ration of food. Fish were counted after 28 days of exposure to HCN, and subsequent feedings were adjusted to the number of fish in each tank. After 56 days individual fish were weighed and randomly thinned to a maximum of 40 fish per chamber. The individual fish were again weighed after 84 days. After 106 days the fish were transferred to a larger test unit consisting of 20 treatment and 5 control chambers, each containing 35 l. The chambers were randomly arranged in groups of five. Each group of chambers was illuminated by two 40-W Vita-Lite fluorescent tubes. One treatment (No. 7) was lost before transfer to the larger apparatus. Of the remaining treatments, eight contained enough fish to be duplicated in the new apparatus so that each chamber contained approximately 20 fish. The three highest treatments each contained 20 fish or fewer and were not duplicated. Two of the three original control chambers were duplicated in the new apparatus. A spawning substrate was provided by placing four 7.5-cm long halves of 7.5- and 10-cm diameter asbestos drain tile in each tank.

The first spawning took place after 149 days. When the first spawning occurred in a chamber, four mature males and three mature females were selected to remain, and two spawning tiles were added. The 20 fish in one of the previously unreplicated treatments were divided between 2 chambers after the first spawning in the treatment.

Egg incubation experiments began after 192 days. When spawning occurred in a chamber, some of the eggs were removed from the tile, and a sample of 50 undamaged eggs was placed in each of two 5.7-cm ID acrylic plastic cylinders covered at one end with 351 μ nylon monofilament screen. Cylinders were suspended in the test water and oscillated at two cycles per minute through an amplitude of 2.5 cm. Eggs not incubated were counted and discarded.

Simulated day length reached a maximum after 183 days and began to decrease after 214 days. After 200 days of exposure, mortality, mostly females, began to occur in the higher treatment levels. Spawning ceased after 256 days.

After 227 days, a 56-day growth and survival experiment was started with second generation larvae hatched in the parent experiment. The test conditions in this experiment were identical to those imposed on the previous generation. Photoperiod, however, was kept constant at the maximum day length of 15 hr, 45 min. Individual treatments were started on different dates, since all treatments in the parent experiment did not produce eggs on the same day. Some parent treatments did not produce fertile eggs in sufficient numbers to continue those treatments through the second generation. Fry which had been spawned and hatched in control chambers were substituted in those treatments.

After 28 days of exposure to HCN, the second-generation fish were measured photographically (Martin 1967) and were randomly thinned to a maximum of 40 per chamber. After 56 days the fish were measured and weighed, and the experiment was terminated.

For statistical analysis, lengths and weights were transformed to logarithms and percentages to angles (Steel and Torrie 1960). Lengths, weights, ages at the onset of spawning, egg production, and hatchability data were subjected to one-way analysis of variance. Treatment means were compared with control means using Dunnett's procedure (two-tailed) with Kramer's modification for unequal replication, at a significance level of $\alpha = 0.05$. Means in tables were calculated from transformed values.

Results

First Generation--

Survival--After 28 days, survival in the first-generation experiment averaged 64 percent in the three controls, and ranged from 80 percent to 11 percent in treatments (Table 20). A significant ($\alpha = 0.01$) correlation coefficient of -0.84 was found between survival and HCN concentration (controls omitted). Lack of treatment replications in this phase of the parent experiment precluded analysis of variance and treatment comparisons.

Over the period between 28 and 56 days, survival was at least 80 percent in all chambers. Survival was at least 95 percent in all chambers between 56 and 84 days (Table 20). Survival in these two periods was not significantly correlated with HCN concentration at the 0.05 level.

Growth--The mean weight of fish in treatments ranged from 130 percent to 65 percent of that in controls after 56 days, and from 128 percent to 86 percent of mean weight in controls after 84 days. Although food was introduced to each chamber according to the number of fish it contained after 28 days, the tendency for variations in cumulative percentage survival to influence the relationship between mean weight after 56 days and HCN concentration can be seen in Table 20. For this reason no analysis of variance was performed.

Reproduction--The length of time from the start of HCN exposure to the onset of spawning averaged 156 days in controls, and ranged from 148 to 206 days in treatment chambers. In no treatment did spawning begin significantly earlier or later than in the controls.

TABLE 20. SURVIVAL AND WEIGHT OF FIRST-GENERATION FATHEAD MINNOWS AFTER 28, 56, AND 84 DAYS OF EXPOSURE TO HYDROGEN CYANIDE*

Treatment	Mean HCN† concentration (μg/l)	Percentage survival‡			Mean weight (g)	
		0–28 d	28–56 d	56–84 d	56 d	84 d
Control A		64	94	100	0.292	0.581
Control B		71	100	100	0.236	0.588
Control C		58	100	100	0.354	0.668
1	5.9	80	100	98	0.205	0.580
2	11.4	59	100	100	0.274	0.676
3	17.9	60	98	95	0.270	0.631
4	24.7	51	100	100	0.296	0.688
5	32.8	46	100	97	0.382	0.785
6	40.5	59	100	100	0.272	0.555
8	57.5	49	97	97	0.190	0.458
9	66.8	49	87	100	0.220	0.528
10	75.3	29	91	100	0.264	0.629
11	88.9	19	80	100	0.199	0.560
12	98.1	11	100	100	0.190	0.627

*Chambers originally contained 80 larvae. Numbers were reduced to a maximum of 40 per chamber after 56-day measurements.

†Eighty-four day period.

‡Survival of fish present at beginning of period.

Total egg production and egg production per female in each chamber are shown in Table 21. Due to the mortality of adult females in the higher treatment levels, the following calculation of egg production per female was required: the number of females surviving during each day of the 107-day spawning period was totaled for each chamber and divided by 107; the quotient was, in turn, divided into the total number of eggs produced in the chamber. Egg production per female in treatments ranged from 72 percent of that in controls to zero (Table 21). Egg production per female was significantly reduced relative to controls in HCN treatments of 19.6 μg/l (Treatment 3) and above.

The number of egg samples incubated in each chamber and the mean percentage hatch are shown in Table 21. Control B was omitted from statistical analysis of hatchability because the mean percentage hatch (20.3 percent) of egg samples spawned and incubated in that chamber was 17 standard deviations lower than the mean percentage hatch in the other four controls (83.9 percent). The mean percentage hatch of egg samples spawned and incubated in treatments ranged from 84.7 percent to 10.0 percent.

Using one-way ANOVA with subsampling, differences in mean percentage hatch between duplicate chambers within treatments were found to be significant ($F_{11,162} = 2.27$, $\alpha = 0.025$). In Treatments 6 and 9, mean percentage hatch was significantly lower than in the controls. The small number of samples incubated in Treatments 8 and 10 decreased the sensitivity of statistical comparisons between those treatments and the controls so that the differences found were not significant. When the parent experiment was terminated, the weights of survivors of either sex in the treatment chambers did not differ significantly from the weights of control fish (Table 21).

F_1 Generation--

Survival--After 28 days survival was generally higher in the F_1 experiment than in the parent experiment. Survival averaged 84 percent in the three controls and ranged from 88 percent to 36 percent in treatments (Table 22). Survival and HCN concentration were not significantly correlated at the 0.05 level. Over the period of 28 to 56 days, all chambers had at least 81 percent survival. Survival from 28-56 days was not significantly correlated with HCN concentration at the 0.05 level.

Growth--Mean length of fish in treatments after 28 days ranged from 116 percent to 64 percent of that in controls. The fish at 26.3 μg/l were significantly longer than control fish, and those exposed to 34.8 μg/l (Treatment 5) and above were significantly shorter than control fish.

Since the numbers of fish in experimental chambers were made approximately equal after 28 days in the F_1 experiment, the effect of HCN on fish size after 56 days is more evident here than in the present experiment. Mean lengths of fish in treatments after 56 days ranged from 105 percent to 81 percent of mean length in controls, and mean weights ranged from 122 percent to 52 percent of that in controls (Table 22). No treatments contained significantly longer or heavier fish than did the controls. Mean lengths and weights of fish at 61.6, 70.5, 95.9 and 105.8 μg/l HCN, respectively, were significantly reduced when compared with controls. The differences between length and weight of the

TABLE 21. EGG PRODUCTION, HATCHABILITY, AND TERMINAL WEIGHTS FOR FIRST-GENERATION FATHEAD MINNOWS EXPOSED TO VARIOUS CONCENTRATIONS OF HYDROGEN CYANIDE*

Treatment		HCN concentration (µg/l) Mean (256 d)	HCN concentration (µg/l) Std. dev.	Eggs per female	Hatchability Mean percentage	Hatchability Egg samples	Mean weight of surviving adults† (g) Males	Mean weight of surviving adults† (g) Females
Control	A			2530	86.2	10	4.78(4)	2.36(3)
Control	B			4483	20.3	23	4.11(4)	2.34(3)
Control	C			3990	87.9	13	3.93(4)	2.59(3)
Control	D			2718	79.4	7	4.47(4)	2.44(3)
Control	E			3660	82.1	16	4.85(3)	2.52(3)
1	A	5.7	1.4	1886	38.8	12	5.24(4)	2.84(3)
	B	5.9	1.3	3138	84.3	11	3.76(4)	2.37(3)
2	A	13.0	1.6	1701	81.2	9	3.89(4)	1.86(3)
	B	12.7	1.8	1989	81.4	13	5.45(4)	2.37(3)
3	A	19.6	3.9	1694**	84.7	11	3.95(4)	3.02(3)
	B	19.6	4.1	1241**	28.0	7	4.67(4)	1.64(2)
4	A	27.1	2.7	1093**	34.8	6	5.22(3)	1.73(1)
	B	27.5	3.4	1640**	43.8	7	4.55(4)	2.39(1)
5	A	36.0	4.0	678**	38.3	6	3.01(4)	2.57(1)
	B	35.6	3.9	1341**	62.9	15	5.06(4)	2.42(3)

- Continued -

TABLE 21. CONTINUED

Treatment		HCN concentration (µg/l) Mean (256 d)	Std. dev.	Eggs per female	Hatchability Mean percentage	Egg samples	Mean weight of surviving adults† (g) Males	Females
6	A	43.7	7.0	2054**	10.0**	18	4.48(3)	2.39(1)
	B	44.7	5.1	194**	15.6**	3	5.52(4)	2.13(2)
8	A	62.5	8.0	74**	11.2	5	5.00(3)	2.26(3)
	B	64.6	9.2	70**	32.0	1	3.84(3)	3.00(2)
9	A	73.1	8.1	573**	19.1**	8	4.60(3)	2.41(3)
	B	72.4	10.5	64**	20.0**	1	3.82(1)	1.96(2)
10	A	81.5	12.1	266**	20.4	4	3.85(3)	2.54(2)
	B‡	79.8	14.1	219**	-	0	5.13(1)	1.92(2)
11§		96.1	9.3	0**	-	0	4.58(4)	1.89(5)
12#		105.4	10.1	0**	-	0	0	2.52(6)

* Except where noted, chambers contained four males and three females when spawning began.

† Parenthesized values are numbers of survivors.

‡ Started after 157 d with three extra males and three extra females from 10A.

§ Contained five males and six females after 106 d; too few for replication.

\# Contained two males and six females after 106 d; too few for replication.

** Values are significantly different from control values according to Dunnett's procedure (two-tailed; $\alpha = 0.05$).

TABLE 22. SURVIVAL, LENGTH, AND WEIGHT OF F_1 FATHEAD MINNOWS AFTER 28 AND 56 DAYS OF EXPOSURE TO HYDROGEN CYANIDE

Treatment	HCN conc.† (µg/l)	Percentage survival‡		Mean total length (mm)		Mean weight after 56 d (g)
		0-28 d	28-56 d	28 d	56 d	
Control A §		85	100	15.0	29.2	0.238
Control B §		83	98	14.2	28.7	0.238
Control C §		84	95	13.6	26.7	0.205
1	5.7	80	100	14.5	28.7	0.234
2	12.2	39	94	14.7	28.0	0.258
3	20.5	60	100	13.9	28.6	0.221
4#	26.3	88	95	16.6‡	29.6	0.277
5	34.8	48	100	12.5‡	27.6	0.184
6§	43.0	64	100	12.0‡	27.1	0.212
7§	52.2	58	98	10.8‡	26.8	0.199
8§	61.6	64	100	11.7‡	26.7‡	0.172‡
9	70.5	56	95	10.5‡	25.9‡	0.160‡
10**	81.0	36	95	10.0‡	24.8	0.163
11§	95.9	64	100	10.4‡	24.8‡	0.150‡
12§	105.8	40	81	9.2‡	22.9‡	0.188‡

*Except where noted, chambers originally contained 80 larvae. Numbers reduced to a maximum of 40 per chamber after 28-d measurements.

†84-d period.

‡Survival of fish present at beginning of period.

§ Larvae spawned and hatched in control chambers of parent experiment.

#Began with 42 larvae.

**Began with 59 larvae.

‡ Values are significantly different from control values according to Dunnett's procedure (two-tailed; $\alpha = 0.05$).

survivors in Treatment 10 at 81.0 μg/l HCN and that of the controls was not significant.

Summary--

Most of the mortality in treatments and controls occurred during the first 28 days of both the parent and F_1 experiments (Tables 20 and 22). Survival during this period was better in the F_1 experiment and was significantly correlated with HCN concentrations only in the parent experiment. The disparity between generations probably was not caused by development of resistance to HCN by the second generation of exposed fish, since fish which had been spawned in controls of the parent experiment and used in Treatments 6, 7, 8, 11, and 12 of the F_1 expermient appear to have been as resistant to HCN as fish which had been spawned in treatments. The staggered starting dates of individual treatments or the fact that no two chambers contained fish from the same spawning may have contributed to the greater random variation in survival in the F_1 experiment.

The combined effects of survival and HCN concentration on early growth of the parental generation make it difficult to interpret treatment-to-treatment differences in mean weight at 56 and 84 days. In the F_1 experiment, where numbers of test fish were reduced after 28 days, 34.8 μg/l HCN and higher significantly reduced length after 28 days of exposure, and 61.6 μg/l and higher significantly decreased length and weight after 56 days. The tendency of fish in treatments to approach the size of control fish between 28 and 56 days, a growth pattern also observed by Leduc (1966), may have been the result of size-dependent physiological or behavioral phenomena or may have been an experimental artifact caused by crowding of the larger fish in the controls and lower treatments. Again, there is no evidence that the fish in Treatments 6, 7, 8, 11, and 12, which had been spawned in controls, were more or less resistant to HCN than the fish that had been spawned in their respective treatment levels.

The number of eggs produced per female was significantly reduced compared to controls at 19.6 μg/l HCN and above. Egg hatchability was significantly lower in Treatment 6 (44.2 μg/l) and Treatment 9 (72.8 μg/l) than in controls.

We estimate that the highest no-effect level of HCN for the fathead minnow lies between 12.9 and 19.6 μg/l, based on statistical evaluation of egg production. The lethal threshold concentration of HCN for juvenile fathead minnows of the test strain at 25°C, pH 8, and 5-7 mg/l DO, derived from acute toxicity bioassays (Table 6) is approximately 120 μg/l. The application factor therefore (no-effect level/LTC) lies between 0.11 and 0.16.

The statistical significance of an effect in the laboratory does not necessarily imply ecological importance. Waller *et al.* (1971), in a computer simulation study, found that a population of fathead minnows subjected to random environmental variation would be driven to extinction by an 80 percent reduction in the number of progeny recruited into the spawning stock. Assuming that recruitment is proportional to fecundity, such a level of reduction could be achieved between 44.2 and 63.6 μg/l HCN on the basis of fecundity effects alone. At these toxicant concentrations, reduction in hatchability and early

survival could also be expected to influence the level of recruitment.

BROOK TROUT

Experimental Design

Adult brook trout were tested in 10 fiberglass tanks (210 x 54 x 54 cm) with a 30-cm standpipe 12 cm from the downstream end. The tanks contained a volume of 340 l. Each of two head tanks fed water to four cyanide treatments and a control.

A diluter system similar to that described by Brungs and Mount (1970), but utilizing a microswitch and two magnetic drive pumps, was used to control water flow. The toxicant dispensing system was similar to the one described by Mount and Warner (1965). The dilution system dispensed 4 l of water every 2.5 min, providing 99 percent replacement in 17 hr.

The adult fish in each tank were allowed to spawn in two boxes 50 x 38 x 15 cm deep described by Benoit (1974). Eggs were removed on the day of spawning from each spawning box and placed in incubation cups made of acrylic plastic cylinders 6.5 cm OD with Nitex screen bottoms. Eggs were incubated in separate diluter systems employing 20-l glass aquaria. The egg cups were oscillated in the test water by means of a rocker-arm apparatus described by Mount (1968). Brook trout fry and juveniles were subsequently kept in the diluter system used for egg incubation. The diluter system was similar to that described for adults, but it utilized two solenoid valves instead of pumps. A water volume of 1.35 l was dispensed every 2.5 min, and 99 percent replacement occurred in 3 hr.

Temperature, pH and free cyanide concentrations in test waters were measured three times per week, and dissolved oxygen was measured twice per week. In all treatments the mean dissolved oxygen concentration ranged from 64-74 percent saturation, the pH from 7.94-8.01, and total alkalinity from 236-239 mg/l as $CaCO_3$. Water temperatures in the adult brook trout experiments were varied on a seasonal basis with 14°C in May; 15° in June-August; 12°C in September; and 9°C in October. All egg and juvenile tests were conducted at 9°C. Eggs were incubated and hatched in the dark. Fluorescent lights were used in all experiments with other stages. Photoperiod was adjusted bimonthly to simulate the day lengths in Evansville, Indiana.

Nineteen-month-old adult brook trout with mean weight of 103.9 g were obtained from the Wisconsin State Fish Hatchery in Osceola, Wisconsin, on April 29, 1975. Ten fish were randomly placed in each experimental tank on the day of collection, and were kept at 14°C and treated 8 hr per day for 3 days with 20 mg/l tetracycline. Water flow was stopped for 8 hr during treatment, and the temperature rose 0.5°C during this period. After treatment the fish were anesthetized with MS-222 for weighing and measuring. Fish were fed dry pellets at a mean rate of 1.3 percent of their wet body weight per day. Five days after fish were placed in the test tanks, the flow of toxicant was started. The fish were weighed and measured at the beginning of each month until they were thinned. On September 25 the adults were randomly thinned to two males and four females per tank, except at 53.9 μg/l HCN where three

females were present. On the same day a spawning box was placed in each tank at the downstream end, and a second box was placed in the middle of each tank on September 30. Spawning boxes were inspected daily for eggs. The total number of eggs, the number of viable eggs, and the number of spawnings were recorded for each test tank. All spawners were sacrificed 3 weeks after the last spawning or egg deposition. The gonads were inspected for development and to determine which fish had spawned. From each spawning of 50 eggs or more, 50 randomly selected eggs were placed in incubation cups, and the remainder, up to 250, were placed in other incubation cups for determination of viability. Viability was reported as the percentage of eggs which developed a neural keel within 12 days at 9°C. Dead eggs were removed daily from the cups. At concentrations where no spawning occurred, eggs from controls were used to determine hatchability, juvenile survival, and growth. When hatching was completed, 25 fry were randomly chosen for growth and survival tests. Weight at hatching was determined from discarded sac fry. The sac fry were kept in the incubation cups for 21 days and then released into the 20-l tanks. Since the hatching period was spread over 3-6 days, the median hatching date was used to establish a growth period of 90 days. The temperature during incubation and growth of juveniles was maintained at 9°C. The fish were measured at hatching and 30, 60, and 90 days after hatching, and were weighed at hatching and 90 days afterward. Length was measured photographically (Martin 1967) from the snout to the end of the caudal peduncle. The juveniles in the growth tests were fed Glencoe pellets two times per day and newly hatched brine shrimp once per day *ad libitum*. The numbers of juveniles were reduced to 20 fish per tank at 60 days.

