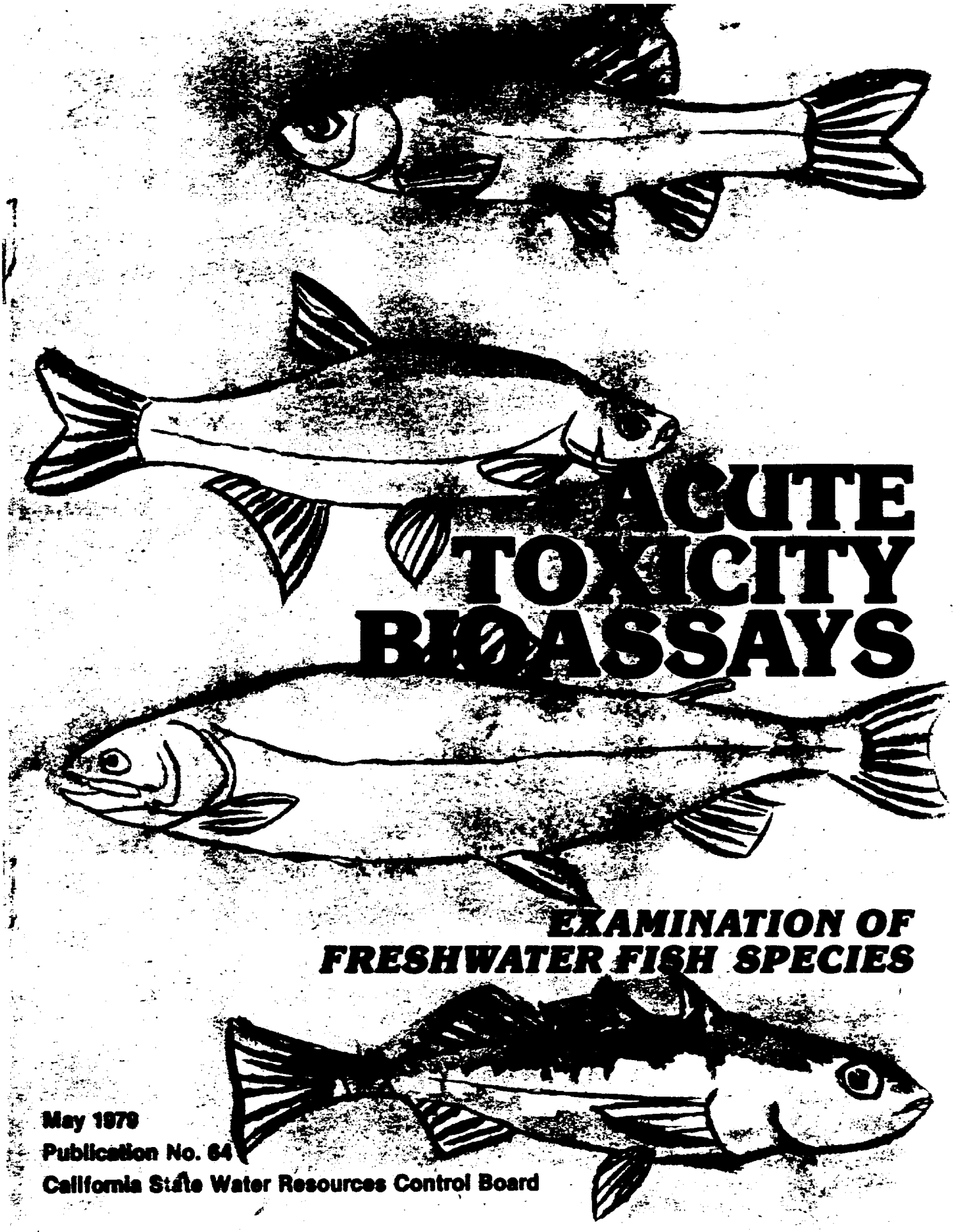

ACUTE TOXICITY BIOASSAYS

EXAMINATION OF FRESHWATER FISH SPECIES

May 1970
Publication No. 64
California State Water Resources Control Board

STATE OF CALIFORNIA
Edmund G. Brown Jr., Governor

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ACUTE TOXICITY BIOASSAYS

EXAMINATION OF FRESHWATER FISH SPECIES

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With A Statistical Analysis

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A Laboratory Report To The

CALIFORNIA STATE WATER RESOURCES CONTROL BOARD

•

May 1979

Publication No. 64

ABSTRACT

Four freshwater fish species were evaluated for use as standardized test fishes in the National Pollutant Discharge Elimination System (NPDES) compliance monitoring program within California. The project was arranged into two parts. Part I was directed to an extensive review and eventual selection of species having the greatest potential for routine applications in a statewide program. Species selected were rainbow trout (Salmo gairdneri), golden shiner (Notemigonus crysoleucas), fathead minnow (Pimephales promelas), and threespine stickleback (Gasterosteus aculeatus). Part II involved extensive testing of the selected species to reference toxicants, salt (NaCl) and sodium pentachlorophenate (PCP), at designated intervals throughout the year to determine reproducibility of results. Species response sensitivity and response consistency to the toxicants revealed a reversal phenomenon. Fathead minnow and golden shiner were more sensitive and consistent with NaCl than rainbow trout and stickleback. The PCP tests showed the exact opposite.

Based on considerations of fish availability, cost and biological response, rainbow trout and either fathead minnow or golden shiner are recommended as primary species for routine testing in cold water (14-18°C) and warm water (20-24°C) conditions, respectively, although some problems with year-round availability of proper-sized specimens need to be resolved. Routine test duration may be reduced to shorter time intervals on specific waste effluents. Routine toxicity tests should use 20-30 individuals per concentration; preferably with concentrations arranged to provide at least three partial responses.

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CONCLUSIONS

1. On the basis of their use in California and elsewhere, rainbow trout, golden shiner, fathead minnow and threespine stickleback were examined to determine their suitability to serve as standardized species for testing toxicity in California's National Pollutant Discharge Elimination System (NPDES) compliance monitoring program.
2. Rankings of the selected species, based on criteria of availability, logistics, costs, laboratory holding success and biological response to reference toxicants, indicate that (a) rainbow trout are best suited for routine testing of toxicity under cold water (14-18°C) conditions; and (b) fathead minnow or golden shiner are best suited for routine testing of toxicity under warm water (20-24°C) conditions.
3. Problems regarding use of stickleback in routine toxicity tests cast some doubt as to their usefulness in California's NPDES compliance monitoring program.
4. Rainbow trout exhibited greatest sensitivity and lowest variability in response to the organic reference toxicant pentachlorophenate (PCP). Fathead minnow and golden shiner exhibited greatest sensitivity and lowest variability in response to the inorganic reference toxicant sodium chloride (NaCl).
5. Trout, minnows, and shiners are, or could be, commercially available (cultured) in sufficient numbers at the right size to meet present and future demand. Stickleback also can be made available on the same basis, but must be secured from wild populations.
6. Use of 4-7 week old fathead minnow and golden shiner may improve testing.

7. Commercial costs of test fish have risen sharply since 1974. The 1978 average cost to conduct a single bioassay test using 60 fish (6 test concentrations at 10 fish per concentration) was $18.00 for rainbow trout, $7.00 for fathead minnow and for golden shiner, and $15.50 for stickleback.

8. The Department of Fish and Game (DFG) may have to provide test fish at cost if commercial supplies are unavailable or costs become unreasonable.

9. The logistics of fish supplier-to-user may be critical in some areas of the State.

10. Quality control at sources of test fish supplies may greatly improve test reliability and usefulness.

11. Routine test duration (96-h) may be reduced to shorter time intervals on a case-by-case basis.

12. Increasing the number of fish per test concentration tends to reduce the interval between confidence limits, thereby improving test reliability.

RECOMMENDATIONS

The following recommendations apply to the conduct of routine freshwater toxicity bioassays for NPDES compliance monitoring programs in California.

1. Rainbow trout should be standardized for testing toxicity of waste effluents under cold water (14-18°C) conditions.
2. Fathead minnow or golden shiner should be standardized for testing toxicity of waste effluents under warm water (20-24°C) conditions. When available, testing should be conducted with 4-7 week old juvenile fish. Use of spawning stock must be avoided.
3. Stickleback could be discontinued for use in routine bioassays. In the event there is strong pressure to continue use of this species, then it should be provisional and restricted to the San Francisco Bay area pending outcome of a study.
4. The DFG should provide test fish at cost if commercial supplies lack suitable quality, become unavailable, or costs become unreasonable.
5. The feasibility of establishing fish culture facilities to provide 4-7 week old fathead minnow and golden shiner year-round should be seriously examined. For this purpose, a pilot facility should be established at the Fish and Wildlife Water Pollution Control Laboratory (WPCL) and funded by the State Water Resources Control Board (SWRCB).
6. Sources of test fish should be located in several strategic areas in the State to better serve testing laboratories.
7. A test fish quality control program should be established to certify fish stocks or waters from which fish stocks are collected. The project should be assigned to the Bioassay Laboratory Certification Program at

WPCL as a primary responsibility. Funding for the program should be provided in the annual contract negotiated between the SWRCB Legal Division and the DFG.

8. Routine test duration should continue to be 96-h; however, based on case-by-case experience, the period could be reduced.
9. Routine tests should be conducted with 20-30 fish per test concentration (in 10 fish replicates); preferably with concentrations arranged to provide at least three partial responses.

INTRODUCTION

The use of fish bioassays is widely accepted as a valid approach to evaluate acute toxicity of waste substances (Doudoroff, et al. 1951; Sprague, 1969-1970; MacLeod, 1972; Martin, 1973). Properly designed and implemented, the test can be invaluable to regulatory agencies to monitor and help control disposal of toxic industrial and municipal wastes.

A major drawback in the use of toxicity bioassays for regulatory purposes has been the response variability of test organisms. Response variation--both interspecific and intraspecific--is inherent in the analysis of biological systems. Also, age, race, and prior acclimation can modify the response characteristics of test organisms and, thus, complicate interpretations. It has been suggested by Lennon (1957, Sprague (1969), and others that many of the problems associated with organism response variability can be corrected through standardization of test animals and test procedures.

The purpose of this study was to examine and evaluate several freshwater fish species commonly used in California for testing acute toxicity of wastes contained in freshwater effluents and to provide recommendations for their application in the State's National Pollutant Discharge Elimination System (NPDES) compliance monitoring program.

BACKGROUND

Toxicity Control Program

Acute toxicity bioassays have been used by California pollution control agencies to help regulate toxic industrial and municipal effluents since 1959. The early effort was confined to the northern part of the State and consisted of evaluating effluent impacts upon aquatic populations of a few selected rivers, estuaries and ocean areas. The California Department of Fish and Game (DFG), operating within its statutory responsibilities to protect aquatic life (Fish and Game Code Section 5650), requested that toxicity limitations be included in the requirements of waste discharge orders issued to dischargers by Regional Water Quality Control Boards (RWQCB). Toxicity limits of effluents discharged to state waters were usually expressed in terms of the 96-h median tolerance limit (TL_m).[1/] Compliance was determined largely by self-monitoring in which tests were conducted by the dischargers and results reported to the RWQCB. Enforcement actions, if necessary, were initiated at the discretion of the RWQCB.

The DFG recommended and revised conditions of bioassay testing based on the most current available information. There was little attempt, however, to standardize procedures or test organisms. Procedures were designed to accommodate specific situations, and test organisms were usually fishes indigenous to the receiving waters. Species commonly used were the threespine stickleback, rainbow trout, and king salmon (Oncorhynchus tshawytscha).

1/ TL_m is defined as the concentration of toxicant that will kill 50 percent of the test organisms at a specified time. This term is no longer in common usage in the current literature--being replaced by the term LC_{50}.

With impetus provided by enactment of the Federal Water Pollution Control Act Amendments of 1972 (PL 92-500), and direction and financial support by the U.S. Environmental Protection Agency (EPA) through Section 106 of the Act, the State Water Resources Control Board (SWRCB) initiated a new program which, in part, would establish the acute toxicity bioassay test as a routine procedure for NPDES permit compliance monitoring throughout the State.

Laboratory Certification Program

The program, initiated in 1973, was designed to provide reliable characterization of wastewater effluents by improving analytical performance of wastewater laboratories. The main thrust was to develop and implement a responsive Laboratory Quality Assurance (LQA) program which involved elaborate inspection, testing, and evaluation of facilities desiring certification. The new LQA program improved the existing State Department of Health Services (DHS) program which had been in effect under statutory authority since 1953. The early DHS program had been concerned only with chemical and microbiological laboratories and suffered from lack of sufficient funding. The new program mandates certification of all analytical laboratories that conduct analyses for NPDES compliance monitoring and provides sufficient funding to implement the accelerated effort. Funds are provided by the SWRCB through separate annual contracts with DHS and DFG. DHS conducts inspections of and evaluates chemical and microbiological laboratories, whereas DFG has responsibility for bioassay laboratories only. Authority to certify all laboratories rests solely with DHS. The DFG provides an advisory function to DHS, recommending actions relative to certification of bioassay laboratories.

The initial effort in the Bioassay Laboratory Certification program was to examine existing practices within bioassay laboratories. The examination revealed a general lack of standardized testing procedures, making it difficult to interpret and compare results. Most laboratory technicians were following procedures outlined in the most current edition of Standard Methods (APHA); however, due to lack of detail in the reference, procedures were broadly interpreted by the analysts. To correct the deficiency, standardized testing procedures designed specifically for California's NPDES compliance monitoring program were prepared by DFG (Kopperdahl 1976) and subsequently approved by the SWRCB as the official guide.

Test Species Selection

At the very onset of the Bioassay Laboratory Certification program it was believed that much of the variability observed in results of similarly conducted bioassay tests was directly attributable to the test organism. It was further believed that statewide acceptance of the test for NPDES compliance monitoring programs would depend upon the use of fish species which would provide both consistent and reliable results for a given set of conditions. Investigators evaluating toxicity in San Francisco Bay (Wilson and Hazel 1971; Esvelt et al. 1971; Brown and Beck 1972) had pointed out that species selection was critical to test reliability and proposed designating the golden shiner as the standardized bioassay test fish for California. In comparative tests, Wilson and Hazel (1971) found that golden shiners displayed greater sensitivity and more consistent results than were observed with the commonly used stickleback.

Considering the prospect of both expanded use of bioassay testing throughout California as an integral feature of controlling toxic waste disposal and the regulatory implications of test results, the SWRCB contracted with the DFG to examine and evaluate candidate freshwater fish species for applicability in the State's NPDES compliance monitoring program. The project was jointly developed by the SWRCB and DFG under Interagency Agreement No. 3-2-77 and was conducted at the DFG Fish and Wildlife Water Pollution Control Laboratory (WPCL) at Rancho Cordova.

Work was arranged into two parts. Part I (Feasibility Studies) was directed to an extensive review and eventual selection of freshwater fish species having the greatest potential for routine application in a statewide bioassay program. Part II (Toxic Response Studies) involved extensive testing of selected species with reference toxicants at designated intervals throughout the year in order to determine both the reproducibility of results on a seasonal basis and the appropriate test duration. Results of this study provide a rationale whereby the SWRCB and RWQCB's can objectively designate test fish species for use in NPDES compliance monitoring programs.

PART I - FEASIBILITY STUDIES

Methods and Results

The purpose of this part of the project was to assess the relative merits of freshwater fish species having greatest potential as a standardized test organism for use in acute 96-h static bioassays throughout California. To attain this objective several tasks were identified in Exhibit A, Interagency Agreement No. 3-2-77. For clarity, each task is restated and is followed by a presentation of the information acquired from this investigation.

Task 1

The following four fish species represent primary candidates for the California standard toxicity bioassay: (1) rainbow trout (Salmo gairdneri Richardson); (2) golden shiner (Notemigonus crysoleucas Mitchell); (3) fathead minnow (Pimephales promelas Rafinesque); and (4) threespine stickleback (Gasterosteus aculeatus Linnaeus). Describe the merits of each as a standard organism as well as less desirable features from known biological data. List other species considered potentially useful as standard test organisms and which might be the subject of future investigations.

Obtaining information about each of the primary test species as well as other potential test species required an extensive review of the biological literature. This was accomplished by physical review and computer-assisted search of the published literature dating back to 1917. This survey revealed several potentially useful test organisms, including both fishes and invertebrates, as well as production and testing methods. This information has been compiled, indexed, and retained at WPCL.

The characteristics of candidate species were compared with developed criteria to identify those organisms useful for bioassay. Other investigators

(Adelman and Smith 1976) have also described selection criteria which closely correspond to qualities deemed necessary by this investigation. The selection criteria of this investigation were:

1. availability of quantities of test organisms of desired size;
2. availability of quality test organisms;
3. current use in bioassay evaluations; and
4. consistent response to toxicants when tested under similar conditions.

A system employing a scale of 1 to 3 was used to rate each species (Tables 1 and 2). Summation of these rating scores described the ability of a species to meet this criteria set and thus the species suitability for bioassay evaluation. This numerical assignment, based on published information, could be changed as new information about a particular species became available. The objective was to consider other species as well as those specified in the Agreement.

The first criterion evaluated available information concerning the potential production of each species. It was assumed that any species which was currently in commercial production (i.e., hatcheries, farm ponds, etc.) fully satisfied the criterion. Those species which had sufficient information on production methods available in the literature but were not currently under production were rated intermediate. Species which could not be commercially cultured or species for which only limited information was available did not meet the criterion.

The second criterion evaluated the potential for quality control in the production of test organisms. To fully meet this criterion an organism must have been

TABLE 1. Evaluation and comparison of seven freshwater fish species to meet basic test-species criteria.

Species	Production	Quality	Current Use	Total
Rainbow Trout	1	1	1	3
Golden Shiner	1	2	1	4
Desert Pupfish (*Cyprinodon macularius*)	2	1	3	6
Fathead Minnow	1	1	2	4
Threespine Stickleback	3	3	1	7
Red Shiner (*Notropis lutrensis*)	1	2	3	6
Bluegill (*Lepomis macrochirus*)	1	2	3	6

Rating System: 1 = fully meets criterion

2 = partially meets criterion

3 = does not meet criterion

TABLE 2. Evaluation and comparison of four common freshwater invertebrate species to meet basic test-species criteria.

Species	Production	Quality	Current Use	Total
Crayfish *Procambarus* sp.	1	1	3	5
Freshwater prawn *Macrobrachium* sp.	1	1	3	5
Water Flea *Daphnia* sp.	2	1	2	5
Mayfly *Ephemeroptera* sp.	2	1	3	6

Rating System: 1 = fully meets criterion
2 = partially meets criterion
3 = does not meet criterion

successfully cultured. Those species which had not been cultured but appeared to offer reproductive characteristics conducive to such culture (ease in collection and propagation of eggs) were rated intermediate. Those species with little or no information on culturing did not meet the criterion.

The third criterion rated the past and present usage of the organism for laboratory bioassay testing. Organisms which had been used in bioassay testing fully met the criterion. Those with little use over the years were intermediate, and those with no use did not meet the criterion. This criterion was designed to measure potential acceptance of the particular species in laboratory testing programs.

The fourth criterion, consistent response to toxicants, was the main purpose of the experimental evaluation portion of this project. Discussion of this criteria will be presented in Part II.

Task 2

List hatcheries, locations, or other areas in the State where the four primary species can be obtained. Determine quantities of suitable size for bioassay at each location. Describe size (length and weight) and age each month of availability.

Two surveys (1974 and 1978) were conducted to identify commercial suppliers of fish for bioassay purposes (Table 3). The 1974 survey, primarily mail contact with licensed commercial fish breeders, indicated that only 11 facilities were interested and capable of supplying suitable freshwater fish for bioassay. We must assume most operators were simply not interested since only 64 of 160 questionnaires were returned.

TABLE 3. Commercial sources of freshwater fish species available for bioassay testing in California. Surveys conducted in 1974 and 1978.

Name and Location	Species	Availability [a/]	Survey Year
Alex Fish Company, San Rafael	RT, GSH, STB, FHM	12	1974,78
Blue Mt. Trout Hatchery, Railroad Flat	RT	4	1974
Chico Game Fish Farm, Chico	FHM	12	1978
Echerdt, R., Los Molinos	GSH, FHM	12	1978
Elk Grove-Florin Catfish Farm, Sacramento	FHM	12	1978
Fish Breeders, Niland	GSH, FHM	12	1974,78
Ft. Bragg Trout Farm, Ft. Bragg	RT	5	1978
Fullerton Tropicals, Fullerton	GSH	12	1978
Golden State Fisheries, Escalon	GSH, FHM	12	1974,78
Hildebrand's Mt. Lassen Trout Farm, Red Bluff	RT	12	1974,78
Ponderosa Bait, Nevada City	GSH	12	1974
Sierra Live Bait, Wilton	GSH	12	1974,78
Smith's Trout Farm, Mt. St. Helena	RT	3	1978
Sticklebacks Unlimited, El Cerrito	STB, GSH, FHM	12	1978
Sweetwater Hatchery, Descanso	RT	5	1978
Triple B-C Ranch, Yermo	FHM	2	1974
Troutmere Hatchery, La Honda	RT	4	1974,78
Van Dyke, Harlan M., Pleasant Grove	RT	12	1974
Whitewater Trout Co., Whitewater	RT	4	1974,78

Species Coding

Rainbow Trout (RT), Golden Shiner (GSH), Fathead Minnow (FHM), Stickleback (STB)

a/ Availability of proper size for bioassay evaluation (number of months available per year).