Growth data were transformed to logarithms and analyzed with one-way analysis of variance followed by Duncan's new multiple range test (Steel and Torrie 1960). One-way analysis of variance was used on the viability and hatch results, with an arcsin square root transformation of the percentages.

Results

Adults--

Growth and Survival--Adult brook trout grew to a mean weight of 229.2 g during 144 days of exposure to cyanide. No significant growth reduction was noted at any concentration ($p > 0.05$). One fish died in each treatment at 53.9, 64.9, and 75.3 µg/l HCN. The deaths occurred after the temperature was lowered to 9°C.

Reproduction--Spawning began on September 27, 1975, in one control tank and in the lowest HCN level, 5.7 µg/l (Table 23). Spawning in other treatments started a few days later, and the peak spawning rate occurred the second week of October. Results from the treatment at 21.8 µg/l HCN were omitted from the spawning analysis because only one female developed eggs in this treatment, and the deposited eggs were infertile.

The number of eggs spawned per female decreased with an increase in HCN concentration above 5.7 µg/l (Table 23). Regression analysis indicated that a significant downward trend occurred with a resulting regression described as $Y = 552.8 - 6.49X$, where: Y = mean number of eggs spawned per female and X = HCN (µg/l). The correlation coefficient between HCN concentration and eggs

TABLE 23. EGG PRODUCTION OF ADULT BROOK TROUT EXPOSED TO HCN FOR 144 DAYS PRIOR TO START OF SPAWNING

HCN (µg/l)	Standard deviation (µg/l)	Sex ratio M/F	Spawning	Percent viability*	Mean eggs spawned per female	Mean weight of females (g)
Control A	-	2/4	5	93.6	502	237.6
Control B	-	2/4	11	93.4	744	225.0
5.7	0.9	2/4	6	89.9	513	198.5
11.2	1.3	2/4	3	78.1	291	238.5
32.3	3.8	2/4	3	72.9	246	156.1
43.6	3.9	2/3	3	86.6	442	258.0
53.9	6.8	2/3	3	64.1	262	213.0
64.9	7.3	2/3	1	0	124	204.5
75.3	8.8	2/2	0	0	0	193.5

* Formation of neural keel

spawned per female was -0.84.

Egg viability was determined for all spawnings, and no fertile eggs were found at 64.9 and 75.3 µg/l HCN. Statistical testing of the viability of all spawned eggs in the remaining treatments showed no significant effects due to cyanide ($p > 0.05$). A composite sperm sample from several untested fish was placed in each of the HCN concentrations used in the spawning tests with no apparent reduction in motility ($p > 0.05$) at any level.

Embryos, Fry and Juveniles--

Growth--Length and weight of fry at hatching were not significantly different ($p > 0.05$) in any treatment or control, but growth of juveniles in HCN was reduced after hatch (Table 24). After 30 days of exposure no significant difference ($p > 0.05$) in length was found between fish exposed to 11 µg/l HCN and fish in the control. Growth in all other treatments was significantly different from control growth ($p < 0.05$). Growth was greater at 5.6 µg/l than in the controls and less at all treatments greater than 11 µg/l HCN. Significant differences in growth ($p > 0.05$) were not found between 22 and 44 µg/l, 33 and 55 µg/l, and 55 and 66 µg/l. At 77 µg/l growth was less than that in the controls and all other treatments. At 60 days the differences in linear growth among treatments were greatest ($F = 53.42$). No significant difference was found at this age between the controls, 11 µg/l, and 22 µg/l ($p > 0.05$).

TABLE 24. GROWTH OF EMBRYO AND JUVENILE BROOK TROUT EXPOSED TO VARIOUS LEVELS OF CYANIDE

HCN µg/l	Stand. dev. µg/l	$\bar{x}$ Wt. at hatch (mg)	Mean length (mm) Hatch	30 d	60 d	90 d	$\bar{x}$ Wt. 90 d (mg)
Control	-	40	13.6	19.6	29.9	41.5	1031
Control	-	42	13.7	20.3	29.3	39.8	910
Control	-	41	13.8	20.1	29.7	41.9	1150
Control	-	39	11.7				
Control	-	38	13.7				
5.6	0.84	43	14.4	20.6	29.8	40.1	937
5.6	0.62	41	12.9	20.4*	31.8*	43.6	1238
5.5	0.50	41	12.7				
5.7	0.50	38	12.6				
11.1	0.86	39	13.1	19.3	29.3	42.4	1051
11.5	2.60	42	13.6	20.6	29.2	44.1*	1219*
11.4	0.72	40					
21.6	1.69	40	12.7	19.7	29.5	41.5	972
22.1	1.52	40	13.0	19.3*	29.5	42.5	1067
33.2	2.60	-	12.0	18.7	26.0	37.1	681
33.4	2.13	38	12.3	18.6*	25.7*	37.4*	762*
32.5	2.15	36	12.8				

- Continued -

TABLE 24. CONTINUED

HCN µg/l	Stand. dev. µg/l	$\bar{x}$ Wt. at hatch (mg)	Mean length (mm) Hatch	30 d	60 d	90 d	$\bar{x}$ Wt. 90 d (mg)
43.6	6.08	40	12.9	19.7 *	26.4 *	36.4 *	688 *
43.5	6.10	40	13.3	18.8	25.4	35.5	651
45.0	8.42	39	13.0				
55.9	4.98	41	12.1	18.0 *	25.0 *	32.8 *	478 *
54.7	7.43	40	12.5	19.0	24.8	32.3	460
55.9	7.68	42	12.2				
66.8	3.37	42	12.6	18.8 *	23.4 *	31.5 *	387 *
67.5	4.40	40	11.0	17.8	23.2	28.8	284
77.0	4.23	40	12.2	16.1 *	21.8 *	25.7 *	196 *
77.4	4.88	37	11.7	16.4	20.0	22.9	127
77.5	3.41	42	12.5				

*Mean significantly different from the control ($p \leq 0.05$).

Length of fish at 5.6 μg/l was significantly greater than that in the controls. Growth was less than the controls ($p < 0.05$) at 33 μg/l HCN and greater concentrations. No significant difference ($p > 0.05$) in length was found between 33 and 44 μg/l and 55 and 66 μg/l, but at 77 μg/l HCN it was less than all other treatments. At 90 days the differences among treatments in length of fish were marked. The lengths of fish in the controls, 5.6 μg/l, and 22 μg/l were not significantly different. Growth at all other treatments was significantly different from one another ($p \leq 0.05$, $F = 41.38$). Growth at 11 μg/l was greater than that in the controls, and growth at 33 μg/l HCN and higher levels was progressively less with increasing concentration. At 90 days, differences in weight among treatments were less extreme, but showed the same pattern of differences as lengths ($F = 37.01$). No difference ($p > 0.05$) was found among the controls, 5.6 and 22 μg/l, between 5.6 and 11 μg/l, and between 33 and 44 μg/l. All treatments of 33 μg/l and greater showed a weight reduction ($p \leq 0.05$) when compared to the controls.

Survival--Survival of embryos and juveniles in each treatment is given in Table 25. Eggs from controls were used in treatments in which no spawning occurred (22, 66, and 77 μg/l HCN). No significant differences ($p > 0.05$) in hatchability of eggs exposed to cyanide was noted because of the high variability between replicates. Survival of 90-day-old juveniles decreased with increasing cyanide concentrations. No significant differences were noted between the control and 5.5, 11, 22, 33, and 44 μg/l ($p > 0.05$). Survival at 55, 66, and 77 μg/l was different from the survival in the control ($p < 0.05$). No difference occurred between 55 and 66 μg/l, but survival at 77 μg/l was different from survival at all other levels ($p \leq 0.05$).

Summary--

Growth was significantly reduced by cyanide at the early life stages, but no differences were noted in adults. During the early life stages from embryo to juvenile, growth was progressively more influenced by the cyanide concentration as age increased. This increase in sensitivity was also shown in acute toxicity tests (see Section IV). The level of cyanide having no adverse effect on the growth of brook trout after hatching is between 22.1 and 33.2 μg/l HCN.

Based on a comparison of size of fish at each time period, growth of juveniles at 5.5, 11 and 22 μg/l HCN was equal to or slightly better than that of the control fish and less at higher HCN concentrations. Comparison of growth increments during three time periods (0 to 30, 30 to 60 and 60 to 90 days) showed that the percentage gain in length at concentrations of 33 to 66 μg/l approached that of the control with an increase in time. This growth pattern was similar to what Leduc (1966) found with coho salmon juveniles. The salmon exposed to cyanide grew somewhat faster than the control fish during the second half of the experiment. This increase in growth after initial impairment was attributed by Leduc to an increase in food conversion efficiency.

Adult mortality occurred in the two highest treatments (64.9 and 75.3 μg/l) when the temperature during the spawning tests was reduced from 12° to 9°C. It was shown in Section IV that a decrease in temperature increases sensitivity

TABLE 25. EFFECT OF HCN ON THE SURVIVAL OF BROOK TROUT EGGS TO HATCHING AND FROM HATCHING TO 90-DAY-OLD JUVENILES; RANGES ARE IN PARENTHESES

Nominal HCN (µg/l)	Mean hatch (percent)	Mean survival from hatch to juvenile (percent)
Control	72.5	98.6
	(6.3-100.0)	(96.0-100.0)
5.5	46.5	100.0
	(22.4-98.0)	
11	70.8	100.0
	(16.5-100.0)	
22*	72.0	94.0
	(68.0-76.0)	(92-96)
33	70.0	100.0
	(52.6-96.0)	
44	72.9	100.0
	(48.7-93.5)	
55	81.3	84.0
	(64.9-89.6)	
66*	86.9	73.5
	(81.3-92.4)	(64.0-83.0)
77*	76.7	30.0
	(51.1-99.1)	(12.0-48.0)

* Data for treatments at 22, 66 and 77 µg/l were from control eggs incubated and hatched at the indicated test treatment concentrations.

of fish to HCN. Mortality of juveniles occurred after the swim-up stage. The level of no adverse effect on survival occurred between 44 and 55 μg/l.

The number of eggs spawned per female was considerably lower at HCN concentrations of 11.2 μg/l and higher. A study reported by Jensen (1971) showed that a 5 percent increase in mortality of zero-age-group brook trout results in a substantial decrease in yield. Jensen's theoretical analysis of the effect of increased mortality rates on yield showed that with 50 percent addition mortality at the zero-age group the brook trout population would become extinct. It is reasonable to assume that a decrease in spawning will also bring about a decrease in the zero-age group unless the spawning population deposits eggs in significantly greater numbers than are needed for maintenance. Therefore, on the basis of the demonstrated effect of cyanide on spawning, a decrease in the abundance of the zero-age class trout may be anticipated (Table 26) in HCN concentrations of 11.2 μg/l and higher.

TABLE 26. PERCENTAGE REDUCTION IN MEAN NUMBER OF EGGS SPAWNED BY FEMALE BROOK TROUT EXPOSED TO HCN

HCN (μg/l)	Mean eggs spawned per female	Percentage reduction in spawned eggs per female: From control A*	Percentage reduction in spawned eggs per female: From lowest HCN conc. (5.7 μg/l)
Control A*	502	--	--
5.7	513	--	--
11.2	291	42.0	43.3
32.3	246	51.0	52.0
43.6	442	12.0	13.8
53.9	262	47.8	48.9
64.9	124	75.3	75.8

* Control A, used for comparison, was the least productive control.

BLUEGILL

Experimental Design

Chronic toxicity tests were conducted with bluegill adults, juveniles, and newly fertilized eggs. Two of the chronic tests (1 and 3) were started from newly fertilized eggs and lasted for 57 days. Test 2 was conducted for

29 days with young-of-the-year bluegills. Test 4 was conducted with first- and second-year spawners. This test ran for 289 days and was carried through 90 days of the succeeding generation.

Adult bluegills were obtained from Marion Lake, Dakota County, Minnesota, and young-of-the-year juveniles for Test 2 from Round Lake, Ramsey County, Minnesota. Adult fish for Test 4 were given prophylactic treatment with 20 mg/l neomycin sulfate at 12°C before placement into test tanks. Two treatments were given 1 week apart for 3 hr per day on each of three consecutive days.

One hundred fifty adults were equally and randomly distributed to 10 test tanks. Water temperatures were raised approximately 2°C per day from 12° to 25°C and maintained at 25°C for 1 week prior to the beginning of the test. Length and weight measurements were taken from fish anesthetized in MS-222 at 0, 70, 140, and 289 days. The number of fish in each tank was reduced to 10 at 140 days, with each tank having an approximately equal sex ratio. Two controls and eight cyanide treatments were used in the adult test. The fiberglass test tanks were 210 x 54 x 54 cm, and a 30-cm standpipe maintained a volume of 340 l. Four liters were dispensed every 3 min to each tank. Ninety-five percent of the test water was replaced every 12 hr. The chambers for tests with eggs spawned in Test 4 were 20 l glass aquaria into which oscillating incubation baskets were placed for hatching tests. Every 3 min 1.35 l was dispensed to each tank. The 95 percent water replacement time was 2.4 hr. The test chambers and replacement times for chronic Test 2, using juveniles, and Tests 1 and 3, using fertilized eggs, were the same as those used for the second-generation experiment of Test 4. In Tests 1, 2, and 3, four controls and eight replicated cyanide treatments were used. The photoperiod for Tests 1 and 2 was 12 hr of light and 12 hr of darkness. Tests 3 and 4 used the photoperiod of Evansville, Indiana. The temperature in all tests was held at 25°C, and water analyses were made three times weekly from each test chamber for HCN, pH, and dissolved oxygen. The water delivery system was similar to that described by Brungs and Mount (1970), and the toxicant dispensing system was similar to that of Mount and Warner (1965).

Fry from the swim-up stage through the first week of feeding were frequently fed boiled, ground egg yolk suspension and brine shrimp nauplii (*Artemia*). For the next 2 weeks they were fed brine shrimp nauplii only three to five times daily, and after 4 weeks their diet was gradually changed to frozen adult brine shrimp. The juveniles in Test 2 were also fed frozen brine shrimp. Adults were initially fed minnows and Glencoe pellets, but later in the test were fed cut beef liver and Purina floating pellets.

Spawning in the adult Test 4 took place on square concrete blocks (45 x 45 x 8.3 cm), each with a saucer-shaped depression 38 cm in diameter and 5 cm deep. Spawning block areas were isolated by black plastic curtains to minimize aggressive behavior. After each spawning the block was removed, keeping water in the depression. The eggs were loosened from the edges with a paint brush and placed in the block depression. Samples of eggs were removed for hatching tests, and the remainder were preserved for counting. Eggs to be hatched were placed into 6.5-cm OD acrylic cylinders with Nitex screen bottoms and oscillated in the test tanks as described by Mount (1968). As many as three spawnings from each test tank were hatched, and fry were held for 90 days to

determine survival and growth rates.

Tests 1 and 3 were each started with eggs from five artificially spawned pairs. The eggs of a single female were dry fertilized by the sperm of one male, and the fertilized eggs from the five pairs were combined. Two hundred eggs were placed in each incubation chamber. The outlet of each chamber was attached to the outlet on the test tanks. The number of holes in the outlet stem of the chamber was adjusted so that the rate of water flow over the eggs could be regulated as described in Section IV. Swim-up fry were released into the 20-l aquaria which held the incubation chambers. Subsamples of 20 sac fry and 20 swim-up fry were taken from each tank for length measurements in Test 1. The number of individuals in each chamber of Test 1 was reduced to 30 and 20 at 29 and 43 days, respectively. In Test 3 the number was reduced to 20 at 30 days. Survival and growth determinations were made at 29, 43, and 57 days in Test 1 and at 30 days (survival only) and 57 days in Test 3. Growth and survival of juveniles were determined at the 29-day termination of Test 2.

Results

Survival--

Survival of adult fish (Test 4) was not markedly affected until the start of the spawning period. Deaths started to occur after a series of behavioral changes which were first noted at the highest HCN concentrations. The first symptoms were a lack of feeding activity and aggressiveness followed by an impairment of ability to judge the distance to food and to capture it. These symptoms were followed by gradual changes in swimming behavior starting with a list to one side, proceeding to spiraling and occasional darting motions, and terminating in a quiescent state, often upside down, on the tank bottom until death. The time from the beginning of this sequence until death was about two weeks. No deaths were observed at 9.8 μg/l HCN and below. Survival of adults when the test was terminated was greater than 84 percent up to 50.2 μg/l HCN and then decreased to 60 and 40 percent at 65.6 and 80.0 μg/l HCN, respectively (Table 27). Early stages of the previously described symptoms were noticable in fish at 50.2 μg/l HCN by the time the test was terminated.