The 1978 telephone survey indicated that 15 (a 36% increase) commercial suppliers are interested and capable of providing bioassay fish. Currently there are seven sources for rainbow trout, golden shiner, and fathead minnow and two sources for stickleback. The rainbow trout sources are conventional hatchery facilities, whereas the golden shiner and fathead minnow sources are bait-fish farms. Stickleback are all collected from the "wild".

Most suppliers (10) are located in northern California, and all four candidate species are available through them. Commercial suppliers in southern California are capable of providing all species except stickleback.

Rainbow trout of desired size are available 3 to 5 months of the year (March-July); however, one supplier indicated trout of suitable size could be provided throughout the year. The other species are always available.

Information provided from the 1974 mail survey on size (length and weight) availability of the various species was insufficient. Respondents either did not specify any size or had difficulty providing a monthly size profile. Those listed in Table 3 indicated in 1974 they could supply fish of the size necessary for a continuous testing program. This was verified verbally in 1978.

Task 3

Calculate the current monthly and annual use of fish by certified bioassay laboratories, and describe regional patterns of demand. Determine whether each of the candidates can be obtained at present in sufficient numbers in each region to be able to supply the demand throughout the year if it were chosen to be the single bioassay organism. Describe any situations where extensive transport may be necessary.

Results of two telephone surveys conducted in 1974 and 1978 to determine usage of bioassay fish for NPDES compliance monitoring programs show substantial increases (Table 4). The 1974 survey, involving contact with fifty certified bioassay laboratories,[2/] indicated that approximately 128,000 fish of various species were used statewide. The most commonly used fish (96%) was the threespine stickleback. This reflects practices in the San Francisco Bay area where the San Francisco Bay RWQCB specified stickleback use for bioassays and requires a number of dischargers to conduct toxicity tests. King salmon and golden shiner were the only other species of consequence, and their combined contribution was only about 4% of the total.

The 1978 survey indicated that bioassay fish usage had more than doubled, and species other than stickleback were being used in greater numbers. Again, stickleback dominated, principally because of their use in the San Francisco Bay area. Although stickleback usage over the four-year period had increased by 41%, they constituted only 63% of the 1978 total as compared to 96% in 1974.

The overall increase in fish usage reflects an increase in waste discharge toxicity requirements, some of which are now being established on southern California dischargers. Approximately 240 waste dischargers are currently required to conduct toxicity bioassays.

2/ Non-public health laboratories approved for water and waste analyses by the California Department of Health--1974 listing.

TABLE 4. Estimated annual quantity of freshwater test-fish species used by certified bioassay laboratories relative to NPDES permit compliance for calendar years 1974 and 1978.

	1974		1978	
Species	No. Labs.	No. Fish	No. Labs.	No. Fish
Stickleback	45	122,500	49	173,000
King Salmon	2	4,000	2	11,000
Golden Shiner	2	1,000	8	37,000
Rainbow Trout	1	100	8	31,000
Fathead Minnow	0	0	13	21,500
Channel Catfish *Ictalurus punctatus*	0	0	1	900
TOTALS	50	127,600	68 [a/]	274,400

a/ In 1978, 68 certified laboratories conducted NPDES bioassays, some using more than one species.

Bioassay laboratory personnel have indicated difficulty in obtaining rainbow trout of sufficient size during the late summer months and, therefore, have turned to other species. Since the principal effort of the few commercial trout suppliers is to raise fish for food and sport and because the bioassay market is only incidental to their operations, year-round commercial supply may not be practical or even probable.

Potential does exist, however, for year-round production of suitably-sized fish (100-300 per lb) at state facilities. Presently, DFG hatcheries produce rainbow trout of suitable size 7 to 8 months each year. With sufficient planning and coordination, this could be lengthened to 12 months (Estey, DFG, pers. comm. 1977). As indicated previously, candidate species other than rainbow trout appear to be available at any time of the year in sufficient numbers to meet current and, most likely, future demand. Transportation of test fish from suppliers to laboratories is not a technical problem.

Task 4

List the average price or cost of obtaining each species asked by private suppliers for the size normally used for bioassay. Describe any significant variable which influences pricing. Note the influence of geographic location, season and suppliers upon pricing.

Prices of bioassay test fish have greatly increased during the past four years for most species as indicated by surveys conducted in 1974 and 1978 (Table 5). The range of costs depends upon the quantity ordered and the distance shipped. As might be expected, the cost per unit decreases with corresponding increases in quantity ordered. The most significant increase has been with rainbow trout where the cost has risen from an average of $.07

TABLE 5. Cost comparisons (per fish) of commercial sources for candidate freshwater test-fish species based on 1974 and 1978 surveys.

	1974			1978		
Species	No. Sources	Cost Range	Average Cost	No. Sources	Cost Range	Average Cost
Rainbow Trout	5	---[a]	$.07	7	NC[b] - $1.05	$.30[c]
Golden Shiner	5	---	.04	7	.02-.30	.12
Fathead Minnow	1	---	.04	7	.02-.30	.12
Stickleback	1	---	.23	2	.12-.30	.26
TOTAL	12			23		

a/ Data not available.

b/ Available only at the hatchery at no charge.

c/ Most costs quoted were in range of $.12-.18.

each in 1974 to $.30 each in 1978. Except for very high priced fish from one dealer ($1.05 each), most rainbow trout can be purchased in the range of $.12 to $.18. One southern California operator will supply trout free of charge FOB hatchery site. The hatchery, however, is in a remote location and use of this source is expected to be minimal. Interestingly, stickleback prices (Ca $.25 each) have not risen sharply during this period.

The average cost of fish to conduct a single, standard, 10-fish-per-concentration, LC_{50} bioassay test (total of 60 fish) in 1978 is $18.00 for rainbow trout (range $7.20 to $63.00), $7.20 for fathead minnow and golden shiner (range $1.20 to $18.00), and $15.50 for stickleback (range $13.80 to $18.00).

Trout can be produced at considerably less cost at DFG hatcheries, although costs have also risen. In 1974 DFG trout production costs were approximately $0.02 per fish (Bruley 1975) increasing to $0.05 in 1978 (Bruley 1978). These costs are substantially less than commercial costs. King salmon costs are not included since the only source of these fish is from a federal facility which provides fish for limited use in specialized cases.

Task 5

Describe optimum procedures for transporting each of the species, rating their relative hardiness in transport, recommended maximum distance of travel and time in transport.

The well being of test fishes during their transport from supplier to laboratory depends on maintaining stress factors at minimal levels. Effective procedures must, therefore, be designed along this concept.

Major stress factors during fish transport include thermal shock, water hardness shock, toxic effects of waste product build-up, dissolved oxygen stress, handling stress, and disease susceptibility. All candidate species can be transported from supplier to areas of need without great difficulty providing they are not crowded, dissolved oxygen is maintained above 5 mg/l, low temperature is maintained, and transit time does not exceed eight hours.

Task 6

Estimate the extent to which each of the present fish stocks can be increased at each location with present facilities, personnel and environmental conditions.

Analysis of written and telephone communication with suppliers of the different species revealed increased supplies of stocks could be developed. Interested suppliers thought they could provide test species either with their existing facilities or would expand to meet the demands if economically feasible. There would be a lag time of approximately one year to set up production. The two Bay area suppliers have expanded their facilities over the past few years and probably will continue to do so as long as the economic incentive is present. The species to be used and numbers required by laboratories for each geographic area must first be determined before details can be planned.

Discussion

Review of the literature on organisms used in bioassay evaluations revealed several potentially useful species for standardization purposes. The four species originally selected for the response evaluation, however, were

considered the appropriate choices for detailed study as they have been used extensively in bioassay testing programs in California and elsewhere. Three of the species, rainbow trout, golden shiner, and threespine stickleback have been used in California for many years. Although not used prior to 1974 in California, the fathead minnow was added for several reasons: The EPA has been closely examining this species; local supplies were available; and because its biological characteristics compared favorably to the three other selected species. As time and need permits, other species, including invertebrates, should be examined for applicability in routine bioassay evaluations. One of the more promising fish species deserving consideration is the red shiner because of its biological characteristics and commercial availability (Table 1).

The Survey of commercial sources revealed that any of the four species selected could be provided in sufficient numbers to meet current demand. Fathead minnow, golden shiner, and rainbow trout are available from suppliers throughout the State. Stickleback are available only from two suppliers who collect them from wild populations.

Most suppliers are located some distance from principal areas of use, and this may be one of the more critical aspects of an expanded program. It would be desirable to locate sources of supply near areas of high use. This could be accomplished by establishing holding facilities at strategic locations from which certified laboratories could draw. The current program, however, does not suffer greatly from lack of centralized fish stocks since procedures for shipping small batches of fish have been developed with some suppliers.

With the exception of rainbow trout the other candidate species are available at the desired size throughout the year. Most commercial suppliers of rainbow trout indicated these fish can be provided only from 3 to 5 months of each year. By contrast, trout of suitable size are available at one of DFG's hatcheries up to 8 months, and perhaps this could be extended to a full year with proper planning.

Costs of bioassay fish from commercial sources have risen sharply during the past four years, and this trend can be expected to continue. Current costs of fish to conduct a single LC_{50}-bioassay vary between $1.20 and $63.00 although most costs appear to fall within a range of $7.20 to $15.50. The most economical fish to test, in terms of commercial costs, are golden shiner and fathead minnow.

Costs of rainbow trout reared at DFG hatcheries have also risen, however, they are considerably less than commercial costs. For example, the 1978 cost for 60 fish used in a routine, 10-fish-per-concentration, LC_{50} test is approximately $3.00, excluding shipping costs.

Comparing costs of State-reared fish with those of commercial suppliers calls attention to an important consideration. Since the State requires these bioassays to be conducted, it is obligated to encourage use of the best quality test fish for the least cost and which can be supplied in the most efficient and practical manner. It has been assumed that commercial suppliers will be able to provide good quality test fish at reasonable cost. However, if these suppliers are unable to provide low-cost, quality fish, then the State may have to assume the role of supplier. Under this circumstance, fish

would be provided to users at production and transport costs. This alternative is not necessarily desirable and would be explored only if commercial participation was lacking or inadequate.

The quality of supplied test fish, in terms of sensitivity response (LC_{50} value) and consistency of the response over time is the most important aspect of this project. This will be the focus of Part II.

PART II - TOXIC RESPONSE STUDIES

Objective and Scope

The fourth criterion identified in Part I (Feasibility Studies) regarding test fish selection--consistent sensitivity response to toxicants when tested under similar conditions--was the basis for the laboratory experimental program. The objective of this part of the study was to examine selected fish species to identify those that display consistent mortality to reference toxicants as determined by 96-hour (h) static bioassays. The four selected test species (rainbow trout, golden shiner, fathead minnow, and threespine stickleback) were compared under conditions paralleling those used by other laboratories conducting routine bioassay evaluations. The basic laboratory program consisted of replicated 96-h bioassay testing of the four species in two toxicants at six time intervals over a one-year period. In addition, selected industrial and municipal wastes were tested. Standard bioassay procedures followed those described by Kopperdahl (1976).

Information obtained during this part of the study also provided an opportunity to examine and evaluate the length of time required to conduct short-term, routine standardized tests and the number of test animals per concentration necessary to provide a reliable estimate of toxicity.

Methods and Materials

Sources of Fish Stocks

The test fish used during the laboratory investigations were obtained from commercial sources identified in Part I (Feasibility Studies). Initially,

TABLE 6. Sources [a/] and costs of individual test fishes used in the reference toxicant testing program.

Test Series	Rainbow Trout		Golden Shiner		Fathead Minnow		Stickleback	
I	D	$0.10	C	$0.03	A	$0.015	B	$0.22
II	D	0.10	C	0.03	A	0.015	B	0.22
III	E	0.02	A	0.02	A	0.015	B	0.22
IV	D	0.10	F	0.04	A	0.015	B	0.23
V	D	0.10	A	0.02	A	0.015	B	0.23
VI	D	0.10	A	0.02	A	0.015	B	0.23

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a/ Code name of facility

A Golden State Fisheries
B Alex Fish Company
C Sierra Live Bait
D Hildebrand's Mt. Lassen Trout Farm
E Department of Fish and Game, American River Trout Hatchery
F Ponderosa Bait

plans were made to purchase fish stocks from different locations to minimize test bias from any one geographic source. During this project, however, commercial sources of fathead minnow, threespine stickleback, and rainbow trout became limited--altering the basic plan. A single source of fish (test lot) was used for each testing period (series), and intermixing of different stocks of the same species was always avoided. The suppliers of fish used in the study and costs of fish are indicated in Table 6.

Transport of Fish Stocks

Initially, fish were transported in insulated containers by truck. During transport, air was supplied continuously to each container by a battery-operated pump. After the second reference toxicant test series, the mode of shipment was changed. With the exception of rainbow trout, fish were packaged in plastic bags (1000 fish in 16 liters of water) which were inflated with compressed oxygen gas and sealed with a twist tie. The fish were then transported by truck to the laboratory. Using the latter method the fish in-transit mortality was held to less than 0.1%. Trout were loaded in 125-liter containers (1000 per container) with approximately 96 liters of water and ice. During transport, tanks were aerated with pumps, and temperatures were maintained at 10-12^{o}C.

Laboratory Handling of Fish Stocks

Fish arriving at the laboratory were allowed to acclimate to holding temperature by placing the shipping containers, with fish, into large (523- to 1,064-liter) holding tanks. The fish were released into the holding tank after the water temperature had equalized (usually in less than one hour).

This procedure reduced stress to the fish during initial laboratory handling. Each holding tank was aerated and supplied with fresh water from the American River. The water was filtered and passed through an ultraviolet treatment unit (Refco Purifier Model RL-120-95), before entering the fish holding tanks to control any diseased organisms originating from the water supply.

The long holding period of individual test fish lots (30-days) and the density of the fish stocks in each holding tank (2,000-3,000) required a vigorous ongoing treatment program against infectious diseases. The DFG Fish Pathology Laboratory recommended a prophylactic treatment which could be applied routinely to incoming lots of fish (Appendix 1). This treatment controlled most external sources of infection through exposure of the fish to various chemical treatments. The routine treatments were periodically evaluated by comparing mortality of treated groups of fish against similar groups which did not receive treatment. Untreated fish rarely survived the second week of holding due to massive disease outbreak; Columnaris was the most prevalent. Treated fish were less diseased, and usually sufficient numbers survived the entire 30-day holding period.

Initially, an attempt was made to hold fish in water near the same hardness as that of their collection point since there was concern that this factor might modify their toxicant sensitivity. The attempt was abandoned, however, when within forty-eight hours all fish groups experienced extremely high mortalities (10-30%). It was then decided to maintain the fish stocks in flow-through tanks, eliminating the inherent adverse problems of static systems such as accumulation of uneaten food and waste products.

A diet which could be fed to all species was required to eliminate possible bias due to different nutritional formulations. Additionally, this diet had to be: (1) readily available in small quantities, (2) nutritionally balanced with the constituents remaining unaltered during reasonable storage time, (3) readily accepted by test fish species with little or no conditioning period, (4) non-fouling in the holding tank water, and (5) reasonably priced. Examination of many different diets resulted in the selection of Tetra-Min(R) (standard diet) staple food. Except for rainbow trout, this diet was maintained throughout the test program. During the initial stages of the study Tetra-Min(R) did not appear to satisfy the nutritional needs of rainbow trout. Consequently, beginning with the fourth reference toxicant test series (November-December 1975) and continuing through the remaining testing, this diet was replaced by Purina Trout Chow(R), a diet specifically formulated for rainbow trout.

Dilution Water

Chemically formulated hard or soft water was used as dilution for each of the tests (Table 7) based upon formulations presented by Kopperdahl (1976). A 1900-liter mixing tank was used to mix de-ionized American River water with the formula constituents. After mixing, hardness and alkalinity were adjusted as necessary.

For ease in moving large volumes, test dilution waters were pumped from the mix tank via overhead lines to the two testing rooms. The remote controlled pump permitted an operator to easily fill test tanks or toxicant mixing

TABLE 7. Chemical characteristics of test dilution waters used during reference toxicant testing program.

Parameter	Soft Water	Hard Water
Alkalinity (mg/l)	30-35	110-120
Hardness (mg/l)	40-48	160-180
pH	7.2-7.6	7.6-8.0
Formulation Quantities (mg/l)		
$NaHCO_3$	48	192
$CaSO_4$ $2H_2O$	30	120
$MgSO_4$	30	120
KCl	2	8

tanks to appropriate volumes. Fathead minnow and golden shiner were evaluated in hard water, and rainbow trout and threespine stickleback were evaluated in soft water throughout the entire reference toxicant testing program.

Basic Test Procedures

Stocks of test fish were held in large flow-through tanks for one week after drug treatment to ensure a healthy stock (Blaxhall 1972). Groups of 250 fish were removed from the holding tanks, transferred to 114-liter acclimation tanks, and held at a density of 1-2 gm fish/liter. Hardness and temperature of the acclimation water were adjusted to match the test conditions before introduction of fish stocks. Acclimation water was circulated through filters to maintain the integrity of the water (Spotte 1970).

After a 3-day acclimation period, groups of fish were selected for replicate bioassays (two aquaria per concentration) using 20-liter glass aquaria as test tanks. Appropriate quantities of toxicants were added to each aquaria and then filled to volume by the overhead dilution water delivery system. In cases where only small volumes were needed, a large graduated cylinder was used to achieve the appropriate dilution level (Steele and Rectenwald 1976).

The fish were handled as little as possible to minimize physical injury. The groups to be acclimated each week were measured volumetrically by weight using the scale method (Leitritz and Lewis 1976). This method eliminated removing too many fish from the holding tank and minimized netting stress. Surplus fish removed from the holding tank and not used for experimentation were discarded. This was done to eliminate, from subsequent tests, any

possible bias that might occur through a modified response to toxicants by the net stressed individuals.

All nets used for transferring fish were first rinsed in tap water to remove any attached material, sterilized in a 1% Wescodyne(R) bath for 30 minutes, and then rinsed for several hours in clean water. This procedure was established to prevent possible cross contamination of fish stocks and subsequent disease problems.

Just before introducing test fish, air lines were provided to each tank and air delivery rates were adjusted to a slow bubble. Dissolved oxygen (D.O.) levels in each tank were monitored, and, once above 5.0 mg/l, 10 test fish were added to each tank. D.O., pH, and temperature were recorded daily. Rainbow trout and stickleback were tested at 14-18°C and golden shiner and fathead minnow were tested at 20-24°C. The two temperature ranges were maintained in separate constant-temperature rooms.

Individual bioassay tests consisted of 6 test concentrations (including control) with 10 fish per concentration conducted over a 96-h time interval. Each test was replicated. Test mortalities were removed and recorded at 24-h intervals. Representative specimens were weighed (W) to the nearest .01 gram and fork length (L) measured to the nearest millimeter. Condition factors (C.F.)[3/] were determined according to the equation in Leitritz and Lewis (1976):

3/ Condition factor (or coefficient of condition) is the ratio of length to weight and serves as an index to the condition of fish (degree of well being).

$$C.F. = \frac{W \times 10^5}{L^3}$$

Reference Toxicant Tests

Standard reference toxicants were selected prior to the experimental evaluations. Based upon preliminary research conducted by Adelman and Smith (1976), sodium chloride (NaCl) and sodium pentachlorophenate (PCP) were selected as the inorganic and organic reference toxicants, respectively. Their investigation demonstrated these chemicals best meet requirements of a reference toxicant according to the following criteria: (1) minimum variability in response of normal fish, (2) rapid detection of abnormal fish by a deviant response, (3) rapid lethal action, (4) simple analytical technique, (5) usable in static or flow-through bioassays, and (6) general ease of laboratory handling. The formulation procedures of the two toxicants are described in Appendix 2.

A 15 parts per thousand (ppt) NaCl stock solution (100% concentration) was used to prepare dilutions for test concentrations. It was observed, however, following the initial week of stickleback testing that the 100% concentration did not always provide sufficient mortality to derive valid LC50 estimates. Consequently, additional quantities of NaCl were added to the stock solution for the stickleback tests. The stock solution was also increased for some of the rainbow trout tests. Concentrations prepared from the augmented stock solution are expressed as percentages above 100%. For example, 135% test concentration is equivalent to 20.25 ppt NaCl. The PCP base strength (100% concentration) was established at 0.5 mg/l. No adjustment of this standard was necessary.

The reference toxicant testing program was arranged into six series; each consisting of a maximum of four consecutive weekly replicated tests involving each of the four fish species and two reference toxicants (Table 8). Test series involving less than four weekly tests were attributed to problems of fish availability. The initial week of testing was designed to establish a general toxicity estimate (LC50) and define the limits of no response (zero mortality) and total response (100% mortality) from which a better estimate could be obtained in subsequent testing. Each consecutive week toxicant concentrations were redefined, as necessary, to provide a more exact toxicity estimate than the value established the previous week. The procedure protocol is shown in Table 9.

Municipal and Industrial Effluent Testing

As an additional comparison of test species response, standard 96-h replicate tests were conducted using municipal and industrial effluents. The municipal waste consisted of grab samples collected from the post-chlorination basin at selected waste treatment plants in the Sacramento area and returned to the laboratory in Cubitainers(R). Initially, the chlorine level was determined chemically. Based on these results, a standard test series was arranged to bracket the reported LC50 of chlorinated effluent for the species to be tested. This procedure was later modified since the majority of test fishes died within the first hour. Subsequent procedure included a preliminary test followed by the standard definitive test (Kopperdahl 1976). Agitation of the test solutions was minimized to prevent loss of volatiles.

TABLE 8. Schedule of testing of candidate freshwater test-fish species to NaCl and PCP reference toxicants during the period May 1975 through May 1976.

Series	Week	Date	Rainbow Trout	Golden Shiner	Fathead Minnow	Stickleback
I	1	5/14 - 20	X		X	
	2	5/21 - 27	X		X	
	3	5/28 - 6/3	X	X	X	X
	4	6/4 - 10	X	X	X	X
	5	6/11 - 17		X		X
	6	6/18 - 24				X
II	10	7/16 - 22	X	X	X	
	11	7/23 - 29	X	X	X	X
	12	7/30 - 8/5		X	X	X
	13	8/6 - 12			X	X
III	15	8/20 - 26	X	X	X	X
	16	8/27 - 9/2		X	X	X
	17	9/3 - 9	X	X	X	X
	18	9/10 - 16	X		X	X
IV	27	11/12 - 18	X	X	X	
	28	11/19 - 25	X	X	X	X
	29	11/26 - 12/2	X	X	X	X
	30	12/3 - 9	X	X	X	X
	31	12/10 - 16				X
V	34	1/31 - 1/6				X
	35	1/7 - 13				X
	36	1/14 - 20	X			X
	37	1/21 - 27	X		X	X
	38	1/28 - 2/4	X	X	X	
	39	2/5 - 11	X	X	X	
	40	2/12 - 18		X	X	
	41	2/19 - 25		X		
VI	47	4/1 - 7				X
	48	4/8 - 14				X
	49	4/15 - 21			X	X
	50	4/22 - 28	X	X	X	X
	51	4/29 - 5/5	X	X	X	
	52	5/6 - 12	X	X	X	
	53	5/13 - 19		X		

TABLE 9. Standard procedure for determining weekly LC50 values during reference toxicant testing program.

Week	Procedure	Example
1	Establish LC50 estimate	Set up Broad Range Tests (% test concentration; 100, 56, 32, 18, 10, 0) by volume of stock solution. Results 74.8% Estimate LC50
2	Redefine concentrations for improved LC50 estimate	Set up Narrow Range of Test Concentrations (100, 87, 75, 65, 56, 0) Results 74.5% Estimate LC50
3	Either repeat Week 2 concentrations or redefine LC50 for improved LC50 estimate	Repeat Week 2 Concentrations Results 79.2% Estimate LC50
4	Same as Above	Same as Above Results 79.2% Estimate LC50

The industrial waste was characterized by a high concentration of NaCl (20%) along with minor unquantified constituents. The grab sample was collected from the effluent line and transported in Cubitainers to the laboratory. A preliminary test preceded the definitive 96-h test.

Data Processing

The LC50 estimates presented in this study are given in terms of percent toxicant concentration relative to the stock solutions. All individual test results were carefully scrutinized for discrepancies prior to deriving toxicity estimates. Tests with major discrepancies, such as control mortalities exceeding 20% or drastic non-linear mortality responses, were designated invalid (Inv.) and discarded from further consideration.

Toxicity estimates of acceptable data were derived by two methods. The preferred method involved a customized program designed for use in a programmable calculator (Tektronix Model 31) which derived LC50 values and 95% confidence limits by probit analysis (Finney 1971). Test results with control losses of 20% or less were adjusted by Abotts Correction Factor (Finney 1971).

The alternative method, straight-line graphical interpolation (Doudoroff et al. 1951), was used only in situations where the probit analysis program failed to accept data. This occurred when tests yielded only two mortality response points, generally 0 and 100%. Results from this approach were not corrected for control mortalities of 20% or less nor were confidence limits established.

Toxicity estimates from the two methods of analysis provide approximately similar values and, therefore, are considered comparable. However, since more data points are considered in probit analysis, greater reliance can be placed on the results obtained from this approach.

In addition to deriving toxicity estimates for individual tests, mortalities of these tests were pooled (by species) to provide various combined LC50 values and 95% confidence limits. Weekly (combined replicates) LC50 and 95% confidence limits values were derived by pooling mortality observations of each replicated test. Series values were derived by pooling mortality observations of all tests conducted during a series. Combined series values represent pooled data of all tests conducted during the year-long program.

Results

Reference Toxicant Tests

Test results for NaCl and PCP reference toxicants are tabulated in Appendix 3 (A,B), respectively. Data are arranged by species for each series and include LC50 estimates and confidence limits (when possible) for both individual and pooled test results. Confidence limits which exceeded the range of concentrations tested are identified as not reported (NR). LC50 values obtained by straight-line graphical interpolation are identified by an asterisk. References to individual test work sheets (maintained at WPCL) are provided in Appendix 3C. Length, weight, and condition factor of fish used in the tests are shown in Appendix 4. Length/weight data were not collected for any of the candidate species during Series I and II, nor for stickleback during the remaining testing.

A total of 355 tests were conducted during this part of the study; 25 were judged invalid due to unacceptable losses in control groups or significant non-linear toxic response. The results of these 25 tests were excluded from consideration in pooled data evaluation. Another 29 tests were originally scheduled but were not conducted due primarily to shortages of acceptable quality fish. This occurred mostly in the latter stages of the earlier series when disease developed rapidly in laboratory holding facilities and affected both quantity and quality of test organisms. Holding mortalities during each series were recorded (Table 10). Fifteen tests failed to yield specific LC50's due either to insufficient (less than 50%) or excessive (greater than 50%) mortality in test concentrations (Appendix 3). The LC50's of these tests are reported as being greater (>) or less (<) than the highest or lowest concentration tested, respectively. These data are included in the pooled evaluations.

Summaries of test results and procedural problems are presented below. The information is arranged by species and includes graphical displays of data summarized from Appendix 3.

Rainbow Trout. Excessive mortality in controls invalidated results of two NaCl tests and four PCP tests and prevented further testing during weeks 3 and 4 in Series II. Sufficient numbers of trout were also unavailable during Series III (week 2) and VI (week 4) of the NaCl tests. Other than these situations, trout held exceptionally well in the laboratory.

The 96-h LC50 distribution pattern over the six test periods is shown in Figures 1 and 2. Figure 1 compares only individual 96-h LC50's with time.

TABLE 10. Holding and acclimation mortalities of candidate freshwater test-fish species in flow-through facilities during period May 1975 through May 1976.

Test Series	Lot	Mortality	Rainbow Trout	Golden Shiner	Fathead Minnow	Stickleback
I & II	No.		2,000	2,000	2,000	1,000
		Holding	40	138	5	302
		Acclimation	0	58	1	25
		Total (%)	2	10	< 1	33
III	No.		1,500	2,000	2,000	2,000
		Holding	26	0	5	557
		Acclimation	26	0	1	36
		Total (%)	3	0	< 1	30
IV	No.		2,000	2,000	2,000	2,500
		Holding	2	1	10	189
		Acclimation	0	0	1	0
		Total (%)	< 1	< 1	< 1	8
V	No.		2,500	3,000	3,000	2,500
		Holding	1	60	2	649
		Acclimation	5	0	0	42
		Total (%)	< 1	2	< 1	28
VI	No.		3,000	3,000	3,000	3,000
		Holding	4	520	46	260
		Acclimation	14	60	21	0
		Total (%)	< 1	20	2	9
Totals	No.		11,000	12,000	12,000	11,000
		Holding	73	719	68	1,957
		Acclimation	24	118	24	103
		Total (%)	< 1	7	< 1	19

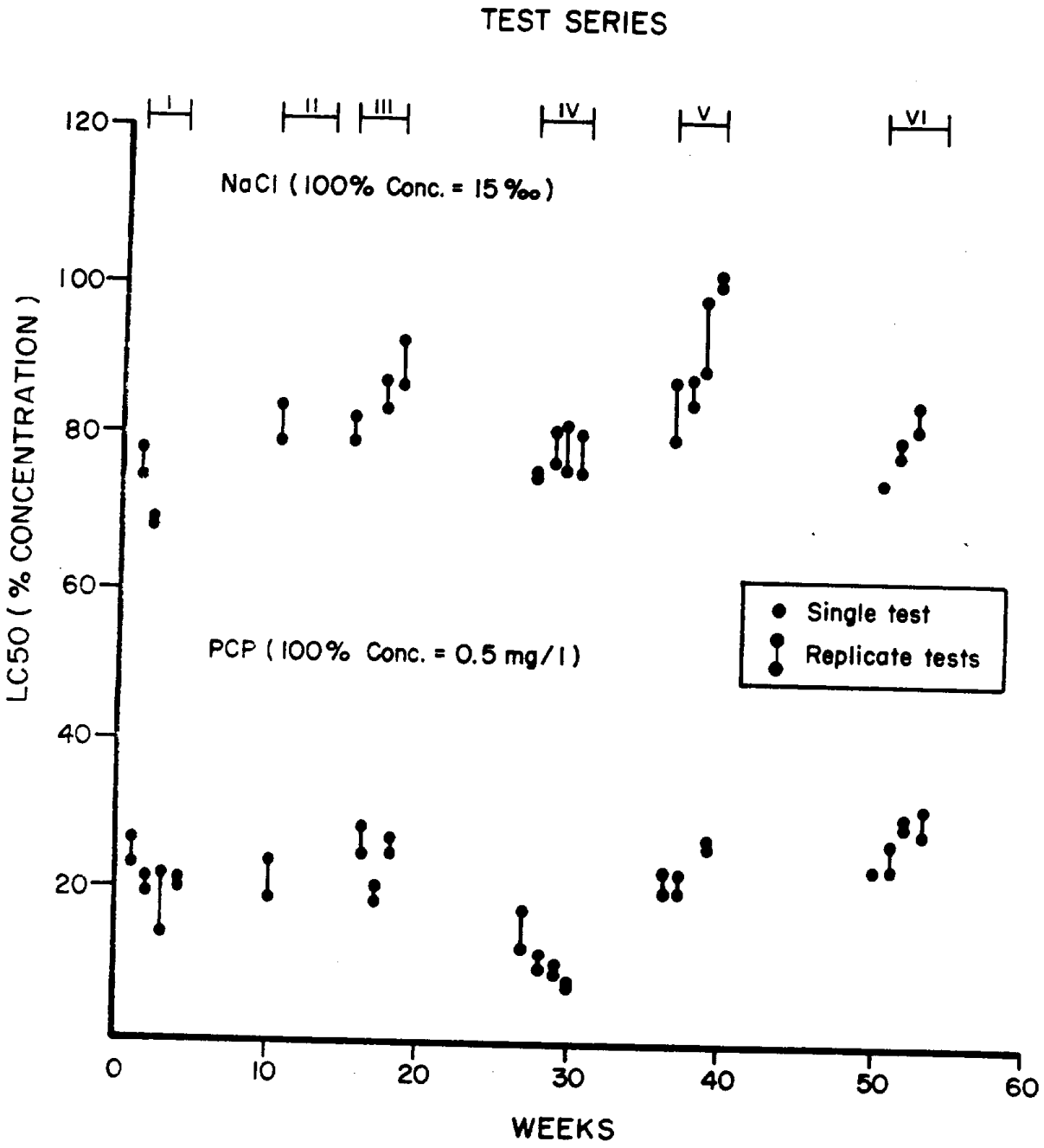

FIGURE 1. Response of Rainbow Trout to Reference Toxicants; Comparison of LC50 Values Derived from Individual 96-h Static Bioassay Tests Conducted from May 1975 through May 1976.

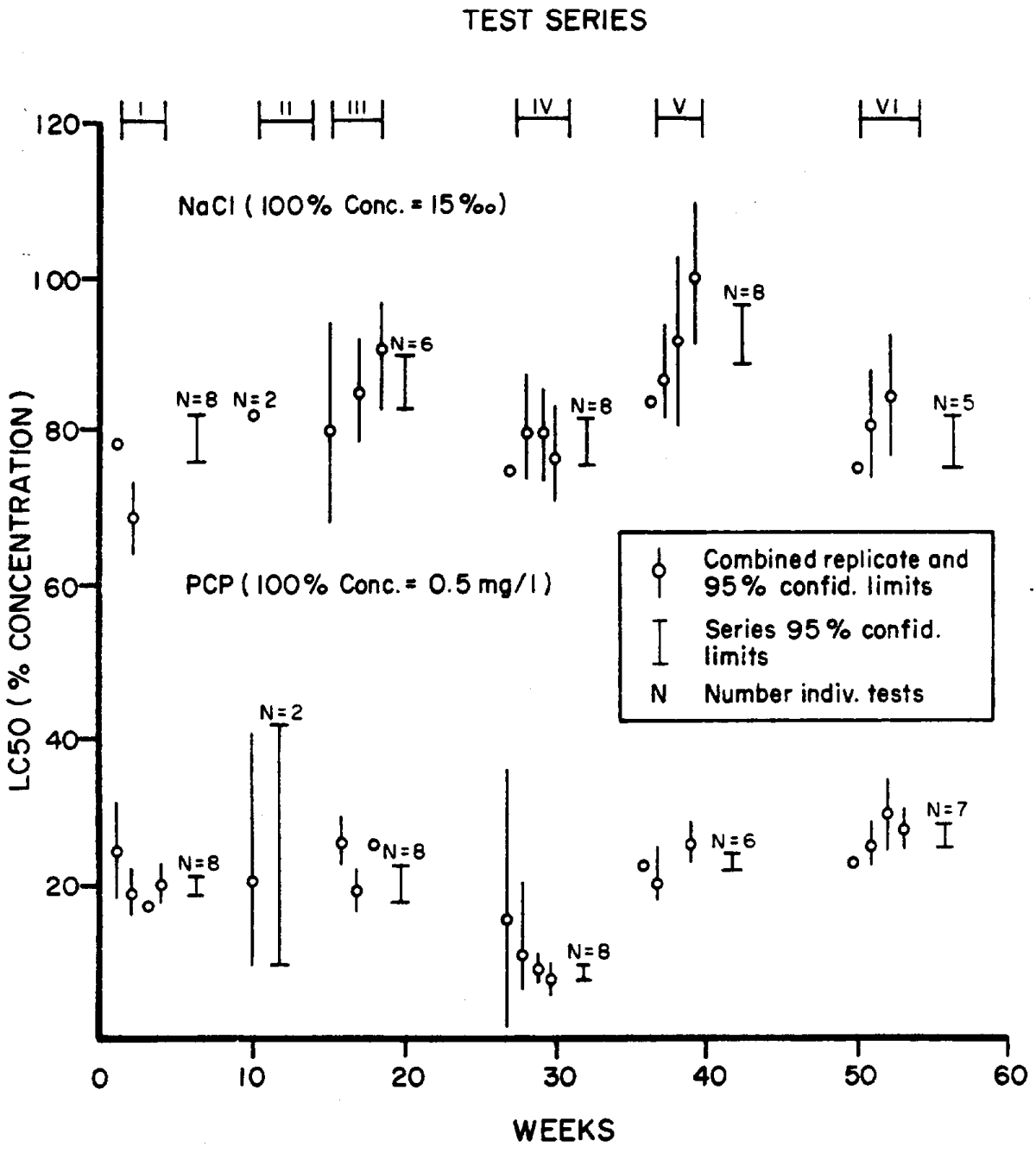

FIGURE 2. Response of Rainbow Trout to Reference Toxicants; Comparison of Combined Replicate 96-h LC50 Values, 95% Confidence Limits for Combined Replicates, and Series 95% Confidence Limits.

Figure 2 compares 96-h Lc50's and 95% confidence limits of combined replicated tests with corresponding series 95% confidence limits.

Mortality respon e of rainbow trout to NaCl varied moderately throughout the testing period (Figure 1). The relative toxicity of each successive series increased or decreased in an alternating pattern. Eighty percent of the observed LC50 values fell within a range of 70-90% NaCl reference toxicant concentration. Agreement between replicates was fair, differing by no more than 9% concentration and averaging 3% concentration. The concentration of the NaCl stock solution was increased during weeks 1, 3 and 4 of Series III and week 4 of Series V to provide an adequate testing range for deriving LC50 values.

Rainbow trout were more sensitive (exhibited LC50 at lower PCP concentrations) and demonstrated a more consistent response to PCP than other species tested. Approximately 80% of the LC50 values fell within a range of 18-31% concentration. Agreement between replicate LC50 values was the best for rainbow trout among all species tested. Replicate LC50 values never differed by more than 8% PCP concentration and the average difference was a 2% concentration.

The low LC50 values observed in Series IV may be attributed to condition of the test fishes. This is supported by comparing Series IV LC50 values (Appendix 3B) with corresponding condition factors (Appendix 4) for this lot of test fish (Figure 3). It is readily apparent that during the 4-week testing period there was a progressive decline in condition factor attended by a progressive increase in fish response sensitivity (low LC50 value).

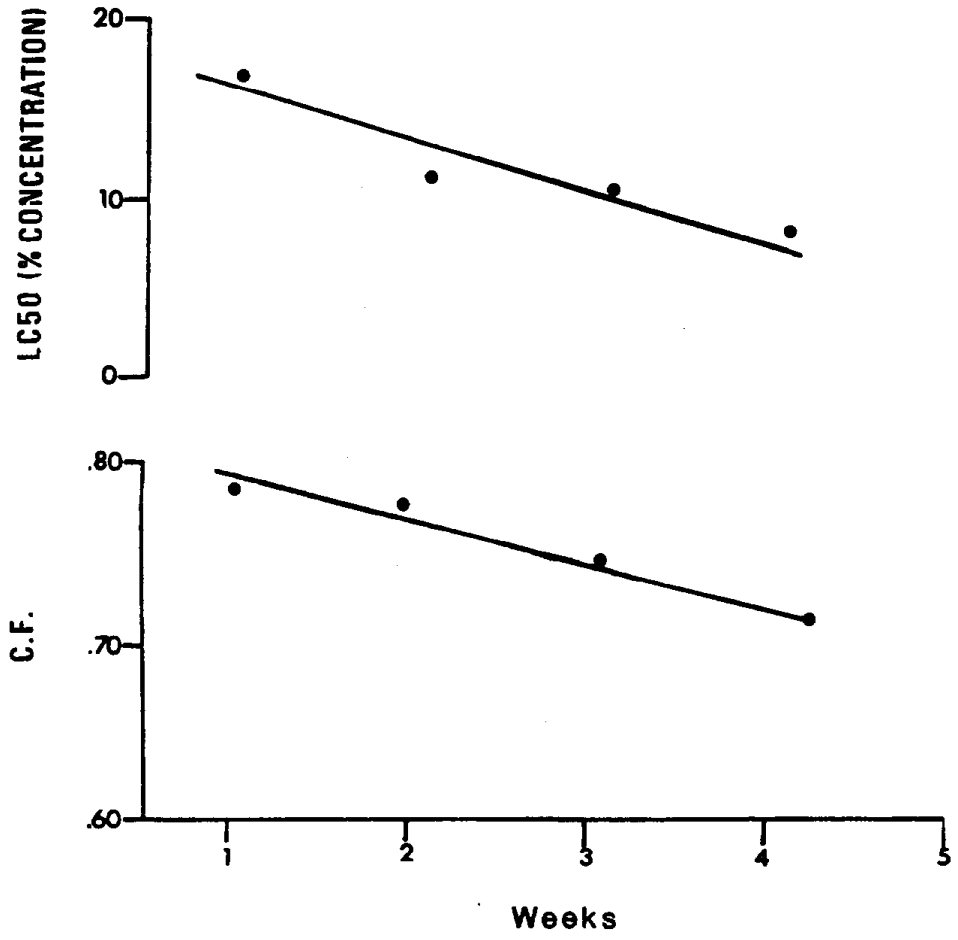

FIGURE 3. Comparison of Condition Factor (C.F.) and Sensitivity (LC50) of Rainbow Trout to PCP During Series IV Reference Toxicant Testing.

Also, the individuals in this test lot were the smallest (and perhaps youngest) of all the trout tested. Sensitivity of trout to many toxicants is size related with the smaller sizes generally being the most sensitive. Either of these factors, condition or size, could explain the results observed in Series IV PCP tests. Interestingly, the response of Series IV NaCl tests did not follow the trend observed in the PCP tests (Figure 1).

Confidence limits of individual tests were often quite broad (Appendix 3) and caused primarily by lack of sufficient, progressive partial responses.[4/] Consequently, individual test results were routinely pooled to provide desired additional partial response data. This approach simplifies display of representative values and permits easy comparison of combined replicate and series results (Figure 2). The dominant feature of this figure (and others to be shown later) is the effect of pooled results on combined replicate and series confidence limits. The series 95% confidence limits are essentially uniform throughout the year for each toxicant (PCP Series II excepted) with PCP responses displaying the narrowest range. The narrow confidence limits depicted in PCP Series I, III, IV, V, and VI reflect combined results of from four to eight tests, most of which closely agree with one another. The broader limits indicated by PCP Series II, for example, is usually indicative of either few tests or a lack of close agreement among tests or both.