In the tests which were started with fertilized eggs (1 and 3), there was considerable variability in survival (Tables 28 and 29). The mean survival in the controls at termination of Test 1 was 18.7 percent (range 9.6 to 29.4). Survival was significantly less than that in controls at 38.6 μg/l HCN, using one-way analysis of variance ($p > 0.05$) with arcsin transformation and Dunnett's procedure (Steel and Torrie 1960). The mean survival for the controls at the termination of Test 3 was 23.3 percent (range 10.0 to 37.5), and survival at 19.4 μg/l HCN and higher concentrations was significantly lower than that in controls. The discrepancy in the no-effect concentration between the two tests (1 and 3) appears to result from the high survival (39.8 percent) at 26.6 μg/l HCN in Test 1, where one replicate had been lost. When the results of this test concentration are omitted the survival trend of the combined tests (1 and 3) indicates that the level of significance determined in Test 3 (19.4 μg/l HCN) fits this trend better than the result from Test 1 (Tables 28 and 29). Most deaths of bluegill fry exposed to HCN occurred within the first 30 days after hatching. No change in mortality rate was observed after 6 weeks. This

TABLE 27. SURVIVAL AND FECUNDITY OF ADULT BLUEGILLS AFTER 289 DAYS, TEST 4*

HCN conc. µg/l†	Survival percent	No. females after thinning‡	No. of mature females §	No. of surviving females	Number of spawnings #	Total eggs spawned	Mean eggs/ spawning	Number eggs/g female
Control	100	5	5	5	8	59,394	7,424	110
Control	100	5	5	5	5	26,791	5,358	54
5.2 (1.1)	100	6	5	6	0	0	0	0
9.8 (1.2)	100	3	3	3	0	0	0	0
20.5 (1.8)	90	6	4	6	0	0	0	0
30.0 (2.4)	90	6	5	6	0	0	0	0
39.7 (4.5)	84	3	3	3	0	0	0	0
50.2 (4.5)	90	5	5	5	0	0	0	0
65.6 (4.0)	60	8	7	6	0	0	0	0
80.0 (4.7)	40	6	6	2	1	6,896	6,896	12

*Mean of all treatments: temperature 24.7°-25.1°C; pH 8.07-8.11; dissolved O_2 6.07-6.26 mg/l; total alkalinity 236.0-236.3 mg/l.
†Mean with standard deviation in parentheses.
‡After thinning at 140 d from 15 to 10 fish per tank.
§Declared mature if any sign of development.
#Spawning defined as the eggs deposited on one spawning site within a 24-hr period.

TABLE 28. PERCENTAGE SURVIVAL OF BLUEGILLS FROM FERTILIZED EGG TO THE 57-DAY JUVENILE STAGE IN VARIOUS HCN CONCENTRATIONS FOR TEST 1*

HCN µg/1 (SD)	$\bar{x}$	Day 29 Percent survival	Day 29 $\bar{x}$	Days 43-57 Percent survival	Days 43-57 $\bar{x}$
Control		30.4		29.4	
Control		17.1	19.4	16.5	18.7
Control		19.8		19.1	
Control		10.2		9.6	
5.2(0.7)	5.3	40.0	23.3	37.3	21.9
5.3(1.5)		6.5		6.5	
14.7(2.0)	15.6	lost	10.3	lost	9.0
15.6(2.2)		10.3		9.0	
26.7(3.7)	26.6	22.7	33.5	lost	39.8
26.6(2.5)		44.2		39.8	
38.5(4.1)	38.6	1.3	1.9†	0	0.7†
38.7(4.9)		2.5		1.3	
49.6(5.3)	49.7	6.9	5.7	3.1	1.6
49.7(6.3)		4.4		0	
60.3(7.2)	61.8	0	0.3†	0	0†
63.2(7.0)		0.6		0	
68.2(9.1)	70.3	1.3	2.3†	0.7	1.3†
72.3(7.8)		3.2		1.9	
80.7(9.7)	81.2	0	0†	0	0†
81.7(8.4)		0		0	

*Means of all treatments: temperature 24.8° to 25.0°C; pH 7.87 to 8.02; dissolved O_2 5.77 to 6.40 mg/1; total alkalinity 237.6 to 238.3 mg/1.

†Mean is significantly different from control ($p \leq 0.05$).

TABLE 29. PERCENTAGE SURVIVAL OF BLUEGILLS FROM FERTILIZED EGG TO THE 57-DAY JUVENILE STAGE IN VARIOUS HCN CONCENTRATIONS FOR TEST 3*

HCN µg/l (SD)	$\bar{x}$	Day 30 Percent survival	Day 30 $\bar{x}$	Day 57 Percent survival	Day 57 $\bar{x}$
Control		41.0		37.5	
Control		20.0	25.0	20.0	23.3
Control		13.0		10.0	
Control		26.5		25.5	
4.8(0.9)	4.8	18.5	18.5	18.5	18.5
5.2(0.6)		lost		lost	
8.9(2.1)	9.1	26.5	17.5	25.0	16.3
9.2(1.1)		8.5		7.5	
19.2(1.7)	19.4	3.0	5.5†	3.0	2.8‡
19.6(1.4)		8.0		2.5	
28.5(4.5)	29.1	9.5	7.8	2.5	2.5‡
29.7(4.7)		6.0		2.5	
38.7(2.3)	39.5	8.5	15.3	3.0	3.8†
40.2(2.4)		22.0		4.5	
49.3(3.3)	49.3	17.5	17.5	13.5	13.5
51.9(2.4)		lost		lost	
61.8(4.1)	62.9	2.0	3.3†	0	0‡
64.0(3.9)		4.5		0	
80.4(2.3)	82.1	0	0†	0	0‡
83.8(6.2)		0		0	

*Mean of all treatments: temperature 24.7° to 25.4°C; pH 8.02 to 8.07; dissolved O_2 5.40 to 6.22 mg/l; total alkalinity 236.6 to 237.4 mg/l.

†Mean is significantly different from control ($p \leq 0.05$).

‡Mean is significantly different from control ($p \leq 0.01$).

observation is supported by results in Test 2, in which no deaths occurred to wild young-of-the-year bluegills up to 53.1 µg/l HCN (Table 29).

Growth--

Analyses of growth data are complicated by effects of low levels of HCN on survival. If HCN does affect growth, the compensation created by the lower densities due to greater mortality in the higher levels seemed to nullify this effect. One-way analysis of variance on lengths and weights (logarithmic transformation, $p > 0.05$) demonstrated no significant differences for any of the chronic tests. The mean initial weight of all adult bluegills in Test 4 was 41.3 g, and mean terminal weight was 117.9 g (Table 30). The mean terminal weight was 0.285 g in Test 1, and 0.127 g in Test 3 (Table 31). In Test 2 the mean initial weight was 0.66 g, and terminal weight was 1.11 g (Table 32).

Reproduction--

Spawning of adult bluegills in Test 4 occurred only in the controls except for an early spawning which took place in the highest HCN level (80.0 µg/l). After spawning started in the controls, the test was continued for 68 days. A total of 13 spawnings occurred in the controls (Table 27); one control had 8 spawnings and the other had 5. The control with 8 spawnings produced 110 eggs/g of female, and the control with 5 spawnings had 54 eggs/g of female. All females in the controls were mature. The single spawning at 80.0 µg/l HCN yielded 12 eggs/g of female. No spawning occurred at 5 µg/l HCN, and no lower levels were tested. These data suggest that levels of HCN below 5 µg/l would probably inhibit the spawning of bluegills as well. Nearly 60 percent of the males and 90 percent of the females in the test had partly or fully developed gonads during the spawning period. Autopsies on the surviving adult bluegills in Test 4 were done when the test was terminated. Weights and condition of gonads and liver were noted. Organ weights and organ-to-body weight ratios did not appear to be related to HCN concentration.

Second Generation Hatching and Survival--

The mean percentage hatch for controls in the second generation of Test 4 was 89 percent. Eggs from the single spawning at 80.0 µg/l were incubated at 75.1 µg/l. Forty-three percent of the eggs hatched, but all of the fry were dead within 2 weeks. The mean survival rates for second generation control fish at 30, 60, and 90 days were 41, 36, and 34 percent, respectively. Means of the total lengths of fish in controls at 30, 60, and 90 days were 14.9, 31.4, and 46.9 mm, respectively.

Summary--

The long-term exposure of bluegills to HCN has demonstrated that, as is the case with fathead minnows and brook trout, no-effect levels are much lower than acutely lethal concentrations. Spawning is completely inhibited at 5.2 µg/l HCN and, presumably, is inhibited to some extent at lower levels. The effect on spawning appears to be both physiological and behavioral, since fish exposed to low levels of cyanide exhibited gonadal development but did not spawn when held in clean water for 2 weeks before final termination of the

TABLE 30. MEAN LENGTHS (CM) AND MEAN WEIGHTS (G) OF ADULT BLUEGILLS EXPOSED TO HCN FOR 289 DAYS, TEST 4

Treat-ment	HCN µg/1 (SD)	Length (cm) Days 0	70	140	289	Weight (g) Days 0	70	140	289
1	Control	13.6	14.4	15.1	17.7	43.4	54.6	71.4	118.5
2	Control	13.5	14.3	15.0	17.6	42.8	54.6	70.4	117.3
3	5.2(1.1)	13.8	14.5	15.1	17.5	44.3	57.1	72.4	117.6
4	9.8(1.2)	13.3	14.2	15.0	18.0	42.5	53.6	70.8	130.8
5	20.5(1.8)	13.0	13.8	14.7	17.3	35.2	48.4	65.1	118.7
6	30.0(2.4)	13.1	13.9	14.6	17.0	40.3	50.0	66.1	122.0
7	39.7(4.5)	13.6	14.2	14.9	18.0	41.9	50.1	67.2	122.5
8	50.2(4.5)	13.3	14.0	14.7	17.5	40.1	49.8	66.8	119.7
9	65.6(4.0)	13.2	13.9	14.6	16.2	38.3	51.6	69.3	85.0
10	80.0(4.7)	13.6	14.2	14.9	17.6	44.3	53.7	67.7	110.5

*Mean temperature all treatments 24.7°-25.1°C (SD 0.3-0.4); pH 8.07-8.11 (SD 0.05); Dissolved O_2 (mg/1) 6.07-6.26 (SD 0.19-0.3); total alkalinity 236 mg/1 (SD 16.1).

TABLE 31. TERMINAL MEAN LENGTHS AND WEIGHTS OF BLUEGILL JUVENILES AT VARIOUS HCN CONCENTRATIONS IN TESTS 1 AND 3

Test 1			Test 3		
Mean HCN conc. (μg/l)	Mean length (mm)	Mean weight (g)	Mean HCN conc. (μg/l)	Mean length (mm)	Mean weight (g)
Control	28.8	0.278	Control	19.9	0.094
Control	29.3	0.296	Control	21.8	0.115
Control	29.2	0.285	Control	24.8	0.184
Control	31.3	0.381	Control	20.4	0.099
5.2	28.4	0.275	4.8	21.4	0.102
5.3	28.8	0.346			
			8.9	19.3	0.077
15.6	30.9	0.362	9.2	25.9	0.210
26.6	27.7	0.251	19.2	26.5	0.302
			19.6	25.2	0.259
38.7	30.5	0.337			
			28.5	29.6	0.388
49.6	25.6	0.201	29.7	27.8	0.326
			38.7	25.2	0.213
			40.2	25.3	0.223
			49.3	21.2	0.124

TABLE 32. PERCENTAGE SURVIVAL, LENGTH, AND WEIGHT OF BLUEGILL JUVENILES IN TEST 2 (MEAN INITIAL WEIGHT = 0.66 G)

HCN conc. (µg/l)	Terminal percent survival	Mean length (mm)	Mean weight (g)
Control	100	42.5	1.166
Control	100	42.4	1.200
Control	100	41.1	1.094
Control	100	41.3	1.099
5.3	100	41.3	1.114
5.3	100	41.8	1.084
11.4	100	40.9	1.108
12.2	100	41.4	1.091
18.5	100	42.5	1.124
20.2	100	41.6	1.091
32.2	100	41.6	1.084
32.4	100	41.5	1.105
36.9	100	41.0	1.083
38.5	100	41.2	1.084
44.4	100	41.6	1.095
45.5	100	42.1	1.144
52.2	100	42.3	1.163
54.0	100	41.7	1.096

experiment. During this period there were two spawnings in the controls. Possibly the long delay past the regular spawning period, as defined by the fish in controls, precluded any spawning. The survival of fry was adversely affected at 15.6-19.4 μg/l HCN, but juveniles were not affected below 53.1 μg/l. The effects of low concentrations of HCN on the growth rates of juveniles could not be measured, because of the overriding influence of crowding on growth rates. Among adults, no significant differences in growth were observed.

In assigning a safe or no-adverse-effect concentration of HCN for bluegills, it is evident that this concentration lies below 5.2 μg/l if a complete life cycle is to occur in cyanide polluted waters. If reproduction and early fry stages are not considered, concentrations of about 50 μg/l may be tolerated at higher temperatures. The seasonal implications of these differences must be considered in pollution abatement. No long-term tests on young-of-the-year or adults were run at low temperatures where acute toxicity tests (Section IV) showed fish to be significantly more sensitive to HCN. The apparently safe levels for juveniles (53.1 μg/l) at 25°C are lethal at lower temperatures. Until further testing determines the long-term sensitivity of fish at low temperatures, the apparently safe levels of cyanide for adults and young-of-the-year should not be used as a basis for protecting these life stages at all times of the year.

INVERTEBRATE POPULATIONS

Variability Between Two Species

An important consideration in selecting a test organism for bioassays is its variability in response. An organism with low variability will give greater precision and make comparisons of test results more reliable. Also, researchers interested in doing tests on two species for studies of competition and predator-prey interactions require test organisms which can both do well under the same test conditions.

The experiments reported here compared the variability of *Asellus communis* Say and *Gammarus pseudolimnaeus* Bousfield and determined if both species produced viable populations under the same conditions when tested in separate tanks.

Experimental Design--

The *Asellus* used in the test were the second generation from adults hand picked from bottom material scooped from Rainy Lake near Ranier, Koochiching County, Minnesota. They were abundant along the shoreline in sheltered coves where decomposing organic material had accumulated. The *Gammarus* were collected with a drift net from a small, spring-fed stream entering the Saint Croix River at Marine on St. Croix, Washington County, Minnesota.

Before being used in experiments, *Asellus* and *Gammarus* were held separately in 25-l (50 x 24 x 25 cm high; water depth, 20 cm) tanks constructed with double strength glass and silicone adhesive. Well water flowed through the aerated tank at 700 ml/min. The dissolved oxygen concentration was maintained at saturation and the temperature at 18°C. A constant supply of fall-

dropped leaves and dead minnows was provided as food. The leaves also provided cover for the test animals. The leaves were soaked in flowing, aerated water for about 30 days prior to use and were a mixture of white poplar, hackberry, and cottonwood. The dead fish were freshly killed or frozen, and it appeared that little feeding was done on the fish until some decomposition and fungus growth had developed.

Water for the tests was adjusted to test temperature and saturated with oxygen in a supply tank and then flowed by gravity to the five individual test chambers. The 20-l glass-silicone chambers were 50 x 25 x 21 cm, and a water depth of 16 cm was maintained. The flow-through system provided 95 percent water replacement in about 3 hr.

To begin the *Asellus* test, 40 individuals were randomly placed in each of 5 test chambers, and 40 were preserved to determine mean and range of total length and the mean weight of the individuals. The same procedure was followed for *Gammarus* in five separate tanks (Table 33). During the test, temperature and pH were determined three times weekly, and total alkalinity and dissolved

TABLE 33. TEST CONDITIONS IN *GAMMARUS* AND *ASELLUS* EXPERIMENTS

Item	*Asellus*		*Gammarus*	
Date collected	November 19, 1974		March 3, 1975	
Laboratory holding (days)	114		10	
Test duration (days)	109		71	
Size at start				
Mean total length (mm)	4.2		13.3	
Range	2.0-6.0		6.0-18.0	
Mean weight (mg)	3.1		23.4	
	Means*	S.D.*	Means*	S.D.*
Temperature (°C)	17.96-18.02	0.13-0.15	18.15-18.23	0.70-0.72
pH	8.01-8.03	0.03-0.04	8.03-8.04	0.03
Dissolved oxygen (mg/l)	7.06-7.33	0.34-0.41	7.53-7.69	0.24-0.30
Total alkalinity (mg/l)	236	2	235	4
Light intensity (lux)	420-506	-	538-646	-

* Range of means and standard deviations are for all tanks in each experiment.

oxygen once per week. Each week all tanks were checked for the appearance of young produced by the original stock. The test was terminated 45 days after the first young were observed, and the contents of each tank were passed successively through three screens (square mesh openings of 6.0, 2.0, and 0.4 mm) to separate debris and organisms into four size groups. The material that passed through the smallest screen contained no organisms and was discarded. The three larger groups were preserved and stained with 10 percent formalin and rose bengal (0.6 g/1). After a few weeks in the stain the organisms were separated from debris and measured. The eggs and young were removed from gravid females and counted. The entire population of adults, eggs, and young was centrifuged at a low speed to remove excess water and weighed.

Results--

Length-frequency diagrams of all organisms in each test chamber were made (Figures 2 and 3). Table 34 lists the population parameters for each test chamber. The term "free individuals" refers to organisms not contained in the brood pouch. Table 35 compares *Asellus* and *Gammarus* on the basis of the mean, standard deviation and coefficient of variability for each of the population indices for the five tanks combined.

The similarity of the general shape of the length-frequency diagrams within each species indicates that the five tanks were fairly well synchronized in terms of reproduction time and growth rates (Figures 2 and 3). Comparison of the mean values for the five tanks of each species (Table 35) shows that the number of free individuals, number of eggs and young in the brood pouch, total number, and total weight were similar for the two species. The values of *Asellus* are all about 25 percent higher than for *Gammarus*, but the coefficients of variability are about half as high as those for *Gammarus*. One exception was the number of eggs and young in the brood pouch. In tank 3 of the *Asellus* test reproduction began slightly earlier, there were many females with empty brood pouches, and the number of free individuals in the 1.5 to 3.0 mm length group was high. In the *Asellus* culture tanks, where stocks have been maintained since November 19, 1974, densities are much greater than in the test tanks. Attempts to culture *Gammarus* at similar densities have failed. Allee (1929) found that the *Asellus* can occur at high densities and remarks that in general they are tolerant of many individuals in a limited space. By contrast, papers by Anderson and Raasveld (1974) and Hynes (1954) report predation and cannibalism for species of *Gammarus*. Oseid (1977) studying the effect of *Asellus militaris** and *Gammarus pseudolimnaeus* on fish eggs found that *Gammarus* consumed live fry and eggs of fish whereas *Asellus* did not. The control tanks of toxicity bioassays of Nebeker and Puglisi (1974) and Oseid and Smith (1974) contained means of 254 and 433 *Gammarus*, respectively, where test conditions other than water supply and tank size were similar. The latter study used water with a higher mineral content and larger tanks. In the present study, the mean weight and mean number of eggs or young per gravid female for *Asellus* and *Gammarus* were very similar, but again the coefficient of variability was greater for *Gammarus*. The mean length of gravid females

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* Keyed out to *Asellus militaris* Hay in Pennak (1953) but to *Asellus communis* Say in Williams (1970).

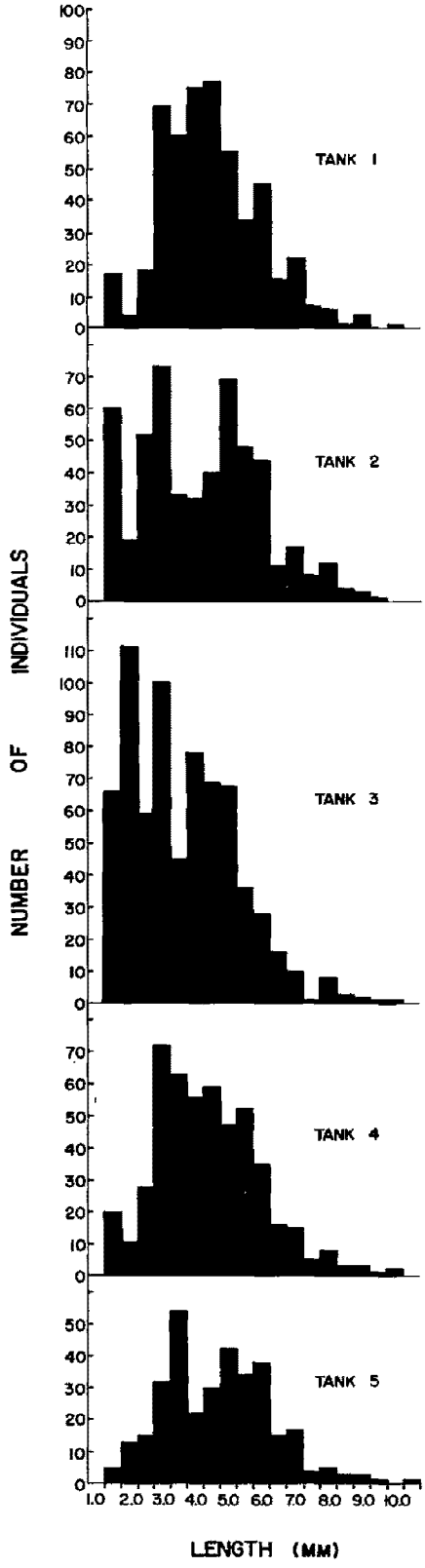

Figure 2. Length-frequency diagrams of all *Asellus* in each test chamber

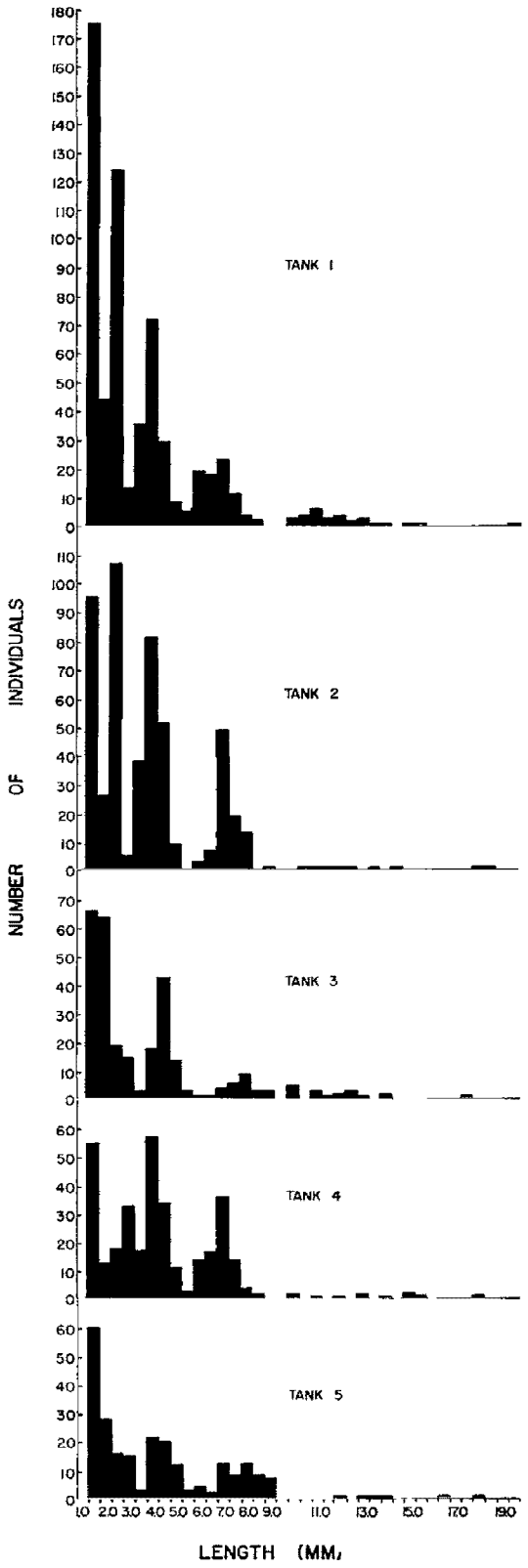

Figure 3. Length-frequency diagrams of all *Gammarus* in each test chamber

TABLE 34. POPULATION PARAMETERS IN *ASELLUS* AND *GAMMARUS* TESTS

	Asellus Tank Number					*Gammarus* Tank Number				
	1	2	3	4	5	1	2	3	4	5
Free individuals	510	526	702	496	334	612	513	289	340	237
Eggs or young in brood pouch	162	120	0	163	103	65	60	117	77	31
Total free individuals plus eggs and young in brood pouch	672	646	702	659	437	677	573	406	417	268
Total weight of free individuals plus eggs and young in brood pouch (g)	1.766	1.538	1.387	1.539	1.026	1.246	1.352	0.617	1.225	0.602
Mean weight of free individuals (mg)	3.46	2.92	1.98	3.10	3.07	2.04	2.64	2.13	3.60	2.54
Mean number of eggs or young per gravid female	23.1	20.0	-	20.4	20.6	21.7	30.0	16.7	19.2	15.5
Mean length of gravid females (mm)	5.50	5.58	-	5.50	5.50	11.8	13.0	11.6	11.8	12.5

TABLE 35. MEANS FOR THE POPULATION INDICES OF FIVE REPLICATIONS

	Asellus			*Gammarus*		
	$\bar{x}$	S.D.	C.V.	$\bar{x}$	S.D.	C.V.
Number of free individuals	514	131	25	298	158	40
Number of eggs and young in brood pouch	110	67	61	70	31	44
Total number of free individuals plus eggs and young in pouch	624	106	17	468	159	34
Total weight of free individuals plus eggs and young in pouch (g)	1.451	0.274	19	1.008	0.367	36
Mean weight of free individuals (mg)	2.91	0.55	19	2.59	0.62	24
Mean number of eggs or young per gravid female	21.0	1.4	7	20.6	5.8	28
Mean length of gravid females (mm)	5.52	0.04	1	12.14	0.59	5

was much smaller for *Asellus*. The growth rates for both species appeared to be similar so that maturity at a small size allowed shorter generation time. After 45 days from the first observation of young, the F_1 generation of *Asellus* had many gravid females, and tanks 2 and 3 had large numbers of F_2 individuals. After the same time *Gammarus* F_1 had not grown to the size of maturity, and only the original stock of individuals, all 10.0 mm or larger, were gravid.

Summary--

These tests indicate that, as an individual test organism, *Asellus* has several advantages. *Asellus* exhibited lower variability than *Gammarus* in six of seven population parameters; therefore fewer replications would be required in experiments with *Asellus* to get the same precision. It can be easily cultured in high numbers in the laboratory and possibly the time to complete a generation is shorter, thereby reducing the time required to do full generation tests. However, apart from variability, the tests also showed that in terms of the population parameters (Tables 34 and 35) the two species produced fairly similar populations. Therefore, when tested in the same test chamber where the two species can interact, both species should have an equal chance to develop a population apart from factors such as competition or predator-prey relations.

Response to Cyanide

The tests described below were designed to determine the effect of cyanide on two invertebrates when exposed separately or simultaneously to the toxicant. *Gammarus pseudolimnaeus* Bousfield was selected because it is aggressive and competitive (Hynes 1954; Anderson and Raasveld 1974; Oseid 1977) and *Asellus communis* Say because it is passive (Allee 1929; Oseid 1977). Short-term tests were done with each species alone, and full-life-cycle tests were done with each species alone and then with the two together.

Experimental Design--

Gammarus were collected 10 days before the beginning of each test from a small, spring-fed stream tributary to the Saint Croix River at Marine on St. Croix, Washington County, Minnesota. The *Asellus* were cultured in the laboratory from an initial group which was collected from Rainy Lake near Rainier, Koochiching County, Minnesota and which provided subsequent generations for all tests. *Asellus* and *Gammarus* were kept in the laboratory at 18°C with the dissolved oxygen concentration near saturation. They were fed pre-soaked deciduous tree leaves and dead fish. The days in the laboratory, the length and weight of organisms at the beginning of each test, and the duration of each test are noted in Table 36. Exposures were made with the apparatus and chemical procedures described above in Section IV. Three acute toxicity tests were performed with *Gammarus* and two with *Asellus*, and the 96-hr LC50 and lethal threshold concentrations were calculated. For each series of chronic exposures (*Asellus* alone, No. 1, and No. 2; *Gammarus* alone; and *Asellus* and *Gammarus* together in the same test tank), two simultaneous but separate tests were conducted.

Nominal test conditions for both acute and chronic exposures were 18°C,

TABLE 36. TEST CONDITIONS FOR SIMULTANEOUS *GAMMARUS* AND *ASELLUS* BIOASSAYS IN HCN

	*Asellus** (1)		*Asellus** (2)		*Gammarus**		*Asellus* and *Gammarus* together	
							Asellus	*Gammarus*
Days in laboratory before testing	300		429		12		360	17
Size at start of test								
Mean length (mm)	6.3		6.7		8.7		6.1	9.7
Length range (mm)	4.0-8.0		5.0-9.5		3.0-14.0		3.5-8.0	2.5-17.0
Mean weight (mg)	7.7		9.2		10.7		7.1	14.1
Duration (d)	115		112		83		98	98
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Temperature (°C)	18.1-18.2	0.1-0.2	18.0-18.1	0.1-0.5	18.0-18.1	0.4-0.8	18.0-18.2	0.1-0.2
pH	7.98-8.13	0.03-0.06	7.92-7.96	0.05-0.10	7.99-8.02	0.04	7.93-7.98	0.04-0.05
Dissolved O_2 (mg/l)	6.03-6.52	0.26-0.42	5.88-6.14	0.26-0.40	6.77-7.00	0.50-0.69	5.70-6.09	0.30-0.41
Total alkalinity (mg/l)	238	3	236	2	236	2	236	1
Light intensity (lux)	481-660	--	506-657	--	452-667	--	431-603	--

*Alone

a pH of 8.0, and 6 mg/l dissolved oxygen. Two 20-W Vita-Lite fluorescent tubes above each tank provided approximately 500 lux at the water surface during the 16-hr daily photoperiod. Chronic tests were started by placing 40 individuals at random into each of the test tanks. From the onset of reproduction, the tests were continued 45 days. At that time all test specimens were preserved, measured, checked for fecundity, and weighed as a group for each tank.

Results--

The 96-hr LC50 at 18°C for *Asellus* was 2,328 µg/l HCN and for *Gammarus* was 175.6 µg/l. The respective LTC values (10-12 days) were 1,834 and 69.6 µg/l (Table 19). The length-frequency graphs for each test tank of the three series are depicted in Figures 4, 5, and 6. Tables 37, 38, 39, and 40 list the mean HCN concentration and standard deviation, and the biological indices for each test tank in the four test series.

TABLE 37. BIOLOGICAL INDICES IN THE CHRONIC TEST SERIES (1) ON *ASELLUS* (BOTH TESTS INCLUDED*)

	Tank number						
	6	6	3	3	5	4	2
HCN (µg/l) mean	0	0	51	110	220	317	432
HCN (µg/l) S.D.	0	0	9	16	19	36	43
Number of free individuals†	1612	2467	262	109	85	56	0
Number of eggs or young in brood pouch	626	855	72	0	60	17	0
Total number of free individuals plus eggs and young in brood pouch	2238	3322	334	109	145	73	0
Total weight (g) of free individuals plus eggs and young in brood pouch	3.703	4.612	0.725	0.292	0.106	0.142	--
Mean number of eggs or young per gravid female	35	34	18	--	20	17	--

* Underlined values are lower than the mean of the controls by more than two times the standard error of the mean

† Those individuals living separate from the brood pouch

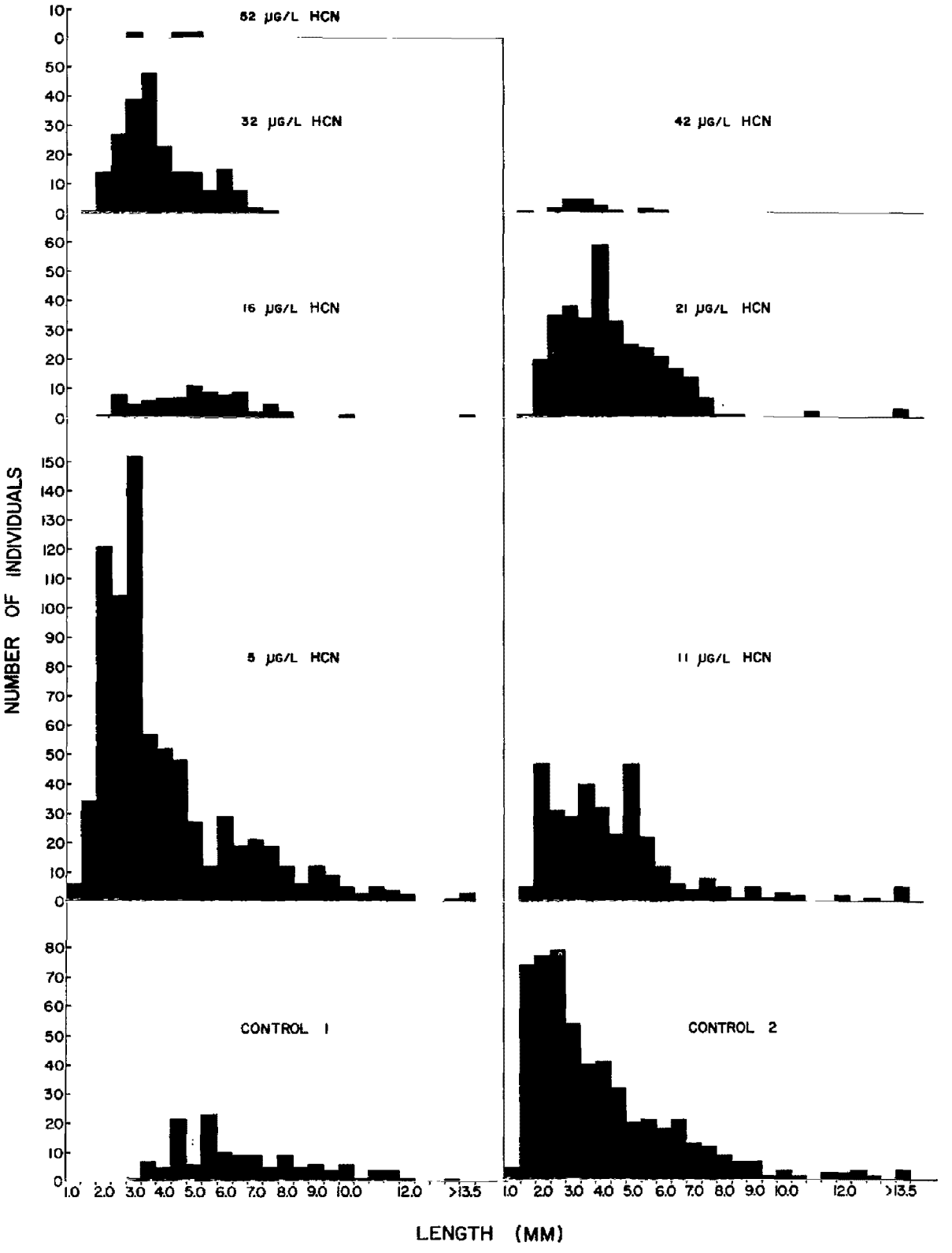

Figure 4. Length-frequency graphs of each test concentration for chronic test--*Gammarus* alone

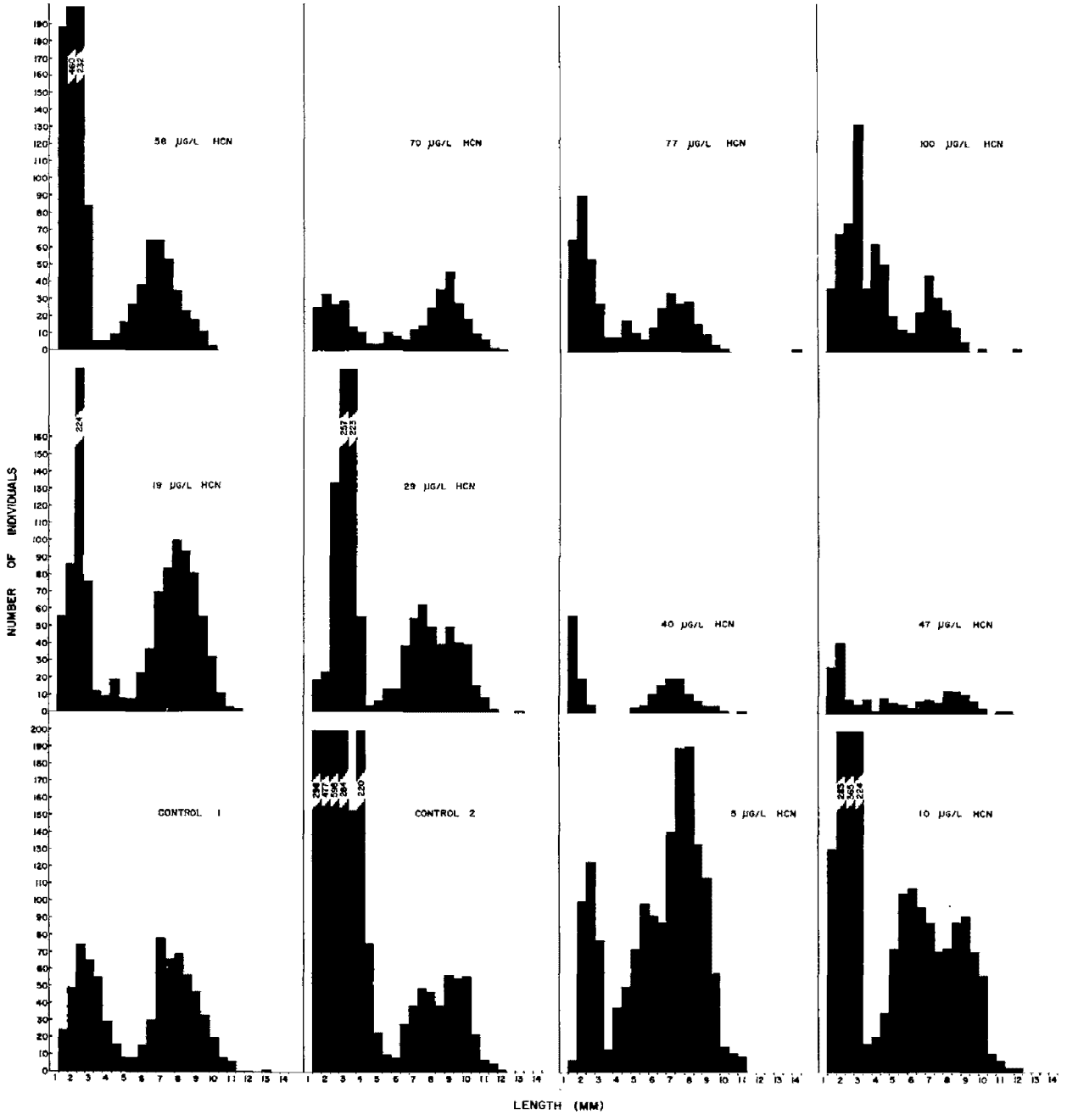

Figure 5. Length-frequency graphs of each test concentration for chronic test--*Asellus* alone (2)