4/ In this context, partial responses are defined as mortalities greater than 0 and less than 100% in any test concentration.

Golden Shiner. Test results with this species were rather disappointing for Series I and II. Abnormal mortalities during holding periods (Table 10) prevented scheduled tests in either toxicant during the fourth week of Series I and II (Appendix 3). Also, commercial unavailability of fish prevented the fourth week of NaCl Series III testing. Seven scheduled tests were not conducted due to both of these problems. In addition, excessive mortalities in controls invalidated results of 14 tests: six NaCl exposures and eight PCP exposures. Three PCP tests failed to produce sufficient mortality to yield an LC50 value.

Mortalities of golden shiner held during the spring months (Series I, II, and VI) are believed to be associated with the species' reproductive season. Sexually active specimens, which constitute much of the testing stock during this season, are apparently in a weakened condition, and their tolerance to additional stresses of holding and testing is quite low. During their reproductive period, golden shiner are highly susceptible to disease, a condition which may be aggravated further by culture practices of fish breeders. Bait-fish operators (primary source of this test species) tend to crowd and handle their stocks excessively. Crowding and handling are major sources of stress which can promote disease outbreak.

Toxicity parameters of individual and combined tests are shown in Figures 4 and 5, respectively. This species demonstrated relatively consistent sensitivity to NaCl; LC50 values range from 61-81% test concentration (Figure 4), and approximately 95% of the observations fall within a range of 70 to 80% test concentration. This consistency results from excellent

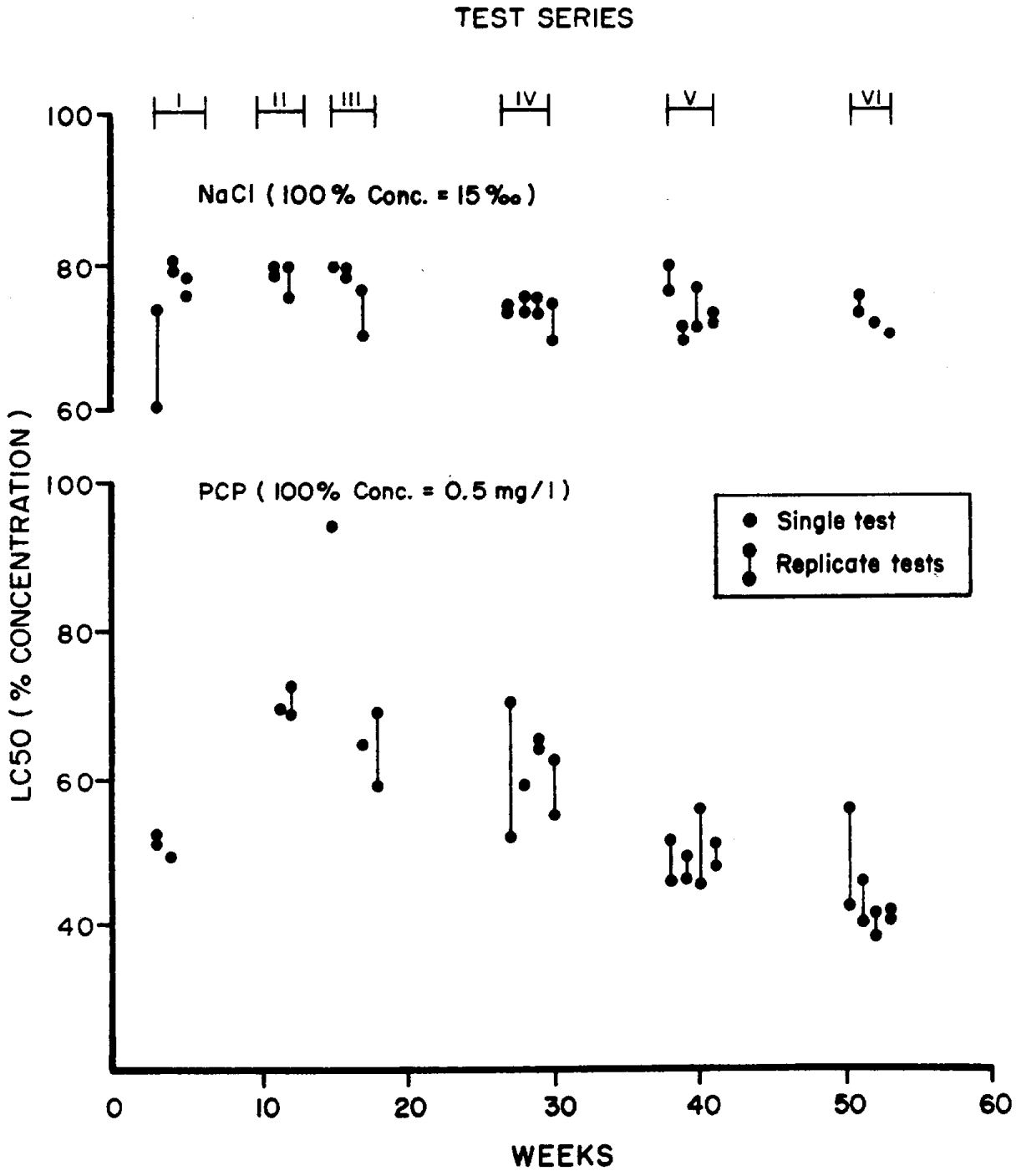

FIGURE 4. Response of Golden Shiner to Reference Toxicants; Comparison of LC50 Values Derived from Individual 96-h Static Bioassay Tests Conducted from May 1975 through May 1976.

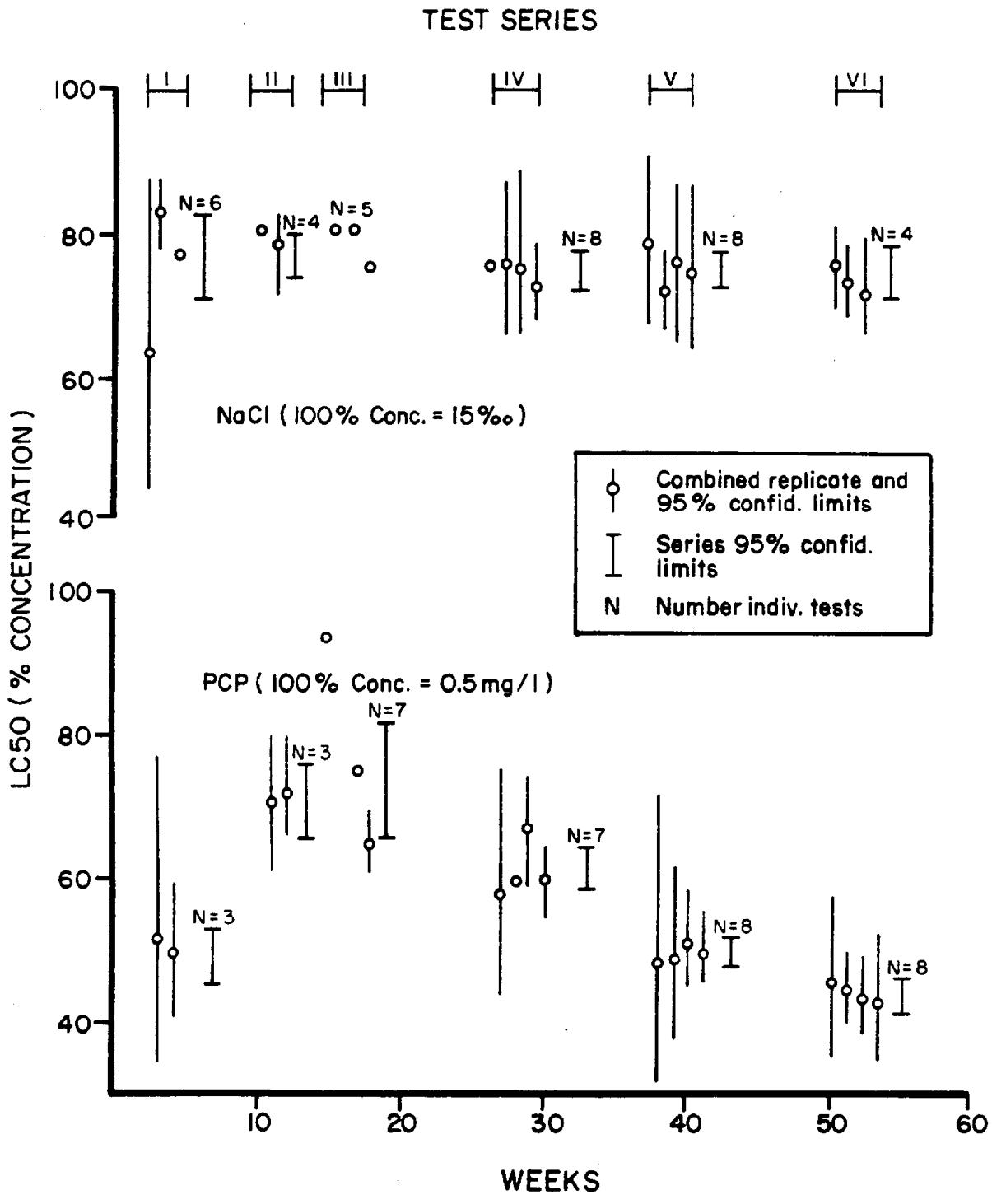

FIGURE 5. Response of Golden Shiner to Reference Toxicants; Comparison of Combined Replicate 96-h LC50 Values, 95% Confidence Limits for Combined Replicates, and Series 95% Confidence Limits.

agreement between replicates in which differences averaged about 3% concentration. The greatest disparity between replicates (14% concentration) occurred in Series I and may reflect the abnormal mortality problem that was prevalent at the time. The combined replicate 95% confidence limits are broad; however, the series confidence limits (Figure 5) are narrower and about the same as those for rainbow trout (Figure 2). Confidence limits for Series III are not available due to lack of data points.

By contrast, the response of golden shiner to PCP was not consistent. An initial upward trend (sensitivity decrease) between Series I and II was followed by a steady decline (sensitivity increase) through Series VI (Figure 4). The overall LC50 range was 39-95% test concentration of which approximately 80% of the observations fell within a range of 41-66% test concentration. Differences between replicates averaged about 6% concentration; the greatest disparity was 19% concentration. The 95% confidence limits of combined replicates are rather broad during the first week of all the series and generally become narrower in succeeding weeks (Figure 5). The series 95% confidence limits varied considerably throughout testing.

Fathead Minnow. The testing program for this species was largely successful and problems were minimal. Only one NaCl and two PCP tests failed to yield sufficient mortality to produce a valid result. In these cases, the test fish survived the highest concentration through the 96-h period even though the same concentrations produced contradictory results during previous tests.

Fathead minnows were easily handled in the laboratory, and the stock used appeared to be of good quality. Holding mortalities were the lowest of all four species (Table 10). Most importantly, no tests were invalidated due to unacceptable losses in the control as these never exceeded 20%.

Response to the two toxicants (Figure 6) was similar to those of the golden shiner tests (Figure 4). Toxicant sensitivity of fathead minnows ranged from 66-85% test concentration for NaCl (Figure 6). Approximately 95% of the NaCl observations fell within a range of 70-81% test concentration, indicating excellent consistency between replicates; differences never exceeded 7% concentration and averaged 2% concentration. NaCl combined replicate and series 95% confidence limits (Figure 7) are distributed similarly to the golden shiner tests (Figure 5). Combined replicate limits are rather broad whereas series limits are narrower with some variation between testing periods.

The response of fathead minnows to PCP (Figure 6) was similar to the pattern of the golden shiner tests (Figure 4). There was a similar decrease in sensitivity between Series I and II followed by a slight but steady increase in sensitivity through Series VI. Sensitivity of fathead minnow ranged from 38-87% test concentration, and approximately 80% of the observations fell within the range of 50-70% concentration. Differences between the LC50's estimates for replicates were as high as 28% concentration and averaged 6% concentration. The 95% confidence limits of combined replicates were broad and varied considerably (Figure 7). Series 95% confidence limits were more uniform and consistent than the golden shiner PCP tests (Figure 5).

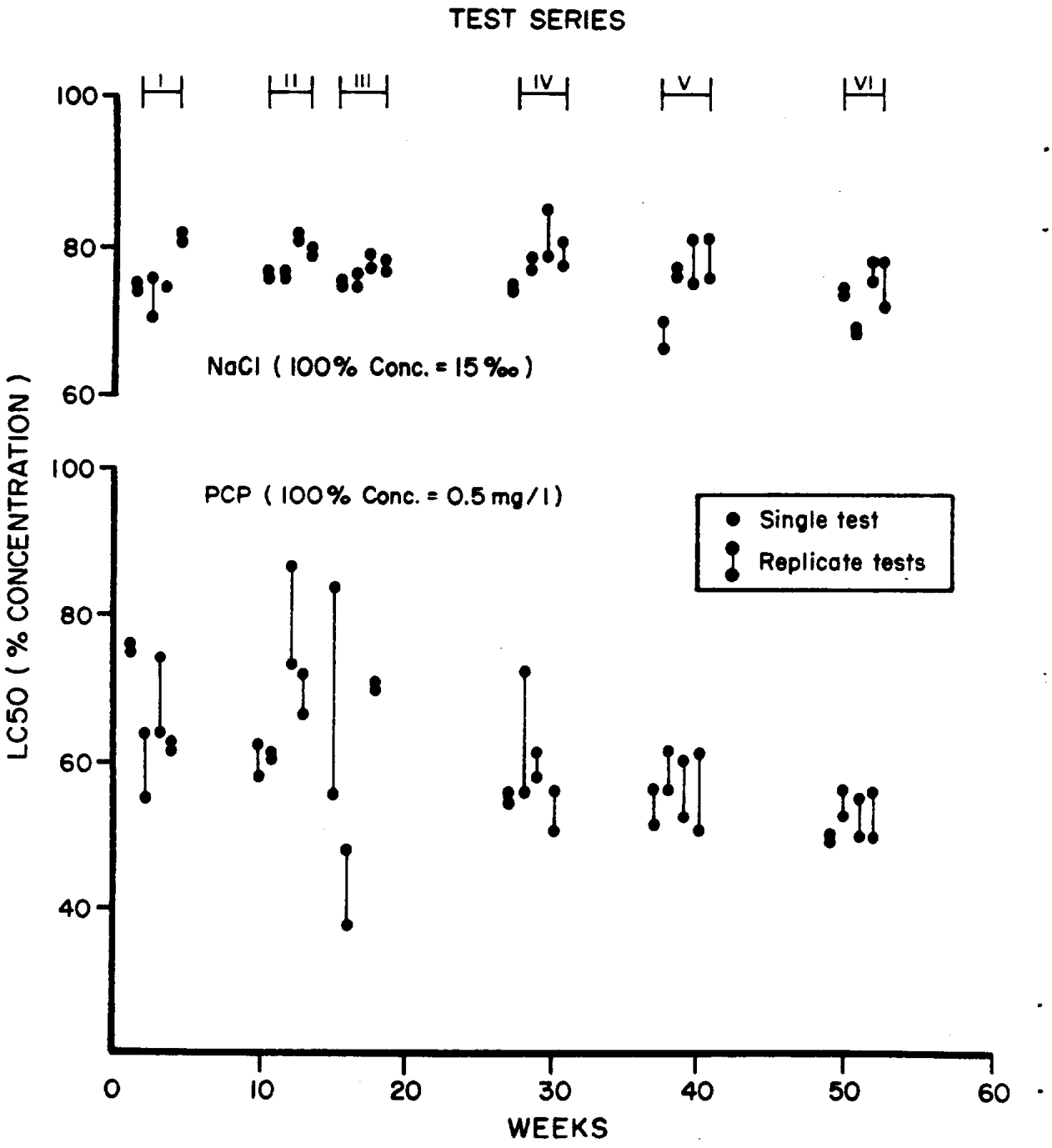

FIGURE 6. Response of Fathead Minnows to Reference Toxicants; Comparison of LC50 Values Derived from Individual 96-h Static Bioassay Tests Conducted from May 1975 through May 1976.

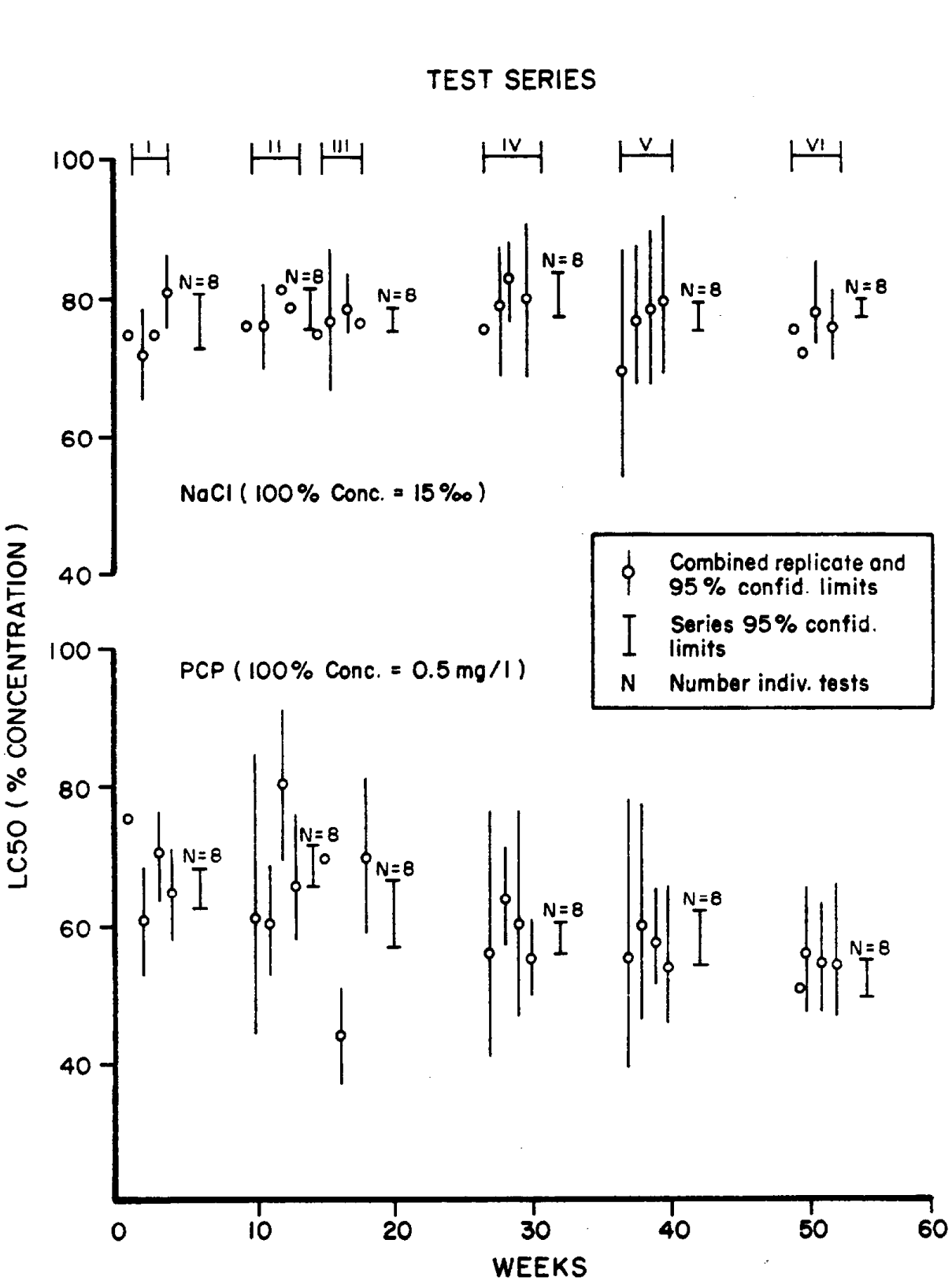

FIGURE 7. Response of Fathead Minnow to Reference Toxicants; Comparison of Combined Replicate 96-h LC50 Values, 95% Confidence Limits for Combined Replicates, and Series 95% Confidence Limits.

Threespine Stickleback. Because of its ability to tolerate high levels of NaCl, this species had to be tested with higher NaCl concentrations than was originally anticipated in order to produce valid results. This was done for all tests following the first week of Series I.

Most of the tests produced acceptable data. The only scheduled tests not conducted were during the final week of Series II when insufficient fish were available for testing. Five tests were invalidated due to excessive mortality or major non-linear mortality response. Three other tests failed to produce an LC50 value either because mortality exceeded 50% in all concentrations tested (1 test) or because mortality did not exceed 50% in any of the concentrations tested (2 tests).

Holding mortalities for this species were consistently the highest of all the species tested (Table 10). Early in the study, we observed that stickleback could not be held for long periods in American River water (WPCL water source), presumably due to this water's relative low total dissolved solids content (approximately 35 mg/l). The incidence of mortality increased in proportion to length of holding time, indicating a corresponding general weakening of individuals. It is believed that the holding problem was directly related to the species inability to properly osmoregulate (adjust body salt balance) during extended periods of exposure to American River water. Stickleback typically inhabit waters of low or moderate salinity although some populations exist in freshwater environments. It is suspected that fish used in this study were collected from diverse locations and environments along the coast (particularly in the vicinity of San Francisco Bay), combined,

and sold as single lots. Upon transfer to WPCL, the fish were able to cope initially with American River water, but gradually, over time, osmoregulatory failure caused losses among the most susceptible individuals. Holding practices of San Francisco Bay area bioassay laboratories lend support to this view. Kopperdahl (DFG, pers. commun.) reports that laboratories which successfully hold stickleback over extended periods use water of approximately 15 o/oo salinity.

In addition, some of the holding losses may have been caused by parasitic infections. Severe cases of the common stickleback tapeworm (Schistocephalus solidus) were often observed in individuals of the test lots. Although precise records were not maintained, it was estimated that tapeworms were apparent in approximately 5% of the test population. The incidence of mildly infected individuals remains unknown (there is no visible evidence of this condition), but conceivably it was quite high. Since routine prophylactic treatments used in the program were ineffective for control of any endoparasites, including S. solidus, use of heavily infested individuals was avoided when possible. In a few situations some were knowingly used due to short supply of fish. Therefore, these problems (osmoregulation failure and parasitic infections), either singly or in concert, are believed to be the prime contributors to the poor stickleback holding record at WPCL during this study.

Stickleback response to the two toxicants (Figures 8 and 9) was similar to that of rainbow trout (Figures 1 and 2). The response to NaCl was erratic whereas a more uniform response was observed in the PCP tests. NaCl response

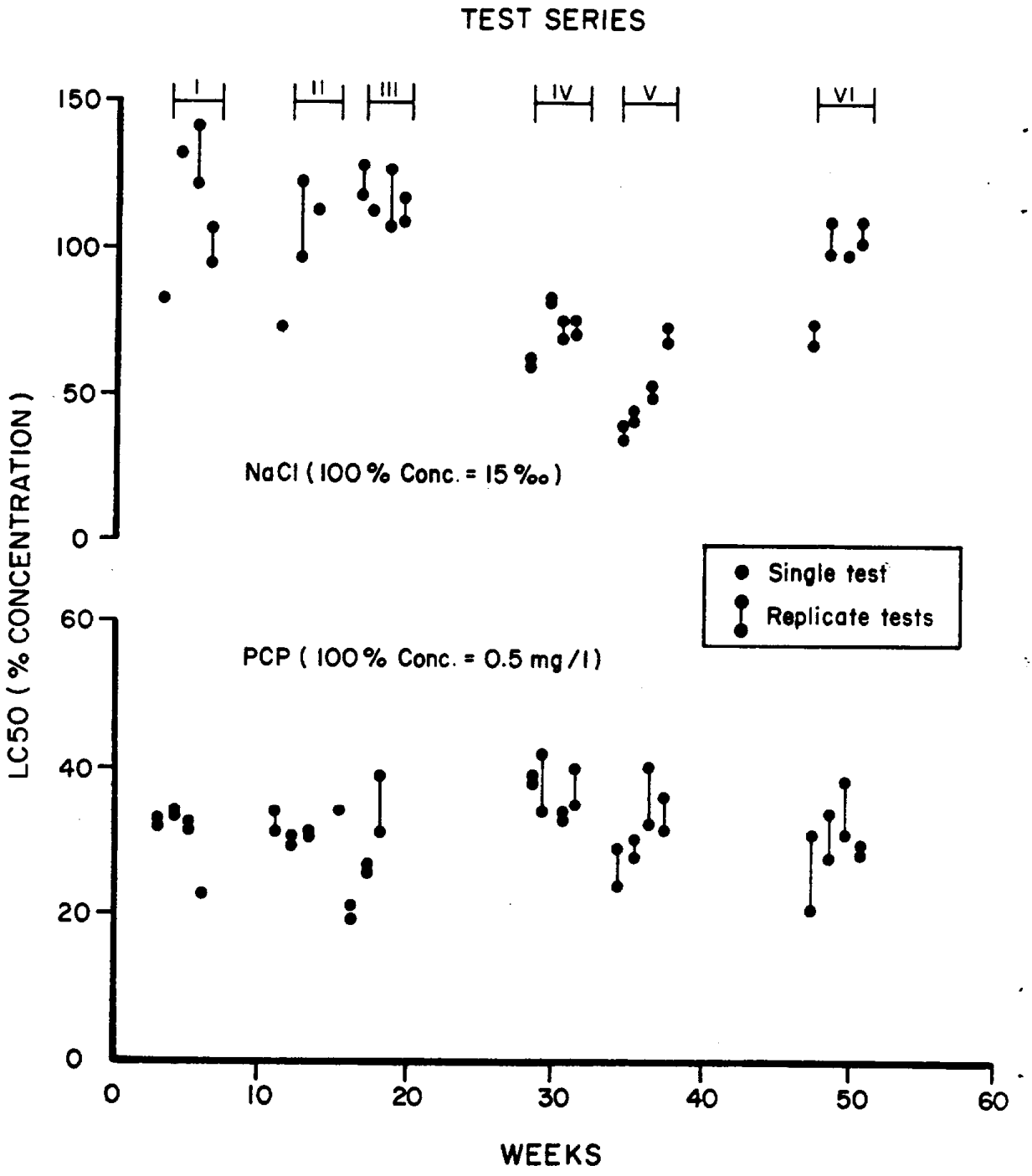

FIGURE 8. Response of Stickleback to Reference Toxicants; Comparison of LC50 Values Derived from Individual 96-h Static Bioassay Tests Conducted from May 1975 through April 1976.

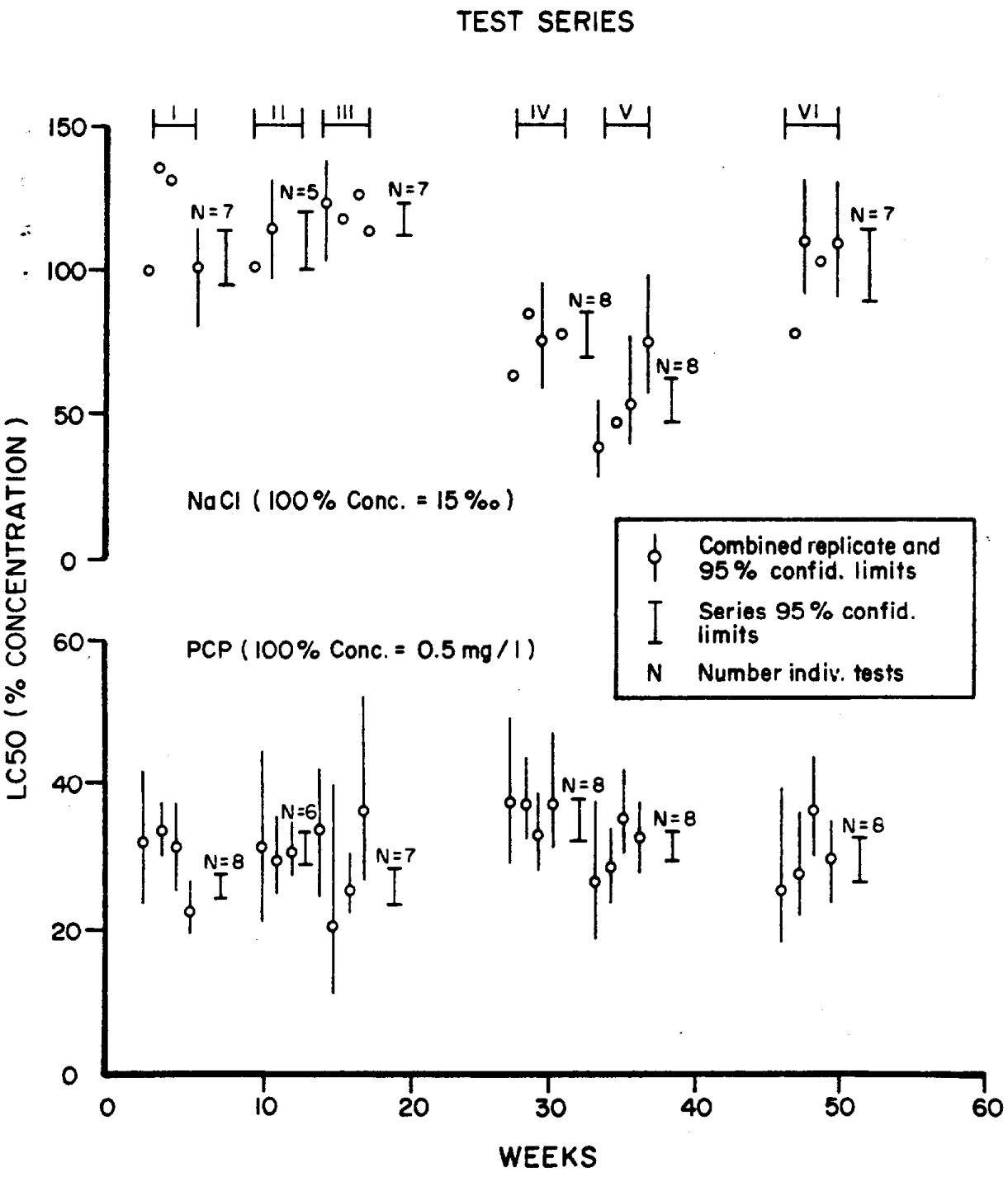

FIGURE 9. Response of Stickleback to Reference Toxicants; Comparison of Combined Replicate 96-h LC50 Values, 95% Confidence Limits for Combined Replicates, and Series 95% Confidence Limits.

consistency was the poorest of all the species tested (Figure 8). The LC50's ranged from 37-140% test concentration; approximately 80% of the observations fell within 48-121% concentration. Two general levels of toxic response are apparent. Series I, II, III, and VI display LC50 values generally greater than 100% concentration whereas Series IV and V LC50 values were generally less than 80% concentration. Agreement between replicates was the poorest of all the species tested, varying by as much as 26% concentration and averaging about 9% concentration. The best agreement between replicates was observed in Series IV and V. The width of the 95% confidence limits of both combined replicates and series (Figure 9) were similar to the rainbow trout tests (Figure 2).

Response to PCP (Figure 8) was similar to that experienced by rainbow trout (Figure 1). The range of LC50 observations was between 20-42% test concentration. Approximately 90% of the values fell within a range of 25-40% concentration. Agreement between replicates was second only to the rainbow trout tests, varying by no more than 9% concentration and averaging about 3% concentration. The width of the 95% confidence limits of both combined replicates and series PCP tests (Figure 9) were uniform and approximated the rainbow trout responses (Figure 2).

Municipal and Industrial Effluent Testing

One industrial and three municipal waste effluents were tested using all four candidate species (Table 11). A total of 32 tests were performed. The first municipal waste tested was toxic to most fish at all concentrations invalidating all but the stickleback tests. Chlorine was the active

TABLE 11. Comparative mortality (LC50 estimates in % concentration) of candidate test species in municipal and industrial wastes.

Date	Toxicant	Replicate	Rainbow Trout	Golden Shiner	Fathead Minnow	Stickleback
11-2-75	Municipal Waste	a	Inv.[a/]	Inv.	Inv.	13
		b	Inv.	Inv.	Inv.	10
11-22-75	Industrial Waste	a	Inv.	65	65	99
		b	87	65	74	96
2-6-76	Municipal Waste	a	4	10	8	14
		b	3	9	9	18
4-29-76	Municipal Waste	a	8	10	10	Inv.
		b	6	8	10	22

a/ Test invalidated due to excessive mortality in experimental concentrations and control groups.

ingredient governing toxicity. Two additional tests, one of each effluent type (industrial and municipal), had excessive mortality in control groups and therefore were invalidated.

For the remaining 24 tests, replicate agreement was excellent with all species for both effluent types. Tested against municipal wastes, rainbow trout and stickleback were the most and least sensitive, respectively. Golden shiner and fathead minnow displayed similar intermediate responses.

Responses to the industrial effluent are interesting in that the observed values closely match those established for the respective species in the NaCl reference toxicant testing program. Since the industrial waste contained a salt concentration of 20 o/oo, it is suspected that the response of each species can be attributed primarily to that component.

Discussion

The primary objective of the experimental investigation with reference toxicants was to determine response characteristics of selected species subjected to standardized toxicity bioassay tests over time. The evaluation of this phase of the study is based primarily on consideration of three factors: laboratory holding, response sensitivity, and response consistency. Data pertaining to each of these factors are first discussed separately and then analyzed and rated collectively.

Laboratory Holding

Success in holding test animals for extended periods without substantial loss or change in toxic response is an important consideration in the standardized species selection process. During the year-long testing program, the six test lots each of fathead minnow and rainbow trout held exceptionally well (less than 1% mortality) while golden shiner and stickleback mortalities were 7% and 19%, respectively (Table 10). Holding response affected test response. Most, if not all, of the invalid tests corresponded to high mortality during holding. Conversely, low holding mortality was consistent with valid tests.

Since completion of the experimental work in 1976, high holding mortalities have been observed at WPCL and other California testing laboratories during the spring-summer months for both golden shiner and fathead minnow whereas rainbow trout continue to hold well. Moreover, fathead minnow suffered greater mortality than golden shiner during the summer-fall months of 1978 at WPCL. During this period, use of fathead minnow was discontinued and replaced by golden shiner because of uncontrollable disease in the former which caused high mortalities in both holding tanks and test controls. Holding losses in several lots of fathead minnow exceeded 30%. Conversely, golden shiner held better (mortalities less than 10%) and yielded consistent results to test toxicants. Discussions with W. Horning II (EPA Newtown Fish Toxicology Station, Ohio) and S. Reynolds (Utah State University) revealed similar experiences with fathead minnow.

Unless this problem can be corrected there appears to be no alternative but to discontinue use of both fathead minnow and golden shiner during spring through fall months whenever the problem arises. As indicated previously, fish used in the testing program were obtained from bait-fish farms, and the predominant fishes available during the spring and summer months were sexually mature. Perhaps if this investigation had used younger, sexually inactive fish, holding success might have been greatly improved. In this regard, Adelman and Smith (1976) recommended that 4-7 week old fathead minnow be used in standard bioassays. Providing golden shiner or fathead minnow or both of this age year-round in California would require some development and this should be investigated. Preliminary culture techniques for producing young fathead minnow year-round have been developed (EPA 1971), and perhaps with some modification, similar techniques could be developed for golden shiner.

Response Sensitivity

Relative sensitivities of the four candidate species to the two reference toxicants during each test series are shown in Figure 10. Data points represent series mean LC50 values (Appendix 3).

Using these data, a species sensitivity rating system was developed to provide a more objective approach for ranking purposes (Table 12). This was accomplished by assigning a reference value of 1.00 to that species having the lowest series mean LC50 value for each toxicant. Numerical values assigned to the other species in the comparison of each series are referenced as percentage increases. For example, in NaCl Series I, fathead minnow exhibited the lowest mean LC50 (77) and accordingly was assigned a reference value of

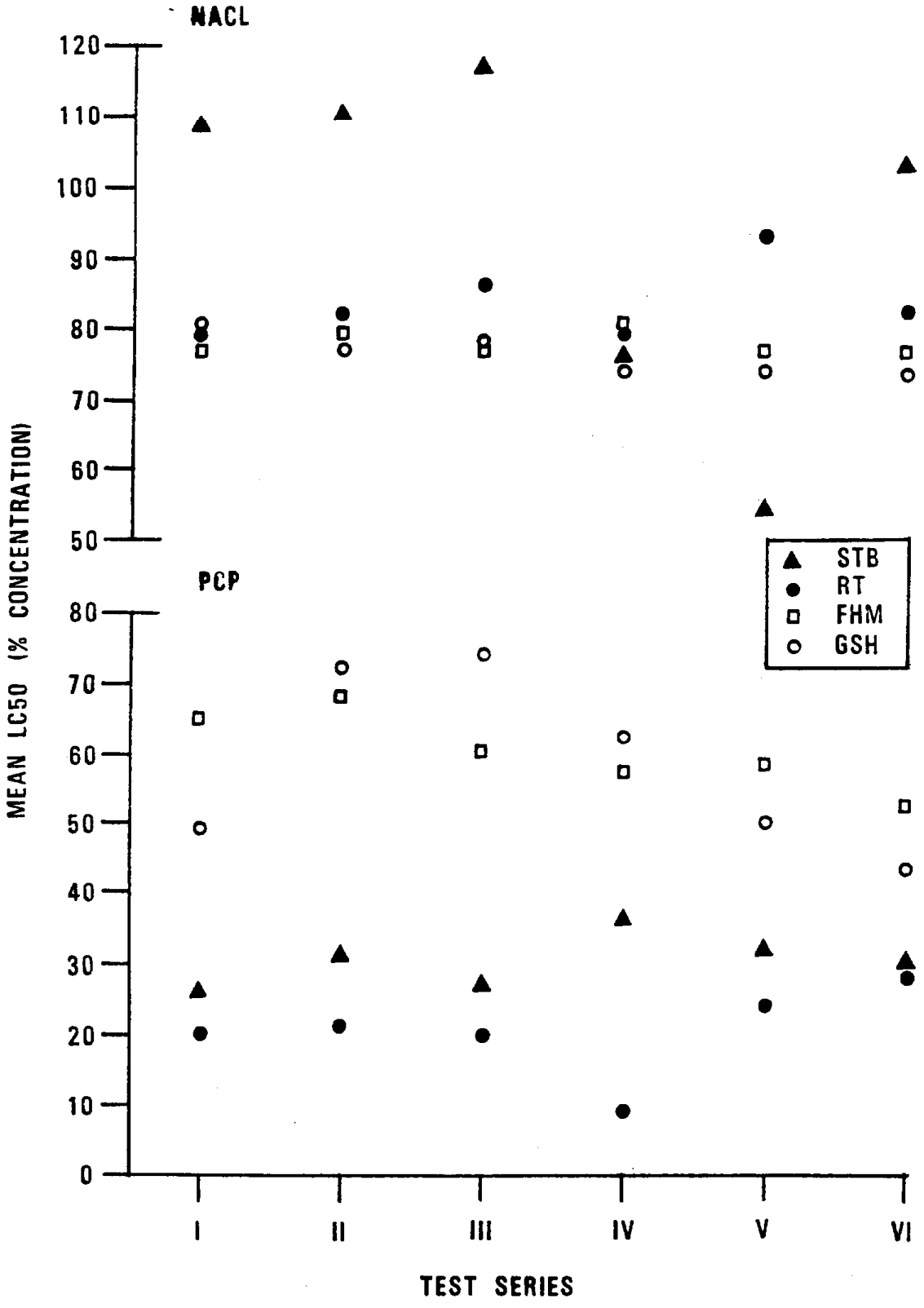

FIGURE 10. Relative Sensitivity of Candidate Freshwater Test Fish Species to Reference Toxicants. Points Represent Series Mean LC50 Values.

TABLE 12. Sensitivity index of candidate freshwater test-fish species exposed to reference toxicants during period May 1975 through May 1976. Values referenced to lowest LC50 (1.00). Rankings are in parentheses.

	Species							
	Rainbow Trout		Golden Shiner		Fathead Minnow		Stickleback	
Test Series	NaCl	PCP	NaCl	PCP	NaCl	PCP	NaCl	PCP
I	1.02(2)	1.00(1)	1.04(3)	2.45(3)	1.00(1)	3.25(4)	1.42(4)	1.30(2)
II	1.06(3)	1.00(1)	1.00(1)	3.43(4)	1.02(2)	3.24(3)	1.43(4)	1.57(2)
III	1.12(3)	1.00(1)	1.01(2)	3.70(4)	1.00(1)	3.00(3)	1.52(4)	1.35(2)
IV	1.07(3)	1.00(1)	1.00(1)	6.89(4)	1.08(4)	6.33(3)	1.03(2)	4.00(2)
V	1.72(4)	1.00(1)	1.37(2)	2.08(3)	1.42(3)	2.42(4)	1.00(1)	1.33(2)
VI	1.12(3)	1.00(1)	1.00(1)	1.54(3)	1.04(2)	1.86(4)	1.41(4)	1.03(2)
Combined	1.10(3)	1.00(1)	1.00(1)	3.11(3)	1.03(2)	3.33(4)	1.20(4)	1.72(2)

1.00. Stickleback, on the other hand, had the highest mean LC50 (109) which is 42% higher than the fathead minnow value, and therefore, 1.42 was assigned as its reference value. In PCP Series 1, rainbow trout was assigned a reference value of 1.00 since this species had the lowest mean LC50 value (20) for the series. By contrast, fathead minnow had the highest value (65), an increase of 325% over rainbow trout, and therefore, 3.25 was assigned as its reference value. In the two examples, reference values of the other species were intermediate.

As shown, some minor shifts in sensitivity occur between species; however, overall, the rankings are clearly defined. In descending order of sensitivity, rankings for the NaCl tests are (1) golden shiner, (2) fathead minnow, (3) rainbow trout, and (4) stickleback. Corresponding PCP test rankings are: (1) rainbow trout, (2) stickleback, (3) golden shiner, and (4) fathead minnow.

Rankings of species sensitivity to the two reference toxicants revealed some interesting relationships. The most significant feature was the response reversal characteristic of the four species. Rainbow trout and stickleback responses are similar to one another with both fishes being most sensitive to PCP and least sensitive to NaCl. The responses of golden shiner and fathead minnow are similar to one another also, but their response to the two toxicants is opposite that exhibited by rainbow trout and stickleback.