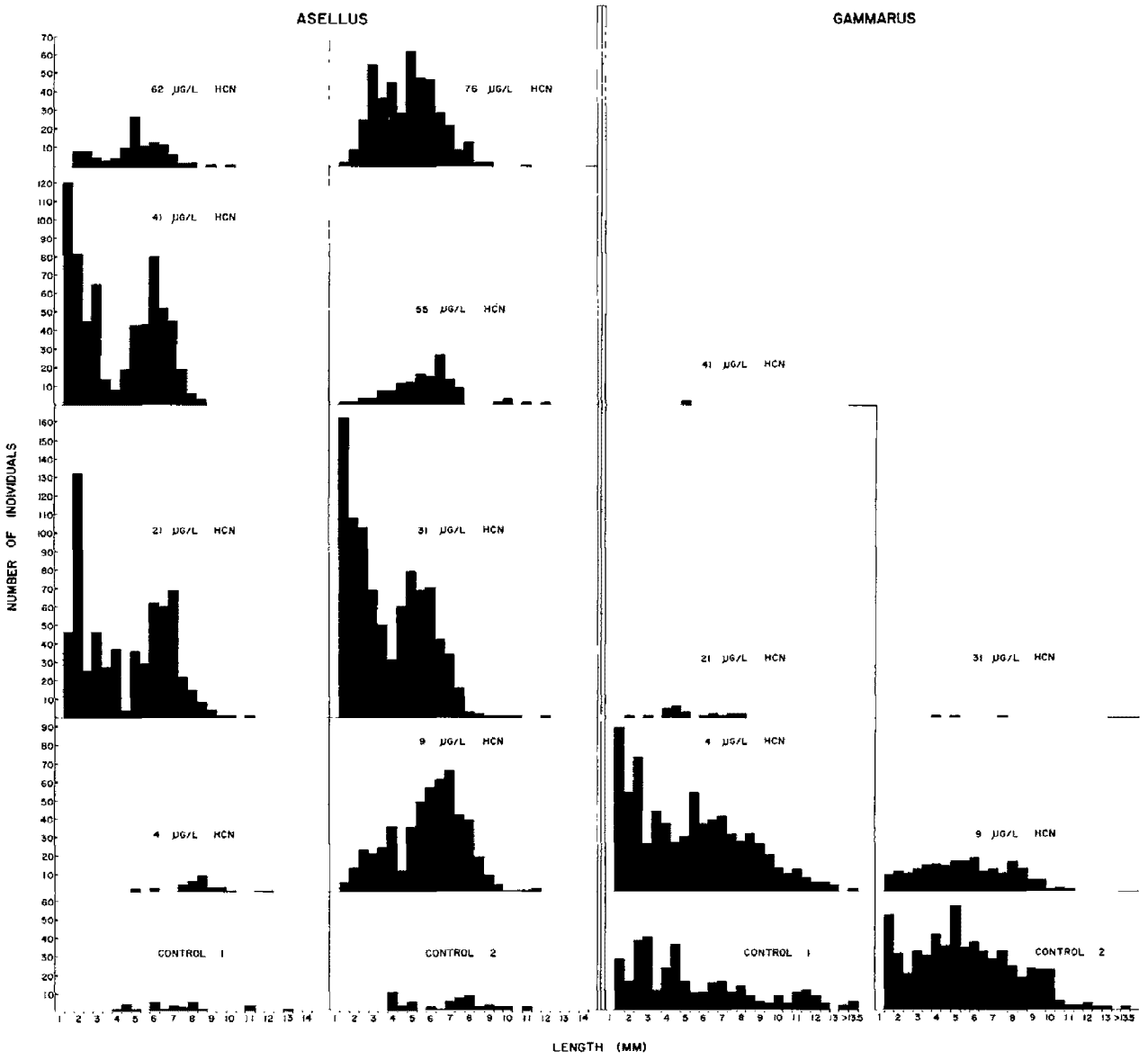

Figure 6. Length-frequency graphs of each test concentration for chronic test--*Asellus* and *Gammarus* together

For the first test series on *Asellus* there was a large reduction of numbers and weight at the lowest tested concentration, 51 µg/l (Table 37). The highest concentration with survival and reproduction was 317 µg/l; there was no survival or reproduction in the range 432 to 2,108 µg/l. For the second series (Table 38) there was survival and reproduction at all concentrations tested (5-100 µg/l). The numbers of free individuals, eggs, and young were variable, making evaluation on the basis of these indices questionable. The standard deviation for total weight was relatively low, and at concentrations of 40 µg/l and higher weights were lower than the mean of the two controls by more than two times the standard error of the control mean. The mean number of eggs per gravid female was reduced only at 100 µg/l. All other indices were within two standard errors of the control mean. The highest no-effect concentration lies between 29 and 40 µg/l, Table 41.

In the series on *Gammarus* (Table 39), numbers of free individuals were variable, but weight was more consistent. At concentrations of 16 µg/l, 32 µg/l and higher, weights were reduced by more than two standard errors of the control mean. The low mean weight in the 16 µg/l treatment was not thought to be the result of cyanide toxicity. The no-effect/effect concentrations were 16 and 21 µg/l on the basis of total number of eggs or young in the brood pouch and mean number of eggs or young per gravid female (Table 41).

The effect of exposing *Asellus* and *Gammarus* in the same test chamber was twofold (Table 40). The *Gammarus* almost eliminated the *Asellus* from the controls and the lowest treatment (4 µg/l HCN), and there was a shift downward of the no-effect/effect concentrations for *Gammarus*. It is felt that predation by *Gammarus* is the factor that almost depleted *Asellus* in the controls and lowest treatment. On the basis of all the indices for *Gammarus*, the highest no-effect concentration lies between 4 and 9 µg/l, values which are lower than when *Gammarus* was exposed alone. Predation by large *Asellus* on small *Gammarus* may account for this reduced no-effect concentration. The highest no-effect concentration for *Asellus* in the same test was between 41 and 55 µg/l, approximately the same as for the two series with *Asellus* alone.

Summary--

The attributes for survival of *Asellus* in a mixed community are their much greater resistance to HCN for short periods (10-12 day LTC value of 1,834 µg/l) and the ability of some fraction of the population to survive and reproduce at high concentrations of HCN (up to 317 µg/l) after a continuous exposure of 115 days. By comparison, *Gammarus* have a 25-fold lower 10-day LTC, a 6-fold lower concentration which permits any survival and a 19-fold lower level permitting reproduction (Table 38). Therefore, the presence of low concentrations (9 to 30 µg/l HCN) probably shifts the competitive advantage from the aggressive and predaceous *Gammarus* to the more passive *Asellus*. *Gammarus* are probably excluded from cyanide polluted areas, which could be satisfactory to them if they were alone, because they are unable to compete with more resistant species whether typically predator or prey. *Asellus* would be benefitted by reduced competition and predation.

TABLE 38. BIOLOGICAL INDICES IN CHRONIC TEST SERIES (2) ON *ASELLUS* (BOTH TESTS INCLUDED*)

	Diluter number											
	3	4	4	3	4	4	3	4	3	4	3	3
HCN (μg/l) mean	0	0	5	10	19	29	40	47	58	70	77	100
HCN (μg/l) S.D.	0	0	1	3	2	5	4	10	8	7	12	12
Number of free individuals†	758	2551	1622	2023	1091	1158	184	183	1335	376	453	655
Eggs or young in brood pouch	154	732	67	578	198	394	0	72	621	124	123	51
Free individuals plus eggs and young	912	3282	1689	2601	1289	1552	184	255	1956	500	576	706
Weight (g) free individuals plus eggs and young	2.495	3.221	6.918	5.466	3.646	3.146	0.488	0.520	1.454	1.418	0.867	0.973
Mean eggs or young per gravid female	19.2	22.2	22.3	22.2	24.8	21.9	--	18.0	20.7	31.0	20.5	17.0

* Underlined values are lower than the mean of the controls by more than two times the standard error of the mean

† Those individuals living separate from the brood pouch

TABLE 39. BIOLOGICAL INDICES IN THE CHRONIC TEST SERIES ON *GAMMARUS* (BOTH TESTS INCLUDED*)

	Diluter number									
	3	4	4	3	4	4	3	4	3	4
HCN (μg/l) mean	0	0	5	11	16	21	32	42	52	64
HCN (μg/l) S.D.	0	0	2	2	3	6	5	7	14	10
Number of free individuals†	137	554	764	331	82	335	214	20	6	0
Eggs or young in brood pouch	6	135	113	13	16	0	0	0	0	0
Free individuals plus eggs and young	143	689	877	344	98	335	214	20	6	0
Weight (g) free individuals plus eggs and young	0.795	1.343	1.705	0.726	0.255	0.629	0.273	0.110	0.033	0
Mean eggs or young per gravid female	3	27	19	13	16	-	-	-	-	-

* Underlined values are lower than the mean of the controls by more than two times the standard error of the mean

† Those individuals living separate from the brood pouch

TABLE 40. BIOLOGICAL INDICES IN THE CHRONIC SERIES WITH *ASELLUS* (A) AND *GAMMARUS* (G) TOGETHER (BOTH TESTS INCLUDED*)

	Species	Diluter number 1	2	1	2	1	2	1	2	1	2
HCN (μg/l) mean		0	0	4	9	21	31	41	55	62	76
HCN (μg/l) S.D.		0	0	1	1	2	5	5	5	11	6
Number of free individuals†	A	27	52	34	519	625	903	644	132	114	437
	G	354	591	745	212	24	3	2	0	0	0
Eggs or young in brood pouch	A	0	0	0	257	470	363	697	127	34	224
	G	334	386	359	25	0	0	0	0	0	0
Free individuals plus eggs and young	A	27	52	34	776	1095	1266	1341	259	148	661
	G	688	977	1104	237	24	3	2	0	0	0
Weight (g) free individuals plus eggs and young	A	0.216	0.419	0.314	2.227	1.734	1.552	1.522	0.568	0.369	1.176
	G	1.965	3.142	3.852	0.941	0.086	0.017	0.011	--	--	--
Mean eggs or young per gravid female	A	--	--	--	19.8	23.5	25.9	25.8	15.9	17.0	18.7
	G	17.6	16.8	17.1	8.3	--	--	--	--	--	--

* Underlined values are lower than the mean of the controls by more than two times the standard error of the mean

† Those individuals living separate from the brood pouch

TABLE 41. COMPARISONS OF THE NO-EFFECT/EFFECT CONCENTRATIONS (μg/l) OF HYDROGEN CYANIDE FOR *ASELLUS* AND *GAMMARUS*

	Asellus			*Gammarus*	
	Exposed alone, Series 1	Exposed alone, Series 2	Exposed with *Gammarus*	Exposed alone	Exposed with *Asellus*
Number of free individuals†	0-51	-	41-55	34-42	4-9*
Number of eggs or young in brood pouch	0-51*	-	41-55	16-21	4-9*
Total number of free individuals plus eggs and young	0-51*	-	41-55	32-42	4-9*
Total weight (g) of free individuals plus eggs and young	0-51*	29-40*	41-55	21-32*	4-9*
Mean number of eggs or young per gravid female	0-51*	77-100	41-55	16-21	4-9*
Highest concentration with any survival	317	-	-	52	41
Highest concentration with any reproduction	317	-	-	16	9

* Those marked with an asterisk were lower than the mean of the two controls for the respective test by at least two times the standard error of the mean

† Those individuals living separate from the brood pouch

SECTION VI

MISCELLANEOUS TESTS

PREDATOR-PREY RELATIONS

At 18°C the green sunfish (*Lepomis cyanellus*) has a greater tolerance of HCN than does the laboratory reared fathead minnow (*Pimephales promelas*). The 96-hr LC50 values were 139 and 125 µg/l HCN, respectively, and the LTC values were 133 and 122 µg/l HCN, respectively. It was considered possible that the lesser tolerance of the fathead may make it more vulnerable to predation by the more tolerant green sunfish. Previous toxicant tests using "Mirex" (Tagatz 1976) and mercury (Kania and O'Hara 1974) exposed the prey, but the predator was not exposed, or was exposed for a much shorter period. In the tests reported here, both the predator and prey were given equal exposure to cyanide.

Experimental Design

The system used to introduce water of controlled temperature, pH, and dissolved oxygen was the same as that described in Section IV. The test tanks were the same as previously described, except that "Nitex" screen partitions across the short side separated the tanks into two equal compartments. Three devices to provide cover were placed in each half. After anesthetizing (150 mg/l MS 222) and weighing, one or two green sunfish were placed in one compartment and 30-60 fathead minnows were placed in the other. The number of fatheads varied depending on their size and the size and number of green sunfish. At the time of the randomization of prey for the test chambers, one additional group was made up and preserved for determination of mean length and weight. For the nine tests the mean size of green sunfish varied from 50 to 88 mm total length and fatheads from 15 to 21 mm total length. Both the predator and prey were fed during the 3 days of pre-exposure acclimation.

The tests were started by dispensing one-fourth of the amount of sodium cyanide required to bring the tanks up to the desired concentration of HCN. Next, the partition was removed that separated the two fish species; then another addition of sodium cyanide was made to bring the HCN concentration up to one-half of the aim. The method and frequency of tests for HCH, pH, temperature, and dissolved oxygen was the same as that described in Section IV. Fatheads which died during the pretest or test periods were removed. After 4 days of interaction the predators and remaining prey were counted, measured, and weighed. The predation rate was calculated as grams of prey eaten for each gram of predator, based on the beginning weights of both predator and prey.

Results

The pH was maintained at 7.9 and the temperature at 18°C. The mean dissolved oxygen concentration varied from 5.5 and 5.9 mg/l. The mean concentration of HCN (μg/l) in each experimental tank for each test and the overall mean concentration in each tank for the nine tests are presented in Table 42. The consumption of minnows in each tank for the nine tests is presented in Table 43. The overall means of these consumption rates ranged from 0.666 g of minnow per gram of sunfish for controls to 0.942 g/g at a nominal HCN concentration of 25 μg/l. The mean consumption rates of minnows in tests at the nominal concentrations of 50 and 100 μg/l were 0.859 and 0.846 g/g of green sunfish. These prey consumption rates, expressed as a percentage of corresponding control values, are given in Table 44.

The variation between prey consumption at similar HCN concentrations in different tests was large. However, it was observed that for a single test the comsumption rate was usually greater in the treatments than in the corresponding control, with minor differences among the four treatments. These tests show that the presence of sublethal concentrations of HCN caused increased predation by green sunfish on laboratory reared fatheads, but do not indicate whether fatheads were easier prey or the green sunfish had greater appetites as suggested by Leduc (1966).

TABLE 42. MEAN HCN CONCENTRATIONS IN EACH EXPERIMENTAL TANK OF EACH TEST AND THE OVERALL MEANS FOR EACH TANK AT DESIGNATED NOMINAL CONCENTRATIONS

Test	Nominal concentration (μg/l) 12.5	25	50	100
1	9.9	24.6	41.5	85.6
2	10.3	16.1	32.7	72.4
3	13.7	20.5	44.2	78.8
4	8.9	23.8	46.5	98.3
5	9.2	26.3	39.0	114.8
6	14.8	30.7	48.3	114.9
7	9.2	25.7	52.7	119.0
8	15.8	29.5	38.8	100.8
9	9.3	24.7	42.9	113.6
Means	11.2	24.7	43.0	99.8

TABLE 43. PREY CONSUMPTION (GRAMS OF PREY CONSUMED PER GRAM OF PREDATOR PRESENT) FOR EACH TEST TANK AND THE OVERALL MEANS FOR EACH NOMINAL HCN CONCENTRATION (WITHIN TEST RANK IN PARENTHESIS)*

Test	Nominal concentration (µg/l) 0	12.5	25	50	100
1	(1) 0.296	(2) 0.298	(4) 0.313	(3) 0.303	(5) 0.354
2	(2) 0.236	(3) 0.323	(5) 0.360	(4) 0.348	(1) 0.222
3	(1) 0.261	(4) 0.370	(3) 0.360	(5) 0.534	(2) 0.314
4	(1.5) 0.803	(1.5) 0.893	(4) 0.988	(3) 0.912	(5) 1.045
5	(3) 1.045	(1) 0.855	(4) 1.083	(2) 0.931	(5) 1.121
6	(1) 0.206	(4) 0.279	(5) 0.307	(3) 0.255	(2) 0.248
7	(3) 0.284	(4) 0.296	(5) 0.315	(2) 0.225	(1) 0.215
8	(1) 1.320	(5) 2.904	(2) 1.452	(3.5) 2.112	(3.5) 2.112
9	(1) 1.452	(2.5) 1.980	(5) 3.300	(4) 2.112	(2.5) 1.980
Means	0.666	0.911	0.942	0.859	0.846

* Ranking of minnow consumptions among HCN concentrations within each test (highest rank - largest consumption).

TABLE 44. PREY CONSUMED FOR EACH TEST TANK AS A PERCENTAGE OF THE CONTROL VALUES AND THE OVERALL MEANS FOR EACH NOMINAL HCN CONCENTRATION

Test	Nominal concentration (μg/l) 0	12.5	25	50	100
1	100.0	100.7	105.7	102.4	119.6
2	100.0	136.9	152.5	147.5	94.1
3	100.0	141.8	137.9	204.6	120.3
4	100.0	100.0	110.6	102.1	117.0
5	100.0	81.8	103.6	89.1	107.3
6	100.0	135.4	149.0	123.8	120.4
7	100.0	104.2	110.9	79.2	75.7
8	100.0	220.0	110.0	160.0	160.0
9	100.0	136.4	227.3	145.5	136.4
Means	100.0	128.6	134.2	128.2	116.8

EXPOSURE TO INCREASING CYANIDE CONCENTRATIONS

In most toxicity tests, exposure to toxicants is continuous and constant. The need to approximate the effects on fish of exposures more closely resembling actual spills is apparent. It has been suggested by Larson and Schlesinger (1977) that the median survival time of fish exposed to chlorine may be expressed as a function of the area under a time-concentration curve. This specific relationship was pursued in an investigation using hydrogen cyanide and fathead minnows.