The reason(s) for the difference in responses of the four species to PCP is unknown. On the other hand, the difference in response to NaCl is attributed to the species osmoregulatory function. Rainbow trout and stickleback are able to withstand wide variations in the salt content of their environment provided they are properly acclimated. Conversely, golden shiner and fathead minnow have a very limited tolerance to saline environments. When their limit is reached, there is uniformity in individual response; death occurs as a result of massive osmoregulatory failure (Adelman and Smith 1976). This would account for their 0 or 100% toxic response often observed between two successive concentrations.

In general, the reference toxicant study reaffirms that response levels among species can be and often are quite different. The surprising observation is the similarity of response between rainbow trout and stickleback. Taxonomically, these species are widely separated. Perhaps the similarity of their ecological niche is the common denominator. Both species are euryhaline and prefer cold water temperatures. On the other hand, the response similarity of golden shiner and fathead minnow is not too surprising. Both inhabit similar environments and are closely related taxonomically.

Response Consistency

Consistency or reproducibility of results is a most important factor to consider in standardized species selection, particularly for regulatory purposes. Tests conducted under identical conditions must yield similar results in order to maintain scientific and legal integrity. Unless reproducible results can be accomplished, sensitivity response levels have little meaning, and most certainly, legal implications are open to questions.

For ranking purposes, response consistency was quantified by determining mean differences between LC50 values derived for replicates, combined replicates, and series in each reference toxicant (Table 13). These values indicate the closeness of agreement between comparisons (the smaller the value, the greater the agreement). Rankings (in parentheses) are based on descending order of consistent response: the least difference (best agreement) for each category is ranked first.

Based on these data, overall rankings are as follows: (1) fathead minnow, (2) golden shiner, (3) rainbow trout, and (4) stickleback in the NaCl tests; (1) rainbow trout, (2) stickleback, (3) fathead minnow, and (4) golden shiner in the PCP tests.

These results clearly indicate that the four species exhibited a reversal in consistent response to the two toxicants. Fathead minnow and golden shiner gave more consistent results than rainbow trout and stickleback in the NaCl tests, but the opposite was true in the PCP tests.

It is important to note that some of the variation in the combined replicates may be artificial, created by the practice of refining tests (changing concentration) each week within a series. In retrospect, a better approach to evaluate consistency over time would have been to bracket the toxic range, use a few more intermediate concentrations, and maintain those concentrations throughout the testing program without refining. Other investigators have come to the same conclusion (Jensen 1972; Hodson et al. 1977).

Finally, the study once again demonstrated that intraspecific response variability can be considerable between replicate tests and through time

TABLE 13. Comparison of response consistency among replicates, combined replicates, and series of candidate freshwater test-fish species exposed to reference toxicants during period May 1975 through May 1976. Numbers represent mean differences in LC50 values.

	Species							
	Rainbow Trout		Golden Shiner		Fathead Minnow		Stickleback	
Comparisons	NaCl	PCP	NaCl	PCP	NaCL	PCP	NaCL	PCP
Replicates	3.33	2.21	3.25	5.73	2.00	6.08	8.82	3.38
Rank	3	1	2	3	1	4	4	2
Combined Replicates	8.00	4.21	3.79	7.16	3.42	8.17	14.78	5.04
Rank	3	1	2	3	1	4	4	2
Series	7.00	6.67	2.67	10.33	2.00	5.67	19.33	4.67
Rank	3	3	2	4	1	2	4	1
Overall Ranking	$\overline{3}$	$\overline{1}$	$\overline{2}$	$\overline{4}$	$\overline{1}$	$\overline{3}$	$\overline{4}$	$\overline{2}$

even when testing is believed to be under strict control (Figures 1, 4, 6, and 8). Use of more test animals and concentrations could alleviate this problem.

Municipal and Industrial Effluent Evaluation

Data gathered during this phase of the experimental program are insufficient to provide a meaningful evaluation (Table 11). In retrospect, perhaps more effort should have been directed to using selected industrial, municipal, and combination wastes as primary reference toxicants. This approach constitutes "real world" situations, precisely the purpose for which the standardized species concept was designed. An effort was made but hardly on a scale necessary to provide sound conclusions. In the event similar studies are conducted in the future, this approach should receive careful consideration and planning.

PROJECT DISCUSSION

Species Selection

The selection process is based upon the combined results of the Feasibility (Part I) and Experimental (Part II) studies. Rating factors and ranking of the four candidate species are shown in Table 14. The information consists of summaries from Tables 1, 3, and 5 (Part I) and 10, 12, and 13 (Part II). Although data are largely objective, the relative weight applied to each factor is a subjective value judgment exercised by the senior author. In this analysis, equal weight was applied to all factors. Ranking is based on summed values of all selection factors. The lowest score is ranked first with the others following in descending order.

Considering first the comparative factors of holding capability and toxicant response in the experimental phase of the study, rankings are: (1) rainbow trout, (2) fathead minnow, (3) golden shiner, and (4) stickleback. Combining this with logistic and economic factors of commercial supply, the overall ranking of species is redefined. The conclusion of this analysis is that fathead minnow, golden shiner, and rainbow trout are all quite close in overall ability to function as standardized test species. The continued use of stickleback for routine testing programs, however, is still open to question.

A strong belief exists among project investigators that the stickleback is not suited for routine NPDES compliance monitoring programs although results of the reference toxicant tests only partially support this view. Response to one toxicant (NaCl) was rather poor; whereas response to the other (PCP) was good. This, of course, presents a dilemma; making it difficult to

TABLE 14. Comparison of rating factors and ranking of candidate freshwater test-fish species.

Rating Factors	Rainbow Trout		Golden Shiner		Fathead Minnow		Stickleback	
Feasibility (Part I)								
Availability	3		1		1		1	
Sources	1		1		1		3	
Costs	2		1		1		3	
Totals	6		3		3		7	
Experimental (Part II)	NaCl	PCP	NaCl	PCP	NaCl	PCP	NaCl	PCP
Sensitivity	3	1	1	3	2	4	4	2
Consistency	3	1	2	4	1	3	4	2
Holding	2		3		1		4	
Totals	10		13		11		16	
Grand Total	16		16		14		23	
Rank	2		2		1		4	

positively accept or reject use of stickleback solely on the basis of this information. Nevertheless, suspicion regarding this species persists. For example, the studies of Wilson and Hazel (1971) indicated that stickleback were less sensitive and more variable to chlorinated waste effluents than golden shiner. Perhaps if these effluents had been used as reference toxicants, the results may have been more definitive. Also, the species is strongly susceptible to tapeworm infections which most likely influences their response pattern; and lastly, since sticklebacks are collected from wild populations, there is concern that stocks from different waters are intermixed and then supplied to users. Erratic response patterns may result from this practice.

Because of these problems and particularly since other species are available for routine bioassay tests, there may be no further reason to continue stickleback use. The greatest impact would be in the San Francisco Bay area (the principal region of use) where many of the area's analysts are partial to stickleback, probably because of long familiarity. However, the test species could be changed without great difficulty or expense providing that waste discharge regulations permit the action. In the event there is strong pressure to continue stickleback use, then this course of action should be carefully evaluated. Results of past testing should be examined and new comparative tests established. Pending outcome of the study, use should be provisional and restricted to the San Francisco Bay area.

Practical Application of Standardized Species

The underlying objective of this study was to reduce the number of commonly used bioassay test species to a minimum--preferably one. Use of a single standardized species is possibly simply by adjusting test procedures, but for

the intended purpose, this does not appear to be biologically sound. Test temperatures would have to be standardized to a single regime which totally ignores regional differences upon which specific testing procedures were established for California. Routine test procedures recognize two basic temperature regimes; cold water (14-18°C) and warm water (20-24°C) which, in essence, differentiate salmonid (salmon and trout) and nonsalmonid (golden shiner, fathead minnow, etc.) waters, respectively. Salmonids are best suited to temperatures of 14-18°C and would be stressed at higher temperatures. Conversely, fishes better adapted to temperatures of 20-24°C would be compromised at lower temperatures. These thermal stresses are an added burden to the response mechanisms of the test fishes and would tend to modify toxicity results. It is important to minimize extraneous stress variables by maintaining test animals in a suitable environment. The test should be concerned with stress derived solely from the toxicant being tested.

We conclude therefore that it is not advisable nor practical, in terms of biological compatability, for any one of the candidate species to be selected as the State's single test animal. The absolute minimum for routine bioassay application in California is two species; one for use in cold water situations and the other for use in warm water situations.

The study clearly distinguished important differences among the four candidates and, therefore, designation of a species for specific application in NPDES related toxicity monitoring programs must take into account their limitations and select accordingly. Standardizing a species for specific application should be based on natural geographic distribution or existing temperature regimes.

Considering the facts of this investigation, we conclude that:

(1) Rainbow trout should be the species of choice for testing wastes discharged to cold waters. Principal areas of use would be northern California and mountain areas of central and southern California.

(2) Fathead minnow or golden shiner should be the species of choice for testing wastes discharged to warm waters or at times when elevated water temperatures favor their use over rainbow trout. The choice should consider source of supply, quality, and costs of test fish. However, once a species has been established for a given area or condition, that species should remain in use until such time as new knowledge requires change. Obviously, if species are frequently changed, comparisons and interpretations will suffer accordingly. Principal areas of use would include southern California and low elevations of central and northern California, particularly during the summer months. A note of caution; care must be exercised from using these species in areas where their accidental escape could create problems to the indigenous fauna. As a routine, the DFG should be consulted in advance of specific species designation to avoid conflicts.

(3) The use of sticklebacks in the State's NPDES compliance monitoring program should be carefully re-examined in terms of quality, costs and sensitivity, and discontinued, if warranted. Pending outcome of the study, use should be provisional and restricted to the San Francisco Bay area.

(4) Finally, we recognize that a problem of year-round availability currently exists for rainbow trout. Also, there is a problem of erratic physiological stress (sometimes quite severe) among fathead minnow and golden shiner adults during their normal spawning season which may require exclusive use of juvenile fish. Success would depend on establishing constant year-round sources of 4-7 week old fish. We believe, however, that all of these problems can be resolved with moderate attention to planning and implementation.

Test Fish Quality Control

Problems associated with the intermixing of stickleback populations and erratic response of golden shiner and fathead minnow spawners clearly demonstrate need for a quality control program. As indicated in this study, species designated for routine testing must be in good condition in order to demonstrate consistent response to waste effluents. Either mixed stocks or reproductive physiology can alter the pattern of toxic response. One approach would be to establish a test fish certification program in which stocks of fish would be inspected periodically at sources of supply to determine their test suitability. Acceptance or rejection of stocks would be based on the ability of a stock to meet specific, well-defined physiological criteria or response limits. Once developed, the program could be implemented and maintained by the Bioassay Laboratory Certification Program conducted by the DFG under auspices of the SWRCB Legal Division.

Supplemental Evaluations

Data gathered during the experimental phase of the study (Part II) permit a cursory examination of two factors important to the conduct of routine bioassay tests: test duration and number of animals per test concentration.

Test Duration

The purpose of this evaluation was to determine if the routine 96-h test period could be reduced to a shorter time interval and still produce acceptable data. Results of each bioassay test were examined and mortalities at 24, 48, and 72-h were compared with mortality observed at test completion (96-h). The percentage of those tests having the same mortality at these designated intervals as that exhibited at 96-h are summarized by species and toxicant (Figure 11). For example, with rainbow trout exposed to NaCl, 26% of all tests showed the same mortality at 24-h as at 96-h, 64% of all tests showed the same mortality at 48-h as at 96-h, and 88% of all tests showed the same mortality at 72-h as at 96-h.

The data indicate that most rainbow trout and fathead minnow tests are completed by 48-h, whereas most golden shiner and stickleback tests are not. The implication is that routine rainbow trout and fathead minnow tests might be reduced to a shorter time interval of 72 or perhaps 48-h, whereas golden shiner and stickleback tests require 96-h. A prudent approach, however, would be to establish 96-h as the initial testing interval (for any species) and then, if warranted, reduce the test interval as experience dictates. Although the effort required to continue the

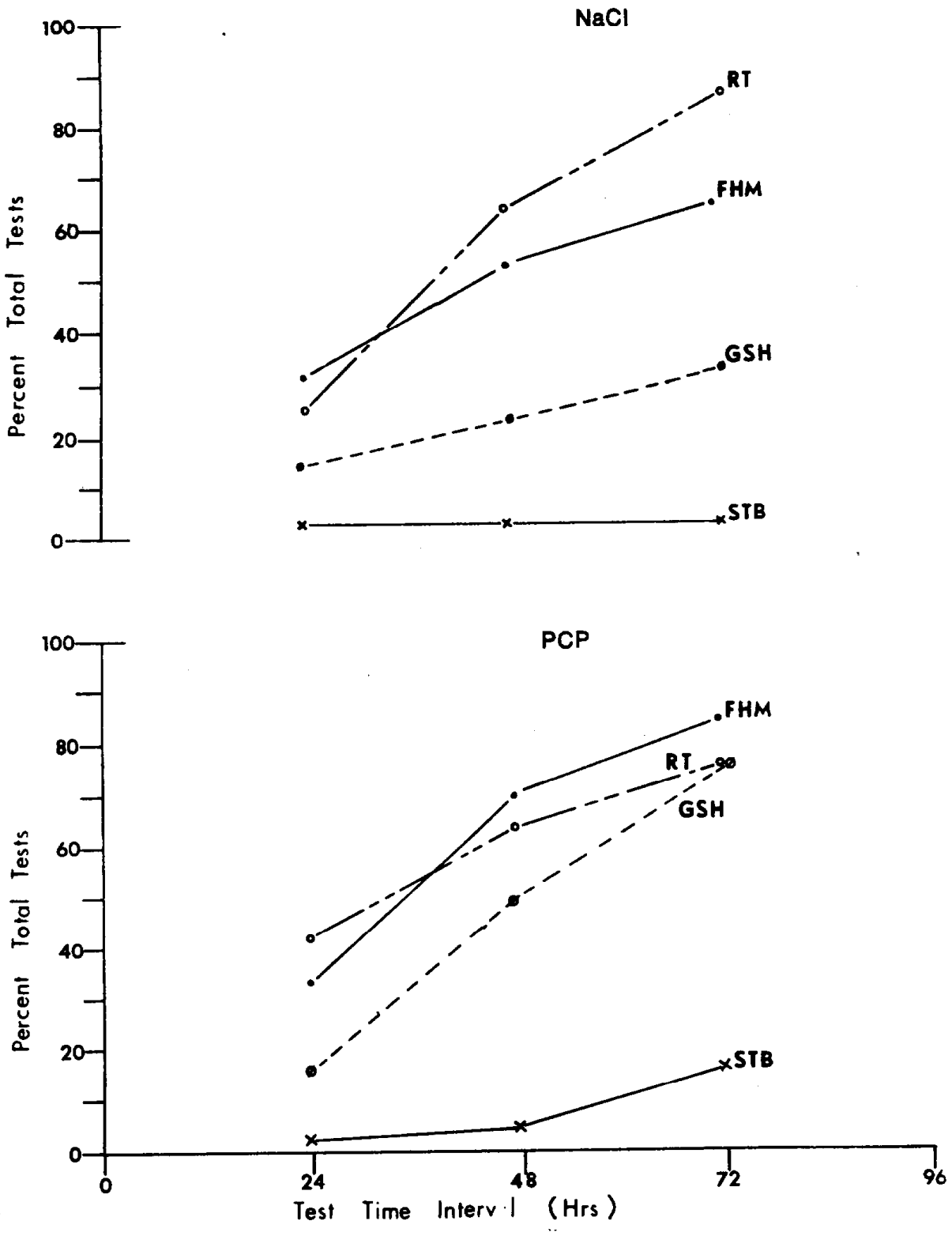

FIGURE 11. Accumulative Percent Agreement of LC50 Estimates At 24, 48 and 72 Hours With Corresponding 96-Hour Estimates.

test to full term is minimal, if it can be demonstrated that a shorter test interval yields essentially identical mortalities as the 96-h test, then there is no reason to continue testing beyond that point.

Number of Fish Per Test Concentration

Most, if not all, NPDES related bioassay tests in California are conducted on the basis of 10 fish per test concentration, including controls. Under this arrangement, each fish represents 10% of the test concentration population and any losses caused by a few nonrepresentative specimens (i.e., unhealthy fish) inadvertently included in the test will obviously bias results. It would appear, therefore, that this bias could be minimized substantially by increasing the test concentration population. By such means, deviant response of nonrepresentative specimens are dampened by the overall response of the remaining test population.

The quantity of fish per test concentration should be of particular concern to waste dischargers who must report test findings to RWQCB's and demonstrate compliance with specific toxicity limits included in their NPDES permit. On occasion, routine NPDES tests have been invalidated due to unacceptable losses in controls (>10%) or severe nonlinear toxic response in test concentrations. It is believed that many of these tests would have been acceptable if the test concentration population had been larger. In short, a larger test concentration population provides better insurance for the discharger against invalidation or erroneous test results caused by deviant responses of a few individuals. The pertinent question is: What is a reasonable number?

To determine the appropriate number of fish per concentration for routine tests, the relationship between confidence limits and number of fish per concentration was examined. This was accomplished by plotting the mean of confidence limit range derived for selected individual and combined tests against corresponding numbers of fish per test concentration (Figure 12). Selected tests included rainbow trout-PCP (Series II excepted for lack of data) and fathead minnow-NaCl. The selected tests serve as examples since the other tests in the study responded similarly. Only data for which confidence limits were established through probit analysis were used (Appendix 3). Individual, combined replicate, and series confidence limits were referenced to 10, 20 and 80 fish, respectively. These are the numbers of fish per test concentration upon which the respective confidence limits are based. Values corresponding to 40 fish were determined by combining results for weeks 1 and 2, and 3 and 4, for each series.

The relationship between confidence limits and numbers of fish per test concentration is curvilinear and similar for both species and toxicants except for relative level. The plots for fathead minnow are approximately twice that for rainbow trout. Sharpest change (67%) occurs between 10 and 40 fish for both species. Beyond 40 fish, confidence limit range reductions do not exceed 12% of the total.

Considering that degree of confidence in test results is related to confidence limit span (the narrower the better) then Figure 12 implies that 60 to 80 fish are needed per test concentration. This, however, is an unnecessary economic hardship in terms of total numbers of fish, aquaria, and space needed,

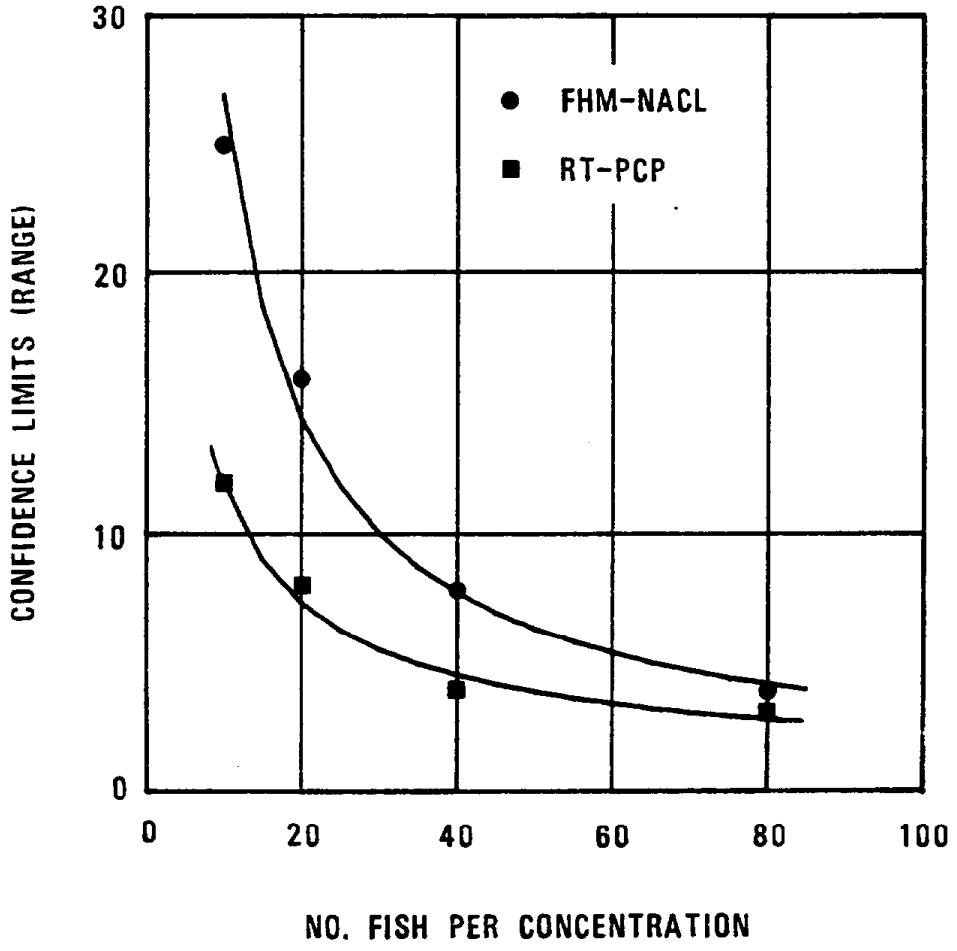

FIGURE 12. Relationship Between Confidence Limits (Range) and Number of Fish Per Test Concentration.

as well as the manpower to service those needs. On the other hand, 10 fish per concentration can contribute to wide confidence limits and, consequently, less confidence in the results. An appropriate compromise would be to use from 20 to 40 fish as the rate of change in the curve is substantially diminished in this range.