Experimental Design

The diluters (Brungs and Mount 1970) and aquaria used for testing are the same as those described in Section IV. Fathead minnows of 9-10 weeks old and reared in a laboratory hatchery were used in the experiments. The tests were conducted at 25°C with oxygen concentrations above 60 percent saturation.

Different rates of HCN increase in test chambers were produced by altering any of the following (Table 45): (1) the initial volume of fresh water in the aquaria, (2) the volume of diluent water dispensed into the aquaria, (3) the concentration of HCN dispensed, and (4) the time interval between introductions of diluent water in successive cycles of the diluter (cycle time).

Each test (except Test 5) was conducted with one control and four treatment levels, using ten test fish in each aquaria. Mortality times were recorded to the nearest minute. HCN samples were taken at 15, 30, 60, 90, and 120 min and every 60 min thereafter until the cyanide concentration peaked. In Tests 1, 2, and 4 the cycle time was the only factor manipulated in changing the time-concentration curve. The initial and final volumes were 20 l in each aquarium. In Tests 7, 8, and 9 the initial aquaria water volumes were 5 l each and the final volumes were 20 l. The volume dispensed to each aquarium with each diluter cycle was reduced from 1.2 l to 0.5 l. The strength of the HCN stock solution also was varied to obtain less convex curves. Essentially straight-line increases in HCN concentration were obtained in Tests 10, 11, and 12 by starting with only 1.5 l in each aquarium and employing the other modifications used in Tests 7, 8, and 9. In addition, the amount of toxicant dispensed to each tank was increased at a geometric rate per unit time (e.g., if 1 ml of toxicant was dispensed for each of 3 diluter cycles, then each of the next 3 cycles would receive 2 ml/cycle, the next 3 cycles 4 ml/cycle, and so on). The three groups of diluter modifications outlined above produced curves of HCN concentration like those in Figure 7 which have been standardized to intersect at the same HCN concentration and point in time.

Test 5 was designed to estimate the latent period (i.e., the minimum amount of time needed to produce mortality regardless of the toxicant concentration. The test was static, conducted at 25°C and lasted 2 hr. Known amounts of sodium cyanide stock solution were pipetted and mixed into 8 of 10 aquaria containing 10 fish each. The other two aquaria were controls. Mortalities were recorded as they occurred. HCN concentrations were recorded initially and at the end of the test. The actual HCN concentrations diminished about 10 percent in 2 hr.

TABLE 45. DILUTER AND AQUARIA CONDITIONS AS RELATED TO WATER TURNOVER TIMES

Test no.	Initial tank volume (l)	Final tank volume (l)	Cycle time (min)	Dispensed volume (ml)	Test duration (min)
1	20	20	6	1,200	270
2	20	20	4	1,200	180
3	20	20	2	1,200	180
4	20	20	2	1,200	180
5	20	20	immediate slug - latent period		180
6	20	20	square slug - removal to fresh water		120
7	5	20	4	500	60
8	5	20	4	500	90
9	5	20	4	500	120
10	1.5	20	4	500	120
11	1.5	20	4	500	120
12	1.5	20	4	500	180

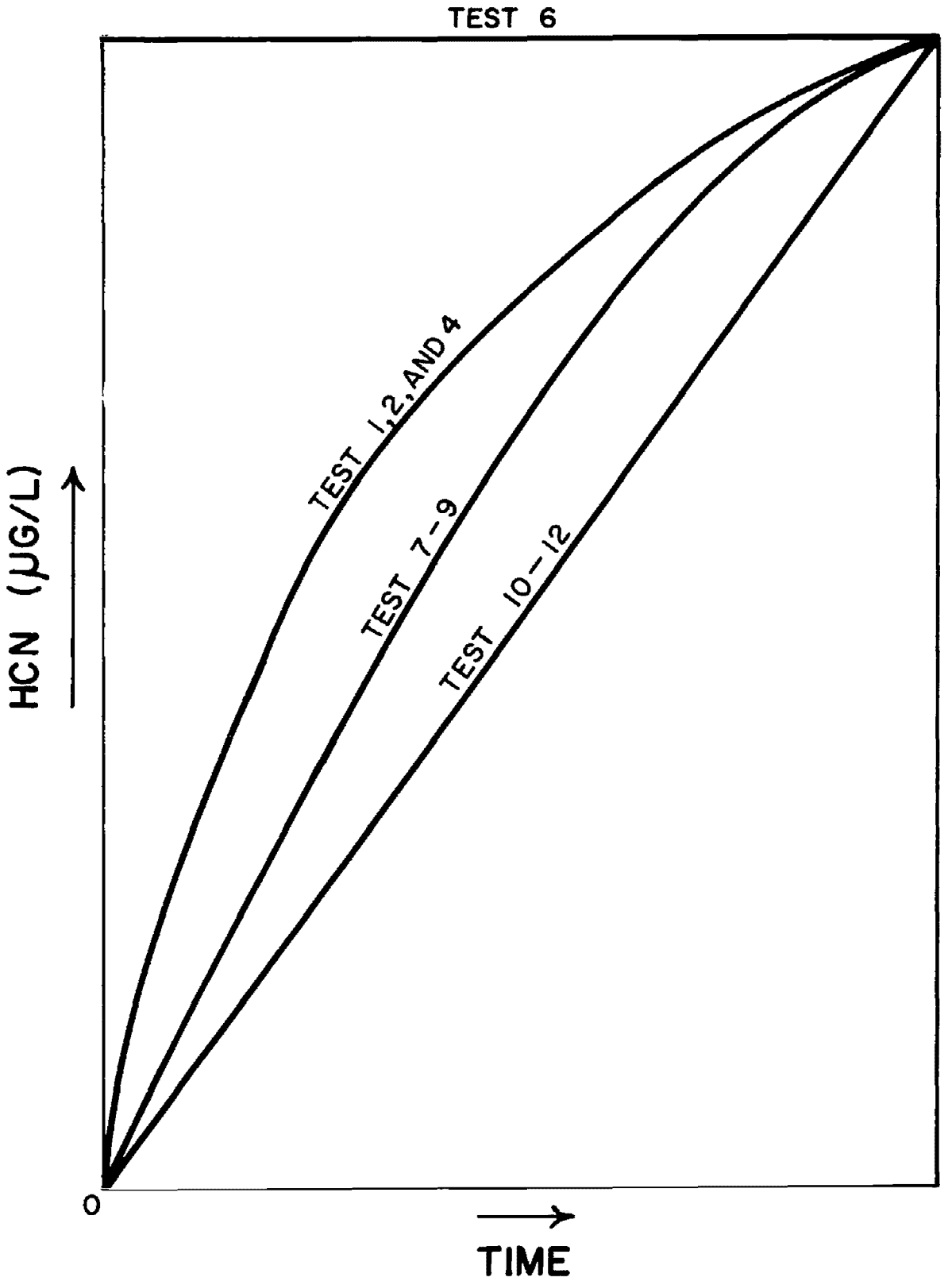

Figure 7. Representative curves for increasing HCN lethal exposure tests normalized to time and concentration

Test 6 was designed to examine the responses of fathead minnows to a given area of "square exposure" (Larson and Schlesinger 1977) at high cyanide concentrations, returning them to fresh water after a specified amount of mortality had occurred. Fish were acclimated for 2 days to test chambers (10 fish per aquarium) before exposure tests. A sodium cyanide stock solution was pipetted into the aquaria at concentrations of up to 1.176 mg/l HCN. The diluter and toxicant dispensers were operated to maintain the HCN concentrations for the test duration. Once the mortality reached 80 percent in any given aquaria, the remaining fish were removed by dipnet to a nearby, identical diluter operating with fresh water only. Any further mortalities were recorded.

Results

It is generally assumed that median survival time exhibits less variability than any other point on the time-mortality curve. Exposure areas bounded by the change in HCN concentration with time from initial exposure to the median survival times are summarized in Table 46. It was anticipated that the area under the time-concentration curve from time zero to the median survival time should be constant regardless of the type of time-concentration exposure curve. In Table 47 the mean areas, standard deviations, and coefficients of variation (i.e., the ratio of the standard deviation to the mean, expressed as a percentage) are given for the various tests, which are grouped according to type of exposure (Test 3 had insufficient mortality). The coefficients of variation ranged for the continuously increasing HCN tests from 20.3 percent for all test concentrations combined (excluding Tests 3, 5, and 6) to 16.6 percent for the combined concentrations of Tests 10-12.

Test 5 indicated a latent period of less than 22 min and somewhere near 15-18 min. Test 6 incorporated a square exposure as opposed to an increasing concentration curve. The areas under the square exposure curves were three to four times as great as the areas under the increasing concentration curves (Table 46), probably because such large proportions of the areas under the square exposure curves lay within the latent period. The coefficient of variability for the areas in the square exposure test was 9.5 percent, less than those for the increasing concentration tests.

Despite a fair amount of variability, there seems to be some support for the concept of toxicity based on area. Therefore, all percentage mortalities were transformed to probits and plotted against the logarithms of their respective expsoure areas, as was done by Larson and Schlesinger (1977). These authors did not carry their data analysis beyond stating that toxicity to fish was a function of area based on the plot of mortality probits versus log area. Regression analysis was performed on the combined data of all tests (excluding Tests 3, 5, and 6), Tests 1, 2, and 4, Tests 7-9, and Tests 10-12. Although a linear relationship is indicated, the multiple correlation coefficients (R^2) were only 0.41 for all combined tests; 0.51 for Tests 1, 2, and 4; 0.42 for Tests 7-9; and 0.49 for Tests 10-12. The variability is compounded from several sources such as HCN determinations, accurate determination of the actual time of death, and the fitting of the mortality data to the time-concentration curves. The R^2 values are not especially high, but the use of areas as a basis for toxicity analysis cannot be disregarded until further

TABLE 46. MAXIMUM HCN CONCENTRATION, MEDIAN SURVIVAL TIME (MST) AND EXPOSURE AREA DERIVED FROM THE TIME-CONCENTRATION CURVES FOR THE INCREASING HCN LETHAL EXPOSURE TESTS

Test no.	HCN (µg/l)	MST (min)	Area (cm^2)*	Test no.	HCN (µg/l)	MST (min)	Area (cm^2)*
1	390	238	189.87	7	1070	57.0	117.08
	596	179	181.02		1423	49.0	129.57
	674	138	140.11		1698	40.5	111.69
	820	146	182.96		2090	38.5	134.09
2	243	-	-	8	593	79	97.69
	348	172	129.19		-	-	-
	401	166	146.89		836	59	102.78
	466	119	106.85		1066	53	122.92
3	227	-	-	9	463	-	-
	293	-	-		619	119	167.09
	362	-	-		713	102	163.33
	430	-	-		905†	68	118.90
4	256	-	-	10	554	-	-
	382	198	184.35		703	124.0	140.16
	464	160	179.26		794	114.0	134.05
	536†	128	164.08		1076	96.5	128.56
5	301	165.0	-	11	933	92.5	104.06
	382	164.0	-		1290	84.5	120.81
	493	107.5	-		1330†	74.5	95.75
	583	88.0	-		1700†	69.0	107.81
	710	69.0	-	12	512	184	152.17
	946	44.0	-		664	166	161.47
	1387	26.5	-		736	154	154.00
	1737	22.0	-		1001	117	126.59
6	606	62.0	587.06				
	797	45.0	560.39				
	920	33.5	481.56				
	1176	26.5	496.13				

* Area based on 1 cm^2 = 40 µg/l HCN x 8 min

† Estimates of the obtained maximum level

TABLE 47. MEAN AREAS, STANDARD DEVIATIONS, AND COEFFICIENTS OF VARIATION FOR THE COMBINED TESTS FROM TABLE 46

Test number	Mean area (cm^2)*	Standard deviation	Coefficient of variation (percent)
All tests combined (excluding 3, 5, and 6)	138.55	28.15	20.3
Tests 1, 2, and 4	160.46	28.25	17.6
Tests 7-9	126.51	23.20	18.3
Tests 10-12	129.58	21.48	16.6
Test 6	531.29	50.55	9.5

* Area based on 1 cm^2 = 40 μg/1 HCN x 8 min

investigations have been concluded which improve on the above conditions of variability. The use of regression in this situation is important in its value as a predictive tool. Should the exposure area to fish of a particular spill be known, the amount of mortality could roughly be predicted from the regression relationship.

INTERMITTENT SUBLETHAL EXPOSURE TESTS

The concentration of toxicants in natural waters frequently fluctuate in a diurnal pattern. In certain situations there may be fluctuations from non-measurable levels to nearly lethal levels. It was the objective of these experiments to compare the sublethal effect of HCN on early growth of fathead minnows in a continuous and two different intermittent exposure situations.

Experimental Design

The sublethal tests were conducted in five identical diluters as previously described for acute tests. The experimental glass chambers measured 25 x 50 x 30 cm high and contained 35 l of test solution. The flow of water through each chamber was at the rate of 250 ml/min, affording 95 percent replacement in about 7 hr. Water temperature was maintained at about 25°C. Aeration in the water reservoir supplying the diluters maintained dissolved oxygen concentrations in the test chambers at approximately 73 percent of saturation. The test chambers were illuminated for 16 hr daily with two 40-W fluorescent tubes 45 cm above the chambers.

Water temperature, dissolved oxygen (DO), pH, and free cyanide concentration in each test chamber were measured three times per week. The HCN concentrations were calculated as previously described. Determinations for each test

tank were averaged and used as the reported values. The toxicant concentrations in the various test tanks followed one of three regimes. In seven tanks the concentrations were held constant. In the remaining 14 treatment chambers, the concentrations fluctuated about two different patterns with seven test chambers for each treatment regime. The overall mean of the maximum concentrations in the pattern of diurnal fluctuation will be used when referring to specific test treatments.

Growth tests were begun by randomly adding 50 newly hatched fathead minnow larvae to each of 21 treatment and 4 control test chambers. Through the first week of exposure, the larvae were fed a fine, hard-boiled egg yolk suspension three times daily. Beginning the second week a supplement of finely ground Glencoe granules was added twice daily. The fry were fed newly hatched brine shrimp (*Artemia*) twice daily beginning the third week, with finely chopped lettuce also being added to the egg yolk suspension. After 30 days of exposure, the test was terminated and the surviving fish were measured and weighed. Mean dry weights were determined after the fish had been dried for 24 hr at 105°C.

Results

The diurnal fluctuations in HCN levels, expressed as percentages of the maximum concentration for each test chamber, under the three treatment regimes are presented in Figure 8. These relationships were determined from three time exposure series which were run during the growth test, and represent the mean patterns for the exposure regimes. The percentages were independent of the maximum HCN concentration, and thus one response curve can be used to represent the fluctuation for all concentrations of each regime. The vertical bars in Figure 8 represent the range of percentages for all test tanks at a given time of the diurnal exposure cycle. The relative exposure areas, compared to 100 percent for continuous and constant concentrations, were 64.9 percent for the pattern of 16 hr on and 8 hr off, and 34.5 percent for the pattern of 8 hr on and 16 hr off. The on and off designations refer to when cyanide was being metered into the test chamber and when it was not, respectively.

The results of 30-day growth tests with fathead minnows exposed to each of three concentration regimes are presented in Table 48. The growth data were analyzed by using linear regression (Table 49) to see if the relationships of fish weight to log HCN concentration for the three exposure patterns were similar and in some manner a function of the continuous exposure response.

Statistical analysis demonstrated that there is no significant difference ($P < 0.05$) between the slopes of the concentration-response regression lines. Therefore, the adverse effects of HCN on early growth of the fathead minnow for the fluctuating diurnal regimes are for each exposure pattern some constant function of the response observed with continuous exposure. This relationship is not directly related to exposure area, mean, or maximum HCN concentration. About all that can be concluded is that as the diurnal exposure period is reduced, the adverse effect of HCN on early growth of the fathead minnow is lessened. This is true even though the maximum concentrations attained in the test chambers with fluctuating concentrations are the same as those sustained in the continuous exposure aquaria.

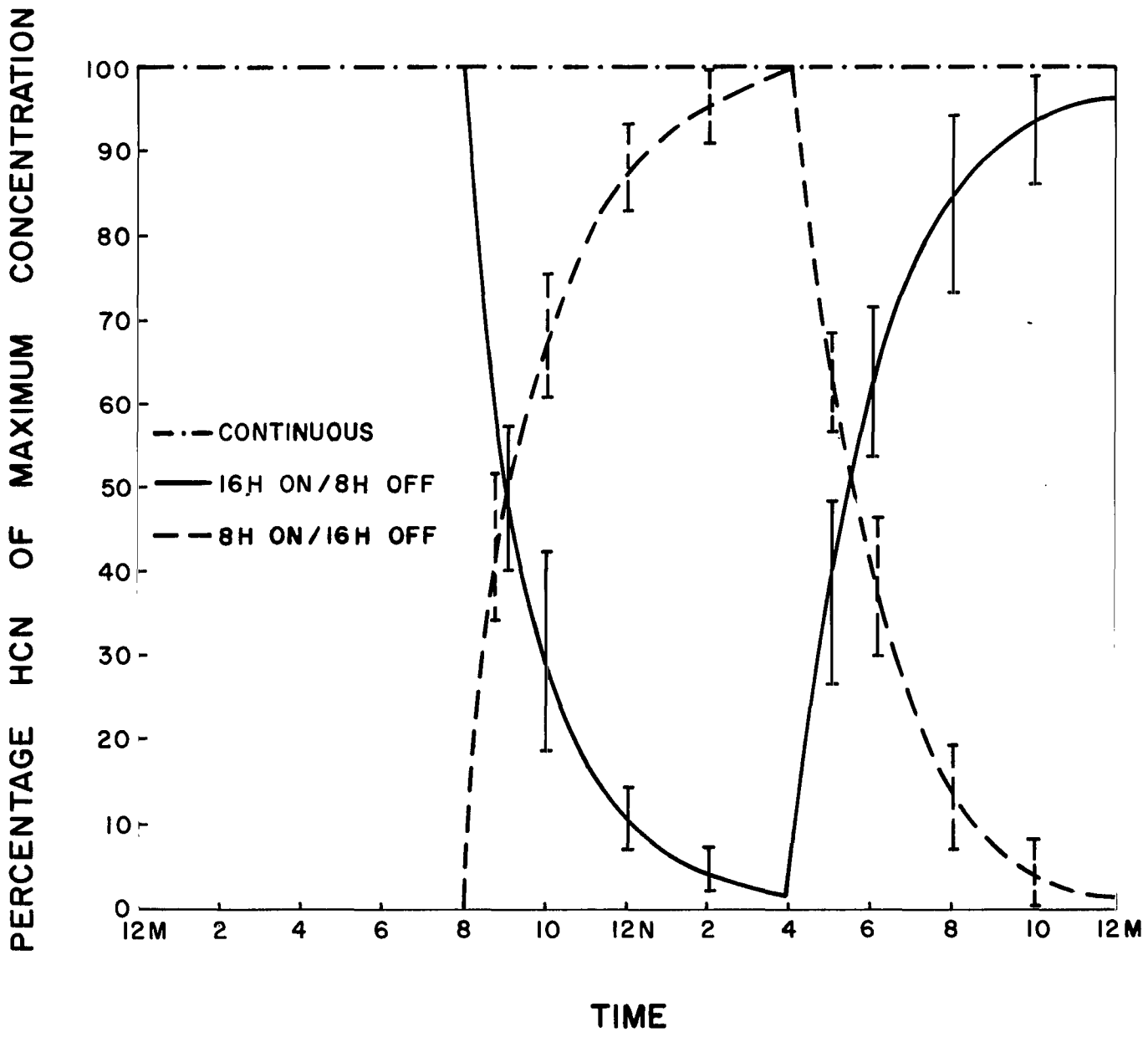

Figure 8. Diurnal fluctuation of HCN levels in test chambers as a function of their maximum concentrations. Ranges indicated by vertical bars.

TABLE 48. GROWTH OF FATHEAD MINNOWS IN 30 DAYS WHEN EXPOSED TO CONTINUOUS OR INTERMITTENT HCN CONCENTRATIONS*

Test solution	HCN† concentration µg/l	Number of survivors	Mean weight (mg) Wet	Dry	Percent‡ of controls
			Controls		
1	-	24	42.5	6.29	-
2	-	26	40.9	6.81	-
3	-	25	32.5	4.84	-
4	-	30	24.9	4.40	-
				5.53§	
			Continuous		
1	20.4	32	41.3	5.16	93.3
2	26.8	30	36.0	4.33	78.3
3	41.8	31	32.0	3.87	70.0
4	43.2	25	19.9	3.16	57.1
5	60.7	26	13.4	2.19	39.6
6	71.4	16	8.69	0.94	17.0
7	82.4	22	9.41	1.09	19.7
			16 hr on - 8 hr off		
1	22.9#	24	29.9	5.20	94.0
2	28.6#	24	35.6	6.34	114.6
3	34.2	29	38.9	5.45	98.6
4	49.8	26	34.0	4.50	81.4
5	57.5	28	30.6	4.26	77.0
6	66.1	24	30.1	3.71	67.1
7	73.8	29	22.3	3.34	60.4

Table 48 Continued

Test solution	HCN† concentration µg/l	Number of survivors	Mean weight (mg) Wet	Dry	Percent‡ of controls
		8 hr on - 16 hr off			
1	23.5#	25	41.8	5.56	100.5
2	33.0#	24	32.3	5.62	101.6
3	45.8	26	30.1	5.23	94.6
4	49.6	26	32.8	5.08	91.9
5	61.6	25	28.9	4.88	88.2
6	63.4	26	27.3	4.54	82.1
7	85.1	27	23.7	3.22	58.2

* Overall mean temperature was 23.5°C; DO, 6.24 mg/l; and pH, 7.99

† Concentrations corresponding to maximum values for intermittent exposures

‡ Percentages based on dry weights

§ Weighted mean based on number of survivors in each replicate

\# Values omitted from regression analysis (Table 49)

TABLE 49. LINEAR REGRESSION ANALYSIS OF THE TOXICANT DOSE RESPONSE CURVES EXPRESSING TERMINAL MEAN DRY WEIGHT IN mg (Y) AND PERCENTAGE TERMINAL MEAN DRY WEIGHT NORMALIZED TO CONTROLS (Y) AS A FUNCTION OF LOG TOXICANT CONCENTRATION IN μg/1 (X)

	Linear regression analysis*					
	Mean dry weight			Normalized percent mean dry weight		
Exposure regime	α	β	R^2	α	β	R^2
Continuous	14.63	-7.070	0.9393	264.5	-127.8	0.9393
16 hr on - 8 hr off	15.01	-6.195	0.9862	271.6	-112.2	0.9866
8 hr on - 16 hr off	17.47	-7.255	0.8923	316.4	-131.4	0.8934

* Values omitted from regression analysis are so indicated in Table 48.

Linear regression equation is $Y = \alpha + \beta(\log X)$.

BINARY MIXTURES WITH HCN

Natural waters receiving wastes often contain several toxic or potentially toxic substances. Relatively few investigations have defined the adverse effect of mixtures of toxicants to fish, especially at the sublethal level. Water quality standards governing the treatment and control of wastes discharged into natural waters should reflect valid water quality criteria that adequately protect valued aquatic populations. When more than one toxicant is present in the same system, their combined, interactive effect can be exerted in such a manner that the collective toxicity may be predictable from the separate toxicities of the individual substances. This interactive effect may occur as similar or independent joint action, and it may be greater or less than predicted or show no interaction. If the response is less than predicted on the basis of no interaction then antagonism occurs. Because of the importance of being able to predict toxicity in natural waters from a knowledge of individual toxic constituents, the purpose of this investigation was to examine the validity of various methods for predicting joint toxicity of binary mixtures of HCN with hexavalent chromium, zinc, or ammonia from lethal and sublethal responses of fish to individual toxic components in the mixtures.

Experimental Design

Newly hatched larvae or juvenile fathead minnows (*Pimephales promelas*) and fry or juvenile rainbow trout (*Salmo gairdnerii* Richardson) were used as test organisms. The minnows were cultured in the laboratory from a strain originating from the U.S. Environmental Protection Agency's Environmental Research Laboratory in Duluth, Minnesota. The minnows were approximately 8 weeks old when used, had mean total lengths of 19 mm, and mean wet weights among survivors of 0.079 g. Numerous lots of juvenile minnows were used during the acute toxicity tests. However, the sensitivity of all minnows to any specific toxicant was demonstrated to be quite uniform throughout the testing period.

Eyed rainbow trout embryos from eggs spawned in late April 1976 were obtained in early July 1976 from the French River State Fish Hatchery, French River, Minnesota. A second lot, spawned in late April 1977, was obtained from the hatchery in early July 1977. The water temperature at collection and during subsequent holding and testing of the trout was 10°C. The trout reared from the 1976 stock were approximately 22 weeks old when used for lethal tests, had mean total lengths of 53 mm, and mean wet weight among survivors of 1.48 g. Fry reared from the 1977 stock were used in the sublethal tests.

The lethal and sublethal tests were conducted in identical diluters as described for other acute tests in Section IV. The experimental glass chambers measured 25 x 50 x 30 cm high and contained 35 l of test solution. Water flowed through each chamber at the rate of 250 ml/min, affording 95 percent replacement in about 7 hr (Sprague 1969).

Water temperature in all tests with fathead minnows was maintained at 25°C and with rainbow trout at 10°C. Aeration in the head water reservoir feeding the diluters maintained dissolved oxygen concentrations in the test chambers at approximately 80 percent saturation. A sulfuric acid solution was auto-

matically dispensed into the reservoir to control the pH of the test water for certain tests. The test chambers were illuminated for 16 hr daily with two 40-W fluorescent tubes 45 cm above the chambers. Stock toxicant solutions of sodium cyanide, sodium dichromate, zinc sulfate and ammonium chloride were prepared with reagent grade chemicals and deionized water. The ammonia tests were conducted at 10°C to retard nitrification and prevent the buildup of adverse nitrite levels in the test chambers.

Water temperature, dissolved oxygen (DO), pH, and toxicant concentrations in each test chamber were measured daily during each lethal test and three times per week during the sublethal tests. Determinations for each test were averaged and used as the experimental toxicant concentration. Free cyanide concentrations were determined as in previous tests. Hexavalent chromium, zinc, and free ammonia concentrations were determined by the diphenylcarbazide, zincon, and direct nesslerization colorimetric procedures, respectively (APHA, *et al.* 1975). The concentration of molecular HCN or NH_3 was calculated for each free cyanide or ammonia determination using the daily pH and temperature measurement and the pK equilibrium constants (i.e., $-\log K_a$) calculated from the equations:

$$pK_{HCN} = 3.658 + 1662/T \quad \text{(Broderius, unpublished data)}$$

$$pK_{NH_3} = 0.09018 + 2729.92/T \quad \text{(Emerson, } et\ al.\text{ 1975)}$$

where T is temperature in degrees Kelvin. The ratio [HCN]/[free cyanide] or [NH_3]/[free ammonia] is taken to be equal to the factor

$$1/(1 + 10^{pH - pK_a}) \text{ or } 1/(1 + 10^{pK_a - pH})$$

respectively. When both the molecular HCN or NH_3 and free cyanide or ammonia concentrations are expressed in the same units and as the molecular form, then free cyanide or ammonia times the appropriate factor will equal molecular HCN or NH_3.

The 96-hr lethal tests were conducted with one control and four treatments in a geometric series. Twenty fathead minnows or 10 rainbow trout juveniles were randomly distributed into each treatment and control chamber. The fish were acclimated to the test chambers for 2-3 days before introduction of the toxicants. During the 24-hr period preceeding and throughout a lethal test the fish were not fed. In each binary experiment the single toxicant tests were completed before the joint toxicant tests. Since all the lethal tests for each pair of toxicants were completed within a few weeks, changes in fish susceptibility within a test period were assumed to be nonsignificant.

Percentage fish mortality after 96 hr of exposure to individual toxicants and to binary mixtures containing fixed proportions of the constituents was used as the response criterion. Estimates of the concentration of toxicant most likely to cause 50 percent mortality (LC50) and toxicity curve slopes after 96 hr of exposure were made by a log-probit analysis computer program (Dixon 1973). All the data from toxicity tests conducted for specific tox-

icants and for their mixtures were composited for probit analysis. The 95 percent confidence limits for LC50 values were computed according to formulas proposed by Litchfield and Wilcoxon (1949) and Finney (1971).

The manner in which the combined effect of mixtures of toxicants was calculated by the toxic unit approach has been outlined by Sprague (1970). With this procedure the proporitonal toxicity of each poison in a mixutre is obtained by dividing the actual concentration in solution by its calculated 96-hr LC50. These values obtained for the two toxicants in a binary mixture are then summed to give the proportion of the 96-hr LC50 in the mixture. The concentrations selected for testing were usually such that each poison theoretically contributed an equal proportion of toxic units to the mixture. The toxic unit-percentage mortality relationships were analyzed by the computer programs as outlined above.

The toxic unit (TU) or sum of biological activity index of joint toxicity can be modified to represent an index of additive toxicity distributed about zero, which represents simple additive toxicity. This additive index as proposed by Marking (1977) is equal to $[(1/TU) - 1.0]$ for $TU \leq 1.0$ and $[TU(-1) + 1.0]$ for $TU \geq 1.0$. Thus toxicity greater than and less than simple additive toxicity is represented by positive and negative index values, respectively. The magnification factor is obtained by adding one to the numerical index value for greater than additive toxicity and taking the reciprocal of the numerical index value for less than additive toxicity. The significance of deviation from zero is determined by substituting selected values of the 95 percent confidence limits for the calculated toxic units into the appropriate additive index formula to establish the deviation in the additive index range. Simple additive toxicity is assumed whenever an index range overlaps zero.

If the linear response curves of individuals to two toxicants are not parallel or if the modes of toxic action of the two constituents are known to be different for toxicants which had similar slopes, then it is possible that the separate responses of organisms to the individual concentrations of each toxicant in a mixture can be summed. The mortality to be expected in such a binary mixture exerting independent joint action (Bliss 1939) or response addition (Anderson and Weber 1977) depends upon the correlation between the susceptibilities of the individual organisms to each toxicant. According to Plackett and Hewlett's (1948, 1952) mathematical models, if the correlation of individual tolerances is completely negative ($r = -1$) so that the proportion of individuals most susceptible to one toxicant alone (P_1) are least susceptible to the other (P_2), then the proportion of individuals expected to respond to the mixture (P_m) can be represented by:

$$P_m = P_1 + P_2 \text{ if } (P_1 + P_2 \leq 1)$$

With no correlation in susceptibility ($r = 0$) the relationship is expressed by:

$$P_m = P_1 + P_2(1 - P_1)$$

If there was a complete and possible correlation ($r = 1$) so that individuals most susceptible to the first toxicant would be correspondingly susceptible to

the second, then

$$P_m = P_1$$

where P_1 is the response to the most toxic constituent in the mixture.

Growth tests were begun by randomly adding 50 newly hatched fathead minnow larvae or 20, 4-week-old rainbow trout fry to each of the 21 treatment and 4 control test chambers. From a subsample of 32 individuals, it was determined that the trout fry initially were 28.5 ± 2.2 mm in mean total length, and 0.180 ± 0.059 g and 0.0278 g in mean wet and dry weight, respectively. The treatments consisted of seven independent cyanide and seven second toxicant levels, and seven binary mixtures.

Through the first week of exposure, the fathead minnow larvae were fed a fine, hard-boiled egg yolk suspension three times daily. Beginning the second week a supplement of finely ground Glencoe granules was added twice daily for the remainder of the test. Beginning the third week, finely chopped lettuce was added to the egg yolk suspension, and newly hatched brine shrimp (*Artemia*) were fed twice daily. During the first two weeks of the trout tests fry were fed twice daily with ground Glencoe starter and three times daily with newly hatched brine shrimp. Beginning the third week, Glencoe No. 1 and Oregon Moist pellets were fed three times per day and brine shrimp twice daily. Food slightly in excess of what the fish consumed in 5 min was added to each chamber. After 30 days of exposure each test was terminated and the surviving fish were measured and weighed individually. Mean weights of fish that had been dried for 24 hr at 105°C were determined for each treatment.

For statistical analysis, individual wet weights were transformed to natural logarithms and subjected to one-way analysis of variance. Treatment means were compared with the overall mean from control replicates using Dunnett's procedure (two-tailed) at a significance level of $P < 0.05$. Linear regression analyses were applied to growth data to determine whether the relationships of mean dry weight or weight normalized to controls versus log toxicant concentration were the same for a toxicant by itself and in a binary mixture.

Results

The test conditions and results of lethal tests using individual toxicants and binary mixtures are summarized in Tables 50 and 51. Probit analysis of data from binary mixture tests, using the log summation of the individual concentrations of toxicants in each treatment mixture, expressed as proportions of their 96-hr LC50 values, as the dose metameter, generally gave 96-hr LC50 toxic unit values of slightly less than 1.0. The hexavalent chromium-HCN mixture was the exception with a 96-hr LC50 toxic unit value of 1.312. Since the 95 percent confidence limits for the toxic unit values do not bracket 1.0 in any case, nor does the additive index range overlap zero, the estimated values for the 96-hr LC50 concentrations differ significantly from that expected from the simple additive toxic unit hypothesis. Therefore, the acute lethal toxicities of various binary mixtures of HCN and Cr, Zn, or ammonia could not be predicted for the ratios tested by summing the fractional toxic-

TABLE 50. MEAN TEST CONDITIONS AND DESCRIPTION OF TEST FISH FOR ACUTE TOXICITY BIOASSAYS OF INDIVIDUAL TOXICANTS AND BINARY MIXTURES. (STANDARD DEVIATIONS IN PARENTHESES)

Toxicant(s)	Fish* species	No. of tests	pH	Temp. (°C)	DO (mg/l)	Test fish mean: Length (mm)	Test fish mean: Wet weight survivors (g)
			Individual Toxicants				
HCN	FM	11	7.76	25.0(0.12)	6.91(0.10)	19.8	0.0762
HCN	RT	6	8.04	10.1(0.06)	8.82(0.09)	52.7	1.407
Cr	FM	6	7.80	25.0(0.18)	6.82(0.09)	20.8	0.0950
Zn	FM	10	7.69	24.7(0.16)	7.12(0.11)	18.0	0.0722
Total ammonia-N	RT	8	7:96	10.0(0.07)	8.74(0.12)	48.7	1.168
NH_3	RT	8	7.96	10.0(0.07)	8.74(0.12)	48.7	1.168
			Binary Mixtures				
Cr-HCN	FM	6	7.90	25.1(0.13)	6.93(0.10)	20.5	0.0988
Zn-HCN(1)	FM	3	7.74	24.8(0.20)	6.95(0.18)	17.6	0.0581
Zn-HCN(2)	FM	4	7.72	24.6(0.15)	7.06(0.08)	18.3	0.0733
(Total ammonia-N)-HCN	RT	4	7.98	10.1(0.04)	8.93(0.05)	57.6	1.852
NH_3-HCN	RT	4	7.98	10.1(0.04)	8.93(0.05)	57.6	1.852

* FM represents fathead minnows and RT, rainbow trout

TABLE 51. DOSE-RESPONSE PARAMETERS, LETHAL CONCENTRATIONS AND ADDITIVE INDICES FOR ACUTE TOXICITY BIOASSAYS OF INDIVIDUAL TOXICANTS AND BINARY MIXTURES (95 PERCENT CONFIDENCE LIMITS IN PARENTHESES)

Toxicant(s)	Fish species	Toxicant conc.* ratios, tox./HCN		Dose-response regression analysis†			96-hr LC50 mg/l or TU§	Additive index and range#
		Aim	Determined	Treatments‡	α	β		
				Individual Toxicants				
HCN	FM	-	-	36	16.75	13.01	0.125 (0.120-0.130)	-
HCN	RT	-	-	15	33.63	23.04	0.0572 (0.0558-0.0586)	-
Cr	FM	-	-	30	-2.787	5.119	33.2 (31.1-35.4)	-
Zn	FM	-	-	32	2.590	5.776	2.61 (2.49-2.75)	-
Total ammonia-N	RT	-	-	23	-9.069	9.063	35.7 (32.5-39.1)	-
NH_3	RT	-	-	23	6.325	8.460	0.697 (0.629-0.773)	-

- continued -

TABLE 51. (continued)

Toxicant(s)	Fish species	Toxicant conc.* ratios, tox./HCN: Aim	Toxicant conc.* ratios, tox./HCN: Determined	Dose-response regression analysis†: Treatments‡	Dose-response regression analysis†: α	Dose-response regression analysis†: β	96-hr LC50 mg/l or TU§	Additive index and range#
Binary Mixtures								
Cr-HCN	FM	266	235±20	20	3.906	9.277	1.312 (1.222-1.408)	-0.312 -0.408 to -0.222
Zn-HCN (1)	FM	20.9	14.3±1.0	10	7.008	13.19	0.704 (0.650-0.763)	0.420 0.311 to 0.538
Zn-HCN (2)	FM	20.9	23.2±2.7	13	6.966	13.87	0.721 (0.692-0.752)	0.387 0.330 to 0.445
(Total ammonia-N)-HCN	RT	624	594±38	10	6.668	20.08	0.826 (0.790-0.864)	0.211 0.157 to 0.266
NH_3-HCN	RT	12.2	12.4±1.1	10	6.401	21.31	0.859 (0.824-0.897)	0.164 0.115 to 0.214

* Ratio of individual toxicant concentrations in different treatments of mixture tests. Aim values are for equal proportions of toxic units.