In this regard, Jensen (1972) compared relative error and sample size in fish bioassays both empirically and theoretically and concluded 20 fish at about six test levels was practical for routine tests. Hodson et al., (1977) examined, through probit analysis, results of contrived and real toxicity data and illustrated how experimental design affects fiducial limits, chi square, slope, and variance of slope. These investigators concluded that 30 fish per concentration was appropriate and that the concentration range should be adjusted to provide for at least five partial responses. They favored replication.

In view of these data, we conclude that routine NPDES tests should include no less than 20 fish nor more than 30 fish per test concentration. Test concentrations should be replicated in groups of 10 fish (two or three aquaria with 10 fish each) and preferably, concentrations adjusted to ensure at least three partial responses. If implemented, we believe these procedures will provide more meaningful test results.

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APPENDIX 1

APPENDIX I. Routine Prophylactic Treatment

Treatment Combinations		Treatment Time
(1) Salt Solution (NaCl)	1.5 or 3.0%	30-minute bath
(2) Formaldehyde Malachite Green	1 : 5000) 2 ppm)	30-minute bath
(3) Acetic Acid Malachite Green	1 : 2000) 2 ppm)	30-minute bath
(4) Terramycin(R)	Water soluble form--0.3 gm) active drug) per gallon) water)	30-minute bath

Procedure

1. The holding tank volume was reduced by one half. This permitted water to be added quickly to dilute the chemical treatment and also reduced the amount of chemicals required.

2. A salt bath was administered within one hour of receiving fish into the laboratory; 1.5% was used for stenohaline (narrow salinity range) species and 3.0% for euryhaline (wide salinity range) species. Following the 30-minute bath, the tanks were flushed with freshwater until the salinity meter registered less and 1 o/oo (ppt).

3. The salt bath was followed with combination treatment (2) or (3). Although either combination would be effective, treatment (2) was used exclusively throughout all test series. If more than 12 hours had elapsed since the salt bath, Step (1) was repeated before the combination treatment.

4. Following these treatments the water was changed completely and a Terramycin(R) bath (4) was administered. After the 30-minute treatment water flow through the tank was started.

The treatment was designed to control several different infestations common to fish from wild or semi-wild habitats: ectoparasites (protozoan and fluke); external bacteria (columnaris disease and gill bacteria); and internal bacteria (Aeromonas and Pseudomonas group).

APPENDIX 2

APPENDIX 2. Reference Toxicant Formulation Procedure

Sodium chloride (NaCl) formulation /100% toxicant concentration = 1.5% or 15 o/oo (ppt)/.

1. The total quantity of NaCl required for each series of replicate tests was based upon a standard dilution series. Initially, this consisted of 100, 56, 32, 18, and 10% toxicant concentrations. The amount of NaCl needed for any one test series was predetermined by the following formula:

   Number of Tanks x volume per tank x concentration per liter (in tanks)

   Example: 10 tanks x 10 liters per tank x 15 gm per liter (100% concentration)
   = 1500 gm per 100 liters

   This calculation was done for each concentration used in a particular test series. The sum of these calculations represented the amount of NaCl needed to make up sufficient toxicant.

2. To produce 100% toxicant solution the required amount of NaCl was weighed out and placed in a 1900-liter polyethylene mixing tank. De-ionized water was added along with the necessary appropriate chemical salts (Table 7) to produce a water of the appropriate hardness (hard or soft). This mixing tank had been previously calibrated (volume per unit depth). The solution was stirred until completely dissolved. The final solution was checked with a salinity meter. If necessary, either the salinity or hardness or both were adjusted.

Sodium pentachlorophenate (PCP) formulation /100% toxicant concentration = 0.5 mg/l (ppm)/.

1. A concentrated stock solution was formulated for each test series by dissolving 1.580 gm of PCP (79% active compound) in 100 ml d-ionized water. This stock solution was then used to prepare an intermediate stock solution.

2. A 1:25 dilution (volume to volume) of the concentrated stock solution was made up using de-ionized water. This was prepared fresh each week of the particular test series. A standard dilution series could be rapidly and consistently set up using this intermediate stock solution. One milliliter of this intermediate stock was added to each liter of dilution water producing a 100% toxicant concentration (0.5 mg/l (ppm)).

3. Subsequently, an appropriate dilution series could be easily formulated by adding specific volumes of this mixture to each tank and then dilution with water. A calibrated depth gauge placed in the center of the test tank permitted rapid, accurate, and repetitive volume regulation.

APPENDIX 3

APPENDIX 3. Results of Reference Toxicant Testing Program

Key

Symbol	
*	LC50 estimate based on straight line graphical interpolation.
>	LC50 estimate greater than highest concentration tested.
<	LC50 estimate less than lowest concentration tested.
---	No Results.
Inv.	Invalid test due to excessive or non-linear mortality.
NT	No test conducted
NR	Confidence limits not reported when exceeding highest concentration tested.
Comb.	Combined (pooled) results.

APPENDIX 3A. Comparative toxicity of NaCl to candidate freshwater test-fish species during the period May 1975 through May 1976. LC50 values and 95% confidence limits (parenthesized) based on 96-h static bioassays are expressed as percent concentration.

Series	Week	Replicates	Test Species: Rainbow Trout		Golden Shiner		Fathead Minnow		Stickleback	
I	1	a	78*	—	61*	(40-95)	75*	—	84	(NR)
		b	75*	—	75*	—	75*	—	>100	—
		Comb.	78	—	63	(45-87)	75	—	96	(NR)
	2	a	68*	—	80*	—	70*	—	132*	—
		b	69	(62-76)	81	(72-91)	76	(62-93)	Inv.*	—
		Comb.	68	(64-73)	85	(79-88)	72	(68-78)	132	—
	3	a	<65	—	79*	—	75*	—	140*	—
		b	65	—	76*	—	75*	—	121*	—
		Comb.	65	—	77	—	75	—	130	—
	4	a	<87	—	NT	—	81	(NR)	96	(68-134)
		b	<87	—	NT	—	81	(73-89)	105	(83-132)
		Comb.	<87	—	—	—	81	(76-86)	99	(79-124)
		Series Estimates	79	(76-82)	80	(75-85)	77	(73-81)	109	(100-120)
		Total Fish	460		340		480		420	
II	1	a	80*	—	Inv.	—	76*	—	>100*	—
		b	84*	—	Inv.	—	76*	—	74*	—
		Comb.	82	—	—	—	76	—	100	—
	2	a	Inv.	—	80*	—	76	(67-85)	99	(77-128)
		b	Inv.	—	80*	—	76	(62-92)	124	(104-148)
		Comb.	—	—	80	—	76	(70-82)	112	(95-131)
	3	a	NT	—	76	(67-85)	81*	—	114	(NR)
		b	NT	—	80	(NR)	81*	—	Inv.	—
		Comb.	—	—	78	(72-84)	81	—	114	(NR)
	4	a	NT	—	NT	—	79	(76-82)	NT	—
		b	NT	—	NT	—	79	(75-82)	NT	—
		Comb.	—	—	—	—	79	(76-81)	—	—
		Series Estimates	82*	—	77	(74-80)	79	(76-82)	110	(99-121)
		Total Fish	120		240		480		300	

APPENDIX 3A. (Continued).

Series	Week	Replicates	Test Species: Rainbow Trout		Golden Shiner		Fathead Minnow		Stickleback	
III	1	a	82*	—	80*	—	75*	—	128*	(106-154)
		b	80	(64-69)	NT*	—	75*	—	120	—
		Comb.	80	(68-94)	80	—	75	—	122	(102-146)
	2	a	NT	—	80*	—	75	(61-92)	Inv.	—
		b	NT	—	79*	—	77	(64-94)	116	(NR)
		Comb.	—	—	80	—	76	(67-87)	116	(NR)
	3	a	87	(75-100)	77*	—	79*	(68-91)	107*	—
		b	84	(74-96)	70*	—	77	—	128	—
		Comb.	85	(79-92)	75	—	78	(75-81)	124	(NR)
	4	a	86	(75-100)	NT	—	77*	—	108*	—
		b	92	(82-104)	NT	—	78*	—	117*	—
		Comb.	90	(83-97)	—	—	77	—	111	—
		Series Estimates	86	(83-90)	78*	—	77	(75-78)	117	(111-123)
		Total Fish	360		300		480		380	
IV	1	a	75*	—	75*	—	75*	—	60	(NR)
		b	75*	—	75*	—	75*	—	64	(NR)
		Comb.	75	—	75	—	75	—	62	(NR)
	2	a	81	(71-91)	76	(64-92)	77	(64-94)	83	(NR)
		b	77	(72-82)	74	(60-92)	78	(64-95)	83	(NR)
		Comb.	80	(74-88)	75	(66-89)	78	(68-89)	83	(NR)
	3	a	76	(67-87)	74	(59-93)	85	(73-99)	78	(58-104)
		b	82	(74-92)	76	(63-93)	78	(64-96)	70	(NR)
		Comb.	80	(74-86)	75	(66-87)	82	(76-87)	74	(57-96)
	4	a	81	(NR)	75	(69-81)	81	(NR)	75*	—
		b	76	(71-82)	70	(54-91)	78	(64-95)	79	—
		Comb.	77	(71-84)	72	(68-78)	79	(68-92)	76	(NR)
		Series Estimates	79	(76-82)	74	(72-77)	80	(77-83)	76	(69-85)
		Total Fish	480		480		480		460	

APPENDIX 3A. (Continued).

Series	Week	Replicates	Test Species: Rainbow Trout		Golden Shiner		Fathead Minnow		Stickleback	
V	1	a	88*	--	77*	(62-96)	70*	(NR)	40	(26-59)
		b	80*	--	80*	--	66*	--	37	(20-69)
		Comb.	84	--	78	(67-90)	68	(53-87)	38	(28-52)
	2	a	85	(78-93)	72	(65-80)	76	(62-92)	43	(NR)
		b	88	(80-97)	70	(63-78)	77	(64-94)	48	(NR)
		Comb.	87	(82-94)	71	(66-76)	76	(67-87)	45	(NR)
	3	a	98	(NR)	77	(64-94)	81	(NR)	55	(32-94)
		b	89	(NR)	72	(57-93)	75	(61-92)	52	(31-88)
		Comb.	92	(81-104)	75	(65-87)	77	(67-88)	52	(38-73)
	4	a	102	(NR)	74	(60-92)	81	(NR)	70	(46-107)
		b	101	(88-115)	73	(58-93)	76	(64-92)	76	(NR)
		Comb.	101	(92-111)	74	(63-86)	78	(68-89)	74	(56-98)
		Series Estimates	93	(89-97)	74	(72-77)	77	(75-79)	54	(47-63)
		Total Fish	480		480		480		480	
VI	1	a	75*	--	Inv.	--	75*	--	80	(NR)
		b	NT*	--	Inv.	--	75*	--	72	(NR)
		Comb.	75	--	--	--	75	--	76	(NR)
	2	a	78	(64-96)	74	(60-92)	70	(NR)	101	(78-131)
		b	80	(72-89)	76	(67-85)	70	(NR)	112	(88-142)
		Comb.	81	(74-89)	75	(69-80)	70	(NR)	110	(92-132)
	3	a	85	(NR)	72	(67-77)	78	(64-95)	101	(NR)
		b	82	(72-92)	Inv.	--	76	(67-85)	Inv.	--
		Comb.	85	(77-93)	72	(67-77)	77	(72-83)	101	(NR)
	4	a	NT	--	Inv.	--	78*	--	112	(90-140)
		b	NT	--	71	(65-78)	72	(66-79)	105	(76-146)
		Comb.	--	--	71	(65-78)	75	(70-79)	109	(90-132)
		Series Estimates	82	(76-83)	73	(70-77)	76	(74-79)	103	(91-116)
		Total Fish	300		240		480		420	
		COMBINED SERIES								
		Estimates	84	(82-86)	76	(75-76)	78	(77-79)	91	(87-95)
		Total Fish	2200		2080		2880		2460	

APPENDIX 3B. Comparative toxicity of PCP to candidate freshwater test-fish species during period May 1975 through May 1976. LC50 values and 95% confidence limits (parenthesized) based on 96-h static bioassays are expressed as percent concentration.

			Test Species							
			Rainbow Trout		Golden Shiner		Fathead Minnow		Stickleback	
Series	Week	Replicates								
I	1	a	26*	(18-37)	55	(52-90)	75*	--	33	(21-52)
		b	23*	--	52	(27-97)	75	(NR)	32	(26-39)
		Comb.	24	(19-31)	52	(35-79)	75	(NR)	32	(24-43)
	2	a	20	(16-24)	50	(41-60)	64*	(53-78)	34	(28-40)
		b	21	(17-25)	Inv.	--	55*	--	34	(29-40)
		Comb.	19	(17-22)	50	(41-60)	60	(52-68)	34	(31-38)
	3	a	14*	--	Inv.	--	64	(54-77)	31	(24-40)
		b	22*	--	Inv.	--	74	(62-87)	33	(26-42)
		Comb.	18*	--	--	--	70	(63-77)	32	(26-38)
	4	a	20	(17-23)	NT	--	63*	(39-94)	<19	--
		b	21	(19-24)	NT	--	62*	--	23	(20-27)
		Comb.	20	(18-23)	--	--	64	(58-70)	23	(20-27)
		Series Estimates	20	(19-21)	49	(46-54)	65	(62-68)	26	(25-28)
		Total Fish	500		130		500		520	
II	1	a	24*	--	Inv.	--	62	(40-94)	34	(22-52)
		b	19	(6-56)	Inv.	--	58	(40-86)	31	(19-50)
		Comb.	21	(10-42)	--	--	61	(43-87)	32	(22-46)
	2	a	Inv.	--	Inv.	--	60	(52-69)	29	(22-39)
		b	Inv.	--	71	(62-81)	60	(52-69)	30	(24-38)
		Comb.	--	--	71	(62-81)	60	(52-69)	30	(25-36)
	3	a	NT	--	74	(61-90)	73*	(64-84)	31	(27-36)
		b	NT	--	70	(62-79)	87*	--	31	(28-34)
		Comb.	--	--	72	(66-80)	79	(69-91)	31	(28-34)
	4	a	NT	--	NT	--	72	(61-85)	NT	--
		b	NT	--	NT	--	66	(55-78)	NT	--
		Comb.	--	--	--	--	65	(57-73)	--	--
		Series Estimates	21	(10-42)	72	(67-78)	68	(64-71)	31	(29-34)
		Total Fish	120		180		480		360	

APPENDIX 3B. (Continued).

Series	Week	Replicates	Test Species: Rainbow Trout		Golden Shiner		Fathead Minnow		Stickleback	
III	1	a	<10	--	95*	--	84*	--	34	(25-45)
		b	<10	--	Inv.*		56	--	Inv.	--
		Comb.	<10	--	95	--	69	(NR)	34	(25-45)
	2	a	28*	--	>56	--	48	(40-58)	20	(8-56)
		b	25	(20-31)	>56	--	38	(22-66)	22	(9-53)
		Comb.	26	(23-29)	>56	--	43	(36-50)	21	(11-41)
	3	a	19*	--	>75	--	>75	--	27*	--
		b	20	(17-24)	66	(46-94)	>75	--	26	(20-32)
		Comb.	20	(18-21)	76	(NR)	>75	--	26	(23-30)
	4	a	26*	--	70*	(62-79)	70*	--	32*	--
		b	25*	--	60*	--	70	(51-94)	39	(27-58)
		Comb.	26	--	65	(61-70)	69	(59-81)	37	(27-52)
		Series Estimates	20	(18-23)	74	(66-83)	60	(56-65)	27	(24-29)
		Total Fish	460		420		480		420	
IV	1	a	13*	(0-41)	72*	--	55*	(34-87)	38*	(28-51)
		b	18	--	53	(45-64)	56	--	39	--
		Comb.	16	(1-36)	58	(44-76)	55	(40-76)	38	(29-49)
	2	a	12*	--	60*	--	56*	--	42	(34-51)
		b	10	--	Inv.*	--	72	(55-97)	35	(29-43)
		Comb.	11	(7-20)	60	--	63	(56-70)	38	(33-44)
	3	a	10*	(8-12)	65*	--	61	(45-82)	33	(26-42)
		b	9	--	66	--	58	(36-94)	34	(27-43)
		Comb.	10	(8-11)	67	(59-75)	60	(46-77)	33	(29-39)
	4	a	8	(5-11)	56*	--	51	(43-61)	35	(27-45)
		b	8	(7-9)	63	(56-72)	58	(50-62)	40	(31-50)
		Comb.	8	(6-9)	60	(55-65)	54	(49-59)	37	(31-44)
		Series Estimates	9	(8-10)	62	(59-65)	57	(55-60)	36	(33-39)
		Total Fish	480		420		480		480	

APPENDIX 3B. (Continued).

Series	Week	Replicates	Test Species: Rainbow Trout		Golden Shiner		Fathead Minnow		Stickleback	
V	1	a	21*	--	52*	--	56	(36-86)	25	(17-37)
		b	23*	--	46	--	52	(27-97)	29	(17-50)
		Comb.	23	--	49	(32-73)	54	(38-77)	27	(19-38)
	2	a	25*	--	43*	(35-69)	57	(40-82)	30	(22-41)
		b	21	--	47	--	61	(41-92)	28	(22-34)
		Comb.	21	(19-25)	49	(38-62)	59	(45-77)	29	(24-34)
	3	a	Inv.	--	56	(46-70)	60	(53-69)	33	(26-44)
		b	Inv.	--	46	(39-54)	53	(42-67)	40	(32-49)
		Comb.	--	--	51	(45-58)	56	(50-63)	36	(31-43)
	4	a	26*	--	49	(44-55)	61	(NR)	32	(26-48)
		b	26	(18-40)	52	(46-58)	51	(40-65)	36	(29-44)
		Comb.	26	(24-29)	50	(46-55)	53	(45-63)	33	(28-38)
		Series Estimates	24	(23-25)	50	(48-52)	58	(54-61)	32	(30-34)
		Total Fish	360		480		480		480	
VI	1	a	24*	—	56*	—	50*	—	22	(NR)
		b	NT*	—	42	—	50*	—	31	(20-49)
		Comb.	24	—	45	(35-58)	50	—	26	(18-40)
	2	a	27*	(22-35)	41	(36-48)	53	(41-70)	28*	(20-39)
		b	24	—	46	(38-55)	56	(40-80)	34	—
		Comb.	26	(24-29)	44	(40-49)	55	(46-65)	28	(22-36)
	3	a	29*	(24-36)	39	(29-52)	50	(42-61)	32	(24-44)
		b	30	—	42	(30-58)	55	(45-67)	39	(32-47)
		Comb.	30	(25-35)	43	(38-49)	53	(46-60)	37	(31-44)
	4	a	31*	—	41	(31-56)	50	(40-63)	29	(22-39)
		b	28	—	42	(32-60)	56	(NR)	30	(23-40)
		Comb.	28	(25-31)	42	(34-52)	53	(45-63)	30	(24-36)
		Series Estimates	28	(26-29)	43	(41-46)	52	(48-55)	30	(27-33)
		Total Fish	420		470		480		480	
		Combined Series								
		Estimates	18	(17-19)	56	(54-58)	60	(59-61)	31	(30-32)
		Total Fish	2340		2150		2900		2740	

APPENDIX 3C. Guide to individual tests (WPCL test number) conducted during the reference toxicant testing program, May 1974 - May 1976.

Test Series	Week	Replicate	Rainbow Trout NaCl	Rainbow Trout PCP	Golden Shiner NaCl	Golden Shiner PCP	Fathead Minnow NaCl	Fathead Minnow PCP	Stickleback NaCl	Stickleback PCP
I	1	a	1	3	25	27	5	7	41	43
		b	2	4	26	28	6	8	42	44
	2	a	13	15	45	47	17	19	61	63
		b	14	16	46	48	18	20	62	64
	3	a	37	39	65	67	29	31	69	71
		b	38	40	66	68	30	32	70	72
	4	a	57	59	--	--	49	51	75	73
		b	58	60	--	--	50	52	76	74
II	1	a	91	89	79	77	85	83	95	93
		b	92	90	80	78	86	84	96	94
	2	a	11	109	99	97	105	103	115	113
		b	112	110	100	98	106	104	116	114
	3	a	---	---	119	117	125	123	131	129
		b	---	---	120	118	126	124	132	130
	4	a	---	---	---	---	139	137	---	---
		b	---	---	---	---	140	138	---	---
III	1	a	190	183	174	172	181	179	187	185
		b	191	184	---	173	182	180	188	186
	2	a	---	192	196	194	204	202	206	208
		b	---	193	197	195	205	203	207	209
	3	a	210	212	217	215	225	223	227	229
		b	211	213	218	216	226	224	228	230
	4	a	231	233	---	235	240	238	242	244
		b	232	234	---	236	241	239	243	245

APPENDIX 3C. (Continued).