† For equation $\hat{Y}_i = \alpha + \beta(\log X_i)$ when $\hat{Y}_i$ is the predicted percentage mortality as probits and X_i is log toxicant concentration in mg/l or toxic units (Dixon 1973)

‡ Number of toxicant concentrations in the regression analysis

§ TU represents toxic units or the proportion of the expected 96-hr LC50 assuming simple additivity

\# Calculations based on 96-hr LC50 in toxic units and 95 percent confidence limits

ities of the particular poisons present in each mixture. In two of the three cases, the mixtures exerted a supra-additive effect (i.e., toxicity was greater than predicted) with the Zn-HCN and ammonia-HCN mixtures being 1.4 and 1.2 times as toxic as predicted, respectively (Marking 1977). The exception was hexavalent chromium whose interaction with HCN seems to be of the infra-additive type since the mixture was 0.76 times as toxic as predicted. It appears that for each fish species the dosage-mortality regression slope value is nearly the same for HCN alone and mixture tests analyzed by the toxic unit approach (Table 51), even when the individual toxicants differ both in potency and in the slopes of their dosage-mortality curves. This result suggests that in both systems HCN exerts its toxic action in a similar manner and is the factor which defines the slopes of the mixture response curves.

For most of the individual toxicant and mixture tests a median lethal threshold concentration could be determined in 96 hr of exposure. However, for hexavalent chromium alone additional mortality of fathead minnows occurred beyond this period. The 10-, 20-, and 30-day LC50 and 95 percent confidence limts, determined under similar test conditions as reported for 96-hr tests with Cr(VI) (Table 50), were 12.4 (11.0-14.0), 5.99 (5.14-6.96), and 4.36 (3.85-4.92) mg/l as Cr, respectively.

In the present study, the slopes of the dose-response lines for components of the mixtures were all dissimilar when tested separately. Parallelism between response curves is apparently a prerequisite to concentration addition as defined by Anderson and Weber (1977), but not for response addition. Therefore, it is possible that this latter model, for toxicants which are thought to primarily affect different biological systems, could predict responses comparable to those observed in the binary mixtures. When this approach was used to analyze the data, it was determined that in every case the expected mortality was significantly less using a Chi square test ($P = 0.05$) than was experimentally obtained. Using the dose-response regression lines for the individual toxicants and assuming the response addition model with no correlation in susceptibility to the two toxicants, a mortality of about 20 percent was predicted among test fish exposed to the Cr-HCN mixture which, in fact, killed about 50 percent. For the Zn-HCN and ammonia-HCN mixtures in which the observed mortality was about 50 percent, the predicted mortality was less than 1.0 percent.

Bliss (1939) has pointed out that for individual toxicants which act independently and have dosage-mortality curves with different slopes, the response to a binary mixture should have some compound discontinuity consisting essentially of two straight line segments. The slopes of these rectilinear segments should also approach the slopes of the regression lines for the individual constituents. This result was not observed for the mixtures tested since in all cases a single straight line adequately defined the dosage-mortality relations. It was also observed that either toxicant could contribute to the toxicity of the mixtures tested even when individually present at a concentration which would result in no mortality if the other were absent. However, responses to toxicants acting upon different biological systems are predicted to be additive only if each toxicant in a mixture is present at a concentration above its respective threshold level. Therefore, it is proposed that the response and concentration addition models (Anderson and Weber 1977) cannot be

used to predict dosage-mortality curves for the mixtures studied.

The results of 30-day growth tests with fathead minnows and rainbow trout exposed to individual toxicants or binary mixture solutions are presented in Tables 52-55. The maximum percentage reduction in mean dry weight (Tables 52-54) for concentrations approaching the incipient lethal level were all about 50 percent, with the greatest being 79 percent for 3.063 mg/l of chromium alone. The growth data were analyzed by linear regression to see if the slopes and intercepts of the relationships defining mean dry weight on log toxicant concentration are the same for a specific toxicant alone in solution and when in a binary mixture. Since F tests show that there is no significant difference ($P < 0.05$) between appropriately paired regression lines defining the relationship of weight to log concentration, no interactive sublethal effect on growth could be demonstrated for the toxicants tested. This similarity of regression lines can be seen in Table 55 by comparing the intercept and slope parameters for individual toxicants with the parameters for those toxicants when in binary mixtures. Hydrogen cyanide in combination with ammonia was the only mixture that seemed to demonstrate some sublethal interactive effect on growth of rainbow trout. When terminal mean dry weight was regressed on log HCN, NH_3, or total ammonia-N concentrations, the weight-concentration relationship for a specific toxicant in the binary mixture indicated in all cases a greater affect than that determined for the corresponding toxicant when tested alone (Table 55). However, the only regression relationships that were statistically different at an acceptable level of significance ($F_{2,10} = 3.67$, $P < 0.063$) were those calculated for the effect of HCN alone compared to HCN when in combination with ammonia. Therefore, the sublethal stress imposed on fish in most binary mixtures studied disrupted physiological functions so that the relationships between concentration and reduction in growth were essentially equal to those when the toxicants were present alone. The adverse joint action of individual toxicants on growth were not predictable according to existing models.

Summary

A thorough understanding of chemical reactions of constituents in a mixture is necessary for prediction of joint effects. Where toxicants chemically combine, prediction of joint toxicity may be most difficult. Chromium and zinc can combine with cyanide to form coordination compounds. However, calculations from stability constants showed that under the conditions prevailing in the mixture solutions, less than 1.0 percent of the cyanide, and even smaller percentages of the metals, would be present as complex ions. Therefore, the toxicants in each of the binary mixtures studied should be in essentially the same form as they were in the solutions where their toxicities were individually determined.

The physiological basis for interaction of toxicants in their effects on fish has received some attention in recent years. As pointed out by Marking (1977), projected theories for the mechanisms of toxicant interaction in mixtures include increase in rate of uptake, formation of toxic metabolites, reduction of excretion, alteration of distribution, and inhibition of detoxification. Lloyd and Swift (1976) studied whether the increased water uptake by fish exposed to high ambient ammonia concentrations was accompanied by an

TABLE 52. GROWTH OF FATHEAD MINNOWS IN 30 DAYS WHEN EXPOSED TO INDIVIDUAL OR MIXTURE SOLUTIONS OF HEXAVALENT CHROMIUM AND HCN*

Test solution	Toxicant concentration mg/l		Number of survivors	Mean weight (mg)		
	HCN	Cr		Wet	Dry	Percent of controls†
			Controls			
1	-	-	23	48.3	9.12	-
2	-	-	37	37.7	6.73	-
3	-	-	36	49.9	9.44	-
4	-	-	25	54.9	11.2	-
				46.9‡	8.91‡	
			HCN			
1	0.0176	-	26	48.5	9.08	101.9
2	0.0202	-	36	50.7	10.0	112.2
3	0.0300	-	27	41.6	7.86	88.2
4	0.0344	-	30	38.4	7.43	83.4
5	0.0517	-	27	33.9§	6.11	68.6
6	0.0643	-	20	30.2§	5.10	57.2
7	0.0842	-	21	22.3§	3.76	42.2
			Cr			
1	-	0.050	27	46.7	9.37	105.2
2	-	0.105	35	31.9§	5.66	63.5
3	-	0.186	40	36.0	6.82	76.5
4	-	0.432	38	36.5	7.11	79.8
5	-	0.803	36	20.4§	3.61	40.5
6	-	1.448	33	19.9§	3.61	40.5
7	-	3.063	22	11.8§	1.91	21.4

TABLE 52 Continued

Test solution	Toxicant concentration mg/l HCN	Toxicant concentration mg/l Cr	Number of survivors	Mean weight (mg) Wet	Mean weight (mg) Dry	Percent of controls†
			Cr-HCN Mixture			
1	0.0149	0.030	28	50.4	9.96	111.8
2	0.0177	0.051	31	42.9	8.19	91.9
3	0.0252	0.100	25	46.9	9.24	103.7
4	0.0278	0.213	28	35.0	6.89	77.3
5	0.0398	0.436	33	30.8§	6.24	70.0
6	0.0460	0.563	34	26.7§	5.35	60.0
7	0.0497	0.796	32	26.6§	4.81	54.0

* Overall mean temperature was 25.3°C; DO, 7.24 mg/l, and pH, 7.79

† Percentages based on dry weights

‡ Weighted mean based on number of survivors in each replicate

§ Values are significantly different from mean of controls according to Dunnett's procedure (two-tailed; $P < 0.05$)

TABLE 53. GROWTH OF FATHEAD MINNOWS IN 30 DAYS WHEN EXPOSED TO INDIVIDUAL OR MIXTURE SOLUTIONS OF ZINC AND HCN*

Test solution	Toxicant concentration mg/l HCN	Toxicant concentration mg/l Zn	Number of survivors	Mean weight (mg) Wet	Mean weight (mg) Dry	Percent of controls†
			Controls			
1	–	–	25	61.2	13.2	–
2	–	–	30	57.5	11.5	–
3	–	–	31	51.3	10.2	–
4	–	–	28	45.8	9.11	–
				53.8†	10.9†	
			HCN			
1	0.0232	–	30	45.1	9.27	85.0
2	0.0286	–	27	42.2	8.22	75.4
3	0.0344	–	23	40.1	7.93	72.8
4	0.0439	–	27	36.9	7.44	68.3
5	0.0535	–	29	32.0§	6.07	55.7
6	0.0545	–	25	27.1§	5.32	48.8
7	0.0865	–	26	26.8§	5.12	47.0
			Zn			
1	–	0.299	33	56.4	11.9	109.2
2	–	0.462	32	46.1	9.28	85.1
3	–	0.465	34	40.9	8.09	74.2
4	–	0.632	26	38.8	7.54	69.2
5	–	0.689	21	37.5	6.86	62.9
6	–	0.759	19	35.9§	7.11	65.2
7	–	0.840	13	42.4	7.92	72.7

TABLE 53. Continued

Test solution	Toxicant concentration mg/l HCN	Toxicant concentration mg/l Zn	Number of survivors	Mean weight (mg) Wet	Mean weight (mg) Dry	Percent of controls†
			Zn-HCN mixture			
1	0.167	0.226	26	56.6	12.2	111.9
2	0.0242	0.288	35	51.9	11.0	100.9
3	0.0283	0.419	29	42.4	8.14	74.7
4	0.0352	0.566	23	46.6	9.39	86.1
5	0.0390	0.655	28	35.8§	6.79	62.3
6	0.0448	0.620	18	44.4	8.89	81.6
7	0.0571	0.863	11	36.4	6.55	60.1

* Overall mean temperature was 25.2°C; DO, 7.01 mg/l; and pH, 7.78

† Percentages based on dry weights

‡ Weighted mean based on number of survivors in each replicate

§ Values are significantly different from mean of controls according to Dunnett's procedure (two-tailed; $P < 0.05$)

TABLE 54. GROWTH OF RAINBOW TROUT IN 30 DAYS WHEN EXPOSED TO INDIVIDUAL OR MIXTURE SOLUTIONS OF AMMONIA AND HCN*

Test solution	Toxicant concentration mg/l HCN	NH_3	Total ammonia-N	Number of survivors	Mean weight (mg) Wet	Dry	Percent of controls†
			Controls				
1	-	-	-	20	815	160	-
2	-	-	-	20	824	154	-
3	-	-	-	19	842	156	-
4	-	-	-	20	863	157	-
					836‡	157‡	
			HCN				
1	0.0086	-	-	20	880	166§	105.7§
2	0.0148	-	-	19	833	154	98.1
3	0.0176	-	-	20	870	163	103.8
4	0.0203	-	-	17	805	149	94.9
5	0.0262	-	-	17	757	136	86.6
6	0.0347	-	-	16	615#	112	71.3
7	0.0440	-	-	6	441#	79	50.3
			NH_3 or total N				
1	-	0.053	2.49	20	907	165§	105.1§
2	-	0.102	5.45	19	747	139	88.5
3	-	0.121	6.53	20	846	156	99.4
4	-	0.171	9.25	20	726	128	81.5
5	-	0.221	11.8	19	672	119	75.8
6	-	0.253	14.5	13	584#	100	63.7
7	-	0.318	18.0	14	459#	76	48.4

TABLE 54. Continued

Test solution	Toxicant concentration mg/l HCN	NH_3	Total ammonia-N	Number of survivors	Mean weight (mg) Wet	Dry	Percent of controls†
			NH_3 or total N-HCN mixture				
1	0.0072	0.057	2.78	20	888	167§	106.4§
2	0.0134	0.098	4.80	19	830	153	97.5
3	0.0161	0.152	6.89	20	755	138	87.9
4	0.0159	0.132	7.57	18	700	125	79.6
5	0.0221	0.161	9.09	19	630	111	70.7
6	0.0248	0.166	9.27	18	625	111	70.7
7	0.0279	0.187	10.6	14	546#	93	59.2

* Overall mean temperature was 10.1°C; DO, 8.0 mg/l, and pH, 7.93

† Percentages based on dry weights

‡ Weighted mean based on number of survivors in each replicate

§ Values were omitted from regression analysis summarized in Table 55

\# Values are significantly different from mean of controls according to Dunnett's procedure (two-tailed; $P < 0.05$)

TABLE 55. LINEAR REGRESSION ANALYSIS OF THE TOXICANT DOSE RESPONSE CURVES EXPRESSING TERMINAL MEAN DRY WEIGHT IN MG (Y) AND PERCENTAGE TERMINAL MEAN DRY WEIGHT NORMALIZED TO CONTROLS (Y) AS A FUNCTION OF LOG TOXICANT CONCENTRATION IN MG/L (X)

	Linear regression analysis*					
	Mean dry weight			Normalized percent mean dry weight		
Toxicant(s)	α	β	R^2	α	β	R^2
			Weight on log HCN			
HCN	-5.053	-8.481	0.9594	-56.70	-95.17	0.9595
Cr-HCN	-6.387	-8.853	0.8469	-71.75	-99.40	0.8468
HCN	-3.590	-7.762	0.9067	-32.81	-71.13	0.9060
Zn-HCN	-5.876	-10.01	0.7332	-53.79	-91.78	0.7331
HCN	-134.4	-165.3	0.9014	-85.71	-105.4	0.9018
NH_3-HCN or total N	-157.0	-162.8	0.9100	-100.3	-103.8	0.9096

TABLE 55. Continued

Toxicant(s)	Linear regression analysis*					
	Mean dry weight			Normalized percent mean dry weight		
	α	β	R^2	α	β	R^2
	Weight on log X - second toxicant					
Cr	3.996	-3.583	0.7992	44.83	-40.23	0.7991
Cr-HCN	4.720	-3.395	0.8987	52.95	-38.12	0.8986
Zn	5.938	-9.798	0.7789	54.47	-89.90	0.7779
Zn-HCN	6.073	-9.021	0.8133	55.73	-82.70	0.8135
NH_3	18.24	-137.5	0.8466	11.63	-87.57	0.8459
NH_3-HCN	-39.67	-193.5	0.7891	-25.46	-123.5	0.7894
Total N	251.3	-131.5	0.8582	160.1	-83.77	0.8575
Total N-HCN	274.0	-170.7	0.9506	174.7	-108.9	0.9508

* Linear regression equation is $Y = \alpha + \beta(\log X)$

increased rate of phenol uptake in ammonia-phenol mixtures. They concluded that the rate of uptake and concentration of phenol in fish muscle is not influenced by elevated ambient ammonia levels nor is the urine flow rate increased for rainbow trout exposed to ammonia-phenol solutions. Therefore, no physiological bases could be found to account for the empirical, additive toxic action of ammonia and phenol. The effects measured in the binary mixture were those corresponding to either ammonia or phenol alone and the presence of one did not appear to enhance the effect of the other.

In the present study, it was thought that the toxic action of the toxicants investigated was dissimilar. However, adverse lethal effects are apparently not independent since each toxicant in our binary mixtures did not act to produce its own separate stress which, when predicting joint toxicity, can be summed according to existing multiple toxicity models. The interactive nature of toxicants studied was also a function of the particular response measured. Therefore, since it has not been established whether the adverse effects of toxicant mixtures on fish can be predicted from individual toxicant effects, a need still exists for development of a valid multiple toxicity approach to evaluate the toxicity of chemical combinations.

SECTION VII

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16. ABSTRACT

Acute and chronic toxicity of hydrogen cyanide to seven fish species and two invertebrates was determined in dynamic flow-through bioassays. The 96-hr LC50 concentrations of HCN for juvenile fish ranged from 57 µg/l for rainbow trout at 10°C to 191 µg/l for field stock fathead minnows at 15°C. The fry and juvenile fish were similar in their sensitivity to HCN with eggs the most tolerant stage. The 96-hr LC50 concentrations for invertebrates ranged from 176 µg/l for *Gammarus* to 2328 µg/l for *Asellus* at 18°C. Long-term tests conducted with fathead minnows demonstrated that the concentrations of HCN having no adverse effect on egg production was between 12.9 and 19.6 µg/l. Chronic tests with brook trout demonstrated that on the basis of spawning success the maximum acceptable toxicant concentration (MATC) was between 5.7 and 11.2 µg/l HCN. Long-term tests with bluegills showed that no spawning occurred at HCN concentrations as low as 5.2 µg/l. Chronic experiments with invertebrates demonstrated that the highest concentration of HCN having no adverse effect was between 29 and 40 µg/l for *Asellus* and between 16 and 21 µg/l for *Gammarus*. Experiments conducted with intermittent and sublethal diurnal exposure regimes demonstrated that adverse effects of early growth of the fathead minnow are lessened as the exposure period is reduced. Using the toxic unit approach, it also was demonstrated that the Zn-HCN and ammonia-HCN mixtures were more acutely toxic and the Cr-HCN mixture less toxic than what would be predicted from simply additive interaction. This report covers a period from November 1, 1973 to September 30, 1978.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS		b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Freshwater fishes	Dissolved oxygen	Predation	06C
Aquatic animals	pH	Exposure time	06F
Toxicity	Growth	Binary mixtures	06T
Cyanide	Reproduction	Intermittent exposures	
Hydrogen cyanide	Bioassay	Diurnal fluctuations	
Behavior			
Temperature			

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