Test Series	Week	Replicate	Rainbow Trout NaCl	Rainbow Trout PCP	Golden Shiner NaCl	Golden Shiner PCP	Fathead Minnow NaCl	Fathead Minnow PCP	Stickleback NaCl	Stickleback PCP
IV	1	a	294	300	289	288	282	284	306	308
		b	295	201	290	293	283	285	307	309
	2	a	334	336	318	320	310	312	342	344
		b	335	337	319	321	311	313	343	345
	3	a	358	360	356	354	350	348	366	368
		b	359	361	357	355	351	349	367	369
	4	a	385	387	380	382	378	376	397	399
		b	386	388	381	383	379	377	398	400
V	1	a	423	425	463	465	443	445	487	489
		b	424	426	464	466	444	446	488	490
	2	a	453	455	491	493	477	475	515	517
		b	454	456	492	494	478	476	516	518
	3	a	483	485	519	521	497	499	531	533
		b	484	486	520	522	498	500	532	534
	4	a	511	513	535	537	525	527	541	543
		b	512	514	536	538	526	528	542	544
VI	1	a	635	636	617	645	623	625	553	555
		b	---	---	618	646	624	626	554	556
	2	a	657	659	671	673	653	655	571	573
		b	658	660	672	674	654	656	572	574
	3	a	681	683	687	689	677	679	599	601
		b	682	684	688	690	678	680	600	602
	4	a	---	699	703	705	693	695	631	633
		b	---	700	704	706	694	696	632	634

APPENDIX 4

APPENDIX 4. Fork length (F.L.), weight (W) and condition factor (C.F.) of rainbow trout, golden shiner and fathead minnow during reference toxicant testing period of August 1975 through May 1976.

		Rainbow Trout			Golden Shiner			Fathead Minnow		
Series	Week	F.L. (mm)	W (g)	C.F.	F.L. (mm)	W (g)	C.F.	F.L. (mm)	W (g)	C.F.
III	1	---	---	---	47.0	1.00	0.96	48.3	1.34	1.19
	2	---	---	---	48.6	1.11	0.97	49.6	1.09	0.89
	3	52.8	1.62	1.10	---	---	---	49.0	1.21	1.03
	4	65.6	3.19	1.13	50.1	1.30	1.03	43.8	1.27	1.52
IV	1	50.8	1.04	0.79	48.9	0.96	0.83	57.5	2.03	1.07
	2	50.2	0.98	0.77	48.9	1.02	0.87	55.5	1.84	1.09
	3	51.8	1.04	0.75	46.6	0.95	0.94	57.4	2.05	1.08
	4	50.7	0.94	0.72	47.2	0.88	0.84	61.1	2.44	1.07
V	1	54.0	1.44	0.91	57.6	1.53	0.80	52.7	1.39	0.95
	2	56.4	1.64	0.91	55.3	1.36	0.80	52.4	1.48	1.03
	3	58.6	2.00	0.99	58.1	1.62	0.83	49.2	1.17	0.98
	4	60.4	2.13	0.97	55.6	1.45	0.84	48.1	1.06	0.95
VI	1	61.5	2.16	0.93	58.4	1.79	0.90	53.0	1.68	1.13
	2	62.8	2.45	0.99	57.9	1.75	0.90	58.8	2.32	1.14
	3	64.5	2.36	0.88	56.4	1.59	0.89	57.9	2.06	1.06
	4	61.4	2.29	0.99	54.7	1.53	0.93	54.2	1.76	1.04

APPENDIX 5

APPENDIX 5. A Statistical Analysis of Data Obtained from Acute Bioassay Tests, by: Alvin D. Wiggins, Ph.D.

1. Acceptability of the DFG LC_{50} Estimates

Data generated by the toxicity trials conducted by the Department of Fish and Game were first analyzed as eight one-factor experiments, one experiment for each of four species of test fish and each of two reference toxicants. The species of test fish were rainbow trout, golden shiner, fathead minnow and three-spine stickleback. The two reference toxicants were NaCl, or sodium chloride, and PCP, or sodium pentachlorophenate. The tests were conducted by preparing a logarithmically graduated series of concentrations of a toxicant and exposing (usually) ten fish to each level of concentration. The cumulative number of dead fish at each concentration was recorded at twenty-four, forty-eight, seventy-two and ninety-six hours. Only the ninety-six hour data are used in both the DFG computations and this statistical analysis. The population parameter of greatest interest was the LC_{50}, or median lethal concentration, at ninety-six hours. Concentrations of each toxicant were expressed in percent. A 1.5% stock solution was taken to be the 100% concentration of NaCl, and a concentration of 0.5 mg/ℓ was taken to be the 100% concentration of PCP. In the actual conduct of the tests, some test solutions exceeded the 100% concentration level.

The principal statistical technique used by the DFG in estimating the LC_{50} was Finney's method of probit analysis (1958). According to the DFG report, the probit analysis was conducted by making use of a prepared statistical program which was written for, and executed upon a Tektronix Model 31 desk calculator. Since this program, as well as the machine on which it was executed, were unavailable to us, we used a slightly different

approach in computing the estimated LC_{50} values and the 95% confidence intervals therefore. Whereas probit analysis is based on the assumption of an integrated normal probability law as an expression of the distribution of tolerance concentrations, a part of the present statistical analysis assumes the logistic function as an expression of this distribution. Using the logistic function, the LC_{50} values were estimated by means of maximum likelihood. The 95% confidence intervals were estimated by means of asymptotic methods.

Despite the different methods used, the LC_{50} values computed by maximum likelihood were in remarkably good agreement with those obtained by the DFG using probit analysis. Accordingly, the DFG estimates of the LC_{50} values were used during the next phase of the statistical analysis.

2. Search for Trends by DFG

The DFG report makes mention in several places of trends (or apparent trends), over time, of the sensitivities of the various species to the reference toxicants. The assertion of a trend is often interpreted (sometimes incorrectly) as an assertion of a linear trend. This is a useful and easily interpretable concept. However the assertion of a lack of trend following statistical significance testing can sometimes be misleading. For this reason, we have chosen to test for a statistically significant time effect rather than test for a significant time trend, the former being more general in the sense that if there is a significant linear trend in time, it will be detected as a significant time effect. Accordingly, we have conducted eight one-factor analyses

of variance, one for each of the four species of fish in each of the two reference toxicants. The model for a typical one-factor, or one-way analysis of variance is as follows:

$$Y_{ij} = \mu_i + e_{ij}, \; j=1,\ldots,n_i, \; i=1,\ldots,6,$$

where Y_{ij} is the DFG-calculated sample LC_{50} value, in percent, for the j^{th} experiment conducted during series i (i=I,II,...,VI), μ_i is the population mean LC_{50} for series i, and the e_{ij} are random error terms, assumed to be independently normally distributed for all i and j, with mean zero and common variance σ^2. For a given species of fish in a given reference toxicant, the null hypotheses tested was

$$H_0 : \mu_i = \mu, \; i=1,\ldots,6.$$

That is, the hypothesis testedwas that the population mean LC_{50} values are the same for all series, I through VI. Since the six series were spaced throughout the year, it was felt that the analysis of variance (ANOVA) provided a fair test of the null hypothesis of no time effect, without committing oneself to a specific form of time trend.

Following a finding of significance, one naturally wishes to know which subgroups of the six test series differ from which other subgroups and in which direction. This has been accomplished through the use of a statistical follow-up procedure known as Scheffé's S-method (Scheffé, H.

1959. The Analysis of Variance. John Wiley and Sons, Inc., New York.) Using the S-method, it is possible to do further significance testing upon subsets of the parameters under investigation. The results of the eight one-way ANOVA's are summarized below by means of standard ANOVA tables and, in cases of significance by a graphical schematic representation of the order relationships of the six LC_{50} values.

2.1 Rainbow Trout. NaCl

Table 2.1 ANOVA Table for Rainbow Trout in NaCl

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	1275.30	5	255.059	9.4005**
Within	732.58	27	27.133	---
Total	2007.88	32	---	---

**Significant at the 1% level.

The experimental mean LC_{50}'s (μ_i's) for the six test series are given in Table 2.2.

Table 2.2 Experimental Mean LC_{50} Values for Rainbow Trout in NaCl

Series (i)	LC_{50}(%) ($\hat{\mu}_i$)	No. Tests (n_i)
I	72.5	4
II	82.0	2
III	85.17	6
IV	77.88	8
V	91.38	8
VI	80	5

Since the F-ratio, 9.4005, with 5 and 27 degrees of freedom (Table 1) is significant, and at the 1% level, we now follow-up with the S-method in an effort to assign the reasons for the significance. Our findings are as follows:

1) μ_1 (the LC_{50} of series I) is significantly smaller than the remaining five.

2) μ_2, μ_3, μ_4 and μ_6 do not differ significantly from one another.

3) μ_5 is significantly larger than the remaining five.

Reference to Table 2.2 shows the following order relationships (smallest to largest) among the six estimated LC_{50} values:

$$\hat{\mu}_1 < \hat{\mu}_4 < \hat{\mu}_6 < \hat{\mu}_2 < \hat{\mu}_3 < \hat{\mu}_5.$$

If we arrange the population LC_{50}'s in the same order and place an underline beneath those sets of parameters which the S-method tells us do not differ significantly, we obtain the following graphical schematic representation:

$$\underline{\mu_1\ \mu_4\ \mu_6\ \mu_2\ \mu_3\ \mu_5}.$$

Thus we would conclude that during the time of year when the tests of series I were being conducted, rainbow trout were significantly more sensitive to NaCl than they were during other series, during series V they were significantly less sensitive than they were during other series, and during test series II, III, IV and VI they did not differ in sensitivity but were intermediate between series I and V.

This analysis is not meant to suggest that there is exclusively a seasonal effect operating here, though that is one of several possibilities. Other explanatory variables should be investigated as possible causal mechanisms, such as variations in pH, temperature, dissolved oxygen, alkalinity, laboratory handling procedures, etc. Such an investigation is beyond the scope of this report.

2.2 Golden Shiner. NaCl

Table 2.3. ANOVA Table for Golden Shiner in NaCl

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	95.703	5	19.1406	1.184 (NS)[1]
Within	468.875	29	16.1681	---
Total	564.578	34	---	---

[1] NS ≡ Not significant at the 5% level.

Table 2.4. Experimental Mean LC_{50} Values for Golden Shiner in NaCl

Series (i)	$LC_{50}(5)$ $(\hat{\mu}_i)$	No. Tests (n_i)
I	75.33	6
II	79.00	4
III	77.20	5
IV	74.38	8
V	74.63	8
VI	73.50	8

Table 2.3 shows a non-significant F-ratio. Following acceptance of the null hypothesis that all population means are equal, the common LC_{50} is estimated to be $\hat{\mu} = 75.43$.

2.3 Fathead Minnow. NaCl

Table 2.5. ANOVA Table for Fathead Minnow in NaCl

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	100.031	5	20.0063	1.7320 (NS)[1]
Within	473.594	41	11.5511	---
Total	573.625	46	---	---

[1] NS ≡ Not significant at the 5% level.

Table 2.6. Experimental Mean LC_{50} Values for Fathead Minnow in NaCl

Series (i)	LC_{50}(%) ($\hat{\mu}_i$)	No. Tests (n_i)
I	76.14	7
II	78.00	8
III	76.63	8
IV	78.38	8
V	75.25	8
VI	74.25	8

The common LC_{50} is estimated to be $\hat{\mu} = 76.45$.

2.4 Three-Spine Stickleback. NaCl

Table 2.7. ANOVA Table for Three-Spine Stickleback in NaCl

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	22,823.2	5	4,564.64	20.5785**
Within	7,541.8	34	221.82	---
Total	30,365.0	39	---	---

**Significant at the 1% level.

Table 2.8. Experimental Mean LC_{50} Values for Stickleback in NaCl

Series (i)	LC_{50}(%) ($\hat{\mu}_i$)	No. Tests (n_i)
I	113.00	6
II	102.75	4
III	117.71	7
IV	74.00	8
V	52.63	8
VI	97.57	7

$$\underline{\mu_5\ \mu_4}\ \underline{\mu_6\ \mu_2\ \mu_1\ \mu_3}$$

The schematic representation above tells us that jointly μ_5 and μ_4 are less than μ_6 (and hence μ_1, μ_2 and μ_3). There is insufficient evidence that μ_5 differs from μ_4, which could be grouped also with μ_6 and μ_2. If we wished to form just two subgroups of series, we would place μ_4 and μ_5 into one group with an estimated joint LC_{50} of $\hat{\mu}_{45} = 63.31$, and μ_1, μ_2, μ_3, μ_6 into the other, with an estimated joint LC_{50} of $\hat{\mu}_{1236} = 108.17$.

Note the extremely large internal variability of stickleback, with an error mean square of 221.816 (from Table 2.7). This is about three times as large as the next largest error mean square (73.0438 for fathead minnow, PCP) and more than 22 times as large as the smallest error mean square (9.84696 for rainbow trout, PCP).

2.5 Rainbow Trout. PCP

Table 2.9. ANOVA Table for Rainbow Trout in PCP

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	1,183.50	5	236.70	24.038**
Within	305.26	31	9.85	---
Total	1,488.76	36	---	---

**Significant at the 1% level.

Table 2.10. Experimental Mean LC_{50} Values for Rainbow Trout in PCP

Series (i)	LC_{50}(%) (μ_i)	No. Tests (n_i)
I	20.88	8
II	21.50	2
III	23.83	6
IV	11.00	8
V	23.30	2
VI	27.57	7

$$\underline{\mu_4\ \mu_1\ \mu_2\ \mu_5\ \mu_3}\ \mu_6$$

The schematic representation tells us that rainbow trout were significantly more sensitive to PCP during Series IV than during any other

series. Furthermore the LC_{50} of Series I is significantly less than that of Series VI. However Series II, III and V can be grouped with either Series I or Series VI. There is insufficient evidence to effect a clear separation.

2.6 Golden Shiner. PCP

Table 2.11. ANOVA Table for Golden Shiner in PCP

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	3,728.22	5	745.644	15.995**
Within	1,258.69	27	46.618	---
Total	4,986.91	32	---	---

**Significant at the 1% level.

Table 2.12. Experimental Mean LC_{50} Values for Golden Shiner in PCP

Series (i)	LC_{50}(%) (μ_i)	No. Tests (n_i)
I	51.67	3
II	71.67	3
III	72.75	4
IV	62.14	7
V	49.63	8
VI	43.63	8

$\mu_6\ \mu_5\ \mu_1\ \mu_4\ \mu_2\ \mu_3$

Here we see that μ_6, μ_5 and μ_1 are significantly less than μ_4, μ_2 and μ_3. However, there is insufficient evidence to effect a clear separation between the Series I LC_{50} and the Series IV LC_{50}.

2.7 Fathead Minnow. PCP

Table 2.13. ANOVA Table for Fathead Minnow in PCP

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	1,320.08	5	264.016	3.614*
Within	2,921.75	40	73.044	---
Total	4,241.83	45	---	---

*Significant at the 5% level, but not at the 1% level.

Table 2.14. Experimental Mean LC_{50} Values for Fathead Minnow in PCP

Series (i)	LC_{50}(%) ($\hat{\mu}_i$)	No. Tests (n_i)
I	66.25	8
II	67.25	8
III	61.00	6
IV	58.13	8
V	56.38	8
VI	52.50	8

μ_6 μ_5 μ_4 μ_3 μ_1 μ_2

All that we can say conclusively in this case is that μ_6 is significantly less than μ_2. However, the intermediate LC_{50}'s, μ_1, μ_3, μ_4 and μ_5 could be grouped either with μ_6 on the low side or with μ_2 on the high side. It is worth noting that with the exception of the reversal of Series I and Series II, there is a steady increase in sensitivity throughout the six series.

2.8 Three-Spine Stickleback. PCP

Table 2.15. ANOVA Table for Three-Spine Stickleback in PCP

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	305.371	5	61.0742	2.986*
Within	777.176	38	20.452	---
Total	1,082.55	43	---	---

*Significant at the 5% level, but not at the 1% level.

Table 2.16. Experimental Mean LC_{50} Values for Stickleback in PCP

Series (i)	LC_{50}(%) ($\hat{\mu}_i$)	No. Tests (n_i)
I	31.43	7
II	31.00	6
III	28.57	7
IV	37.00	8
V	31.63	8
VI	30.63	8

μ_3 μ_6 μ_2 μ_1 μ_5 μ_4

Here we see that sensitivity was greatest during Series III and least during Series IV. Series I, II, V and VI can be grouped with either Series III or Series IV, however.

3. Comparison of Species

It will be instructive to examine the data from another aspect. In particular we now wish to compare species across time. To this end we again adopt the mathematical model of the one-way analysis of variance:

$$Y_{ij} = \xi_i + e_{ij},\ j=1,\ldots,n_i,\ i=1,\ldots,4,$$

where, similarly to the previous analysis, Y_{ij} is the DFG-calculated sample LC_{50} value, in percent, for the j^{th} experiment conducted on species i and ξ_1, ξ_2, ξ_3 and ξ_4 are the population mean LC_{50} values for rainbow trout, golden shiner, fathead minnow and three-spine stickleback, respectively. Finally, the e_{ij} are random error terms, assumed to be independently normally distributed for all i and j, with mean zero and common variance, σ^2. For a given test series (I through VI) and a given reference toxicant (NaCl or PCP), the null hypothesis tested was

$$H_0 : \xi_i = \xi,\ i=1,\ldots,4.$$

That is, the hypothesis tested was that the population mean LC_{50} values are the same for all species of fish tested. As in Section 2, we use the Scheffé S-method as a follow-up procedure following a finding of

significance in any of the twelve one-way layouts. The results of the twelve one-way ANOVA's are summarized below by means of standard ANOVA tables and, in cases of significance by a graphical schematic representation of the order and grouping relationships of the four species LC_{50} values.

3.1 Series I, NaCl

Table 3.1. ANOVA Table for Series I, NaCl

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	6,438.64	3	2,146.21	14.6727**
Within	2,779.19	19	146.273	---
Total	9,217.83	22	---	---

**Significant at the 1% level.

Table 3.2. Experimental Mean Values for Series I, NaCl

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. Tests (n_i)
1 (R.T.)	72.50	4
2 (G.S.)	75.33	6
3 (F.M.)	76.14	7
4 (St.)	113.00	6

$\underline{\xi_1\ \xi_2\ \xi_3\ \xi_4}$

The calculated F-ratio in Table 3.1 above tells us that there are significant differences among the four species with respect to their sensitivities to NaCl during Series I. Our follow-up procedure, the Scheffé S-method, as summarized by the order and grouping relationship below Table 3.2, tells us that stickleback is responsible for the rejection and that there are no statistically significant differences among rainbow trout, golden shiner and fathead minnow. The estimated grouped mean LC_{50} value for the three latter species is $\hat{\xi}_{123} = 75.00$.

3.2 Series II, NaCl

Table 3.3. ANOVA Table for Series II, NaCl

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	1,801.75	3	600.583	5.7014**
Within	1,474.75	14	105.339	---
Total	3,276.50	17	---	---

**Significant at the 1% level.

Table 3.4. Experimental Mean LC_{50} Values for Series II, NaCl

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. Tests (n_i)
1 (R.T.)	82.00	2
2 (G.S.)	79.00	4
3 (F.M.)	78.00	8
4 (St.)	102.75	4

$\underline{\xi_3\ \xi_2\ \xi_1}\ \xi_4$

Again we have significance, and again we see that one possible grouping is $\{\xi_1,\xi_2,\xi_3\}$ vs. ξ_4 alone, although ξ_1 could also be grouped with ξ_4.

3.3 Series III, NaCl

Table 3.5. ANOVA Table for Series III, NaCl

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	7,765.67	3	2,588.56	94.1391**
Within	604.938	22	27.4972	---
Total	8,370.61	25	---	---

**Significant at the 1% level.

Table 3.6. Experimental Mean LC_{50} Values for Series III, NaCl

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. Tests (η_i)
1 (R.T.)	85.17	6
2 (G.S.)	77.20	5
3 (F.M.)	76.63	8
4 (St.)	117.71	7

$\underline{\xi_3\ \xi_2\ \xi_1\ \xi_4}$

3.4 Series IV, NaCl

Table 3.7. ANOVA Table for Series IV, NaCl

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	125.594	3	41.8646	1.7273 (NS)[1]
Within	678.625	28	24.2366	---
Total	804.219	31	---	---

[1] NS ≡ Not significant at the 5% level.

Table 3.8. Experimental Mean LC_{50} Values for Series IV, NaCl

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. Tests (n_i)
1 (R.T.)	77.88	8
2 (G.S.)	74.38	8
3 (F.M.)	78.38	8
4 (St.)	74.00	8

$\underline{\xi_4\ \xi_2\ \xi_1\ \xi_3}$

There are no statistically significant differences among the four species. The estimated common LC_{50} value is $\hat{\xi} = 76.16$.

3.5 Series V, NaCl

Table 3.9. ANOVA Table for Series V, NaCl

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	6,076.84	3	2,025.61	27.2794**
Within	2,079.13	28	74.2545	---
Total	8,155.97	31	---	---

**Significant at the 1% level.

Table 3.10. Experimental Mean LC_{50} Values for Series V, NaCl

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. Tests (n_i)
1 (R.T.)	91.38	8
2 (G.S.)	74.63	8
3 (F.M.)	75.25	8
4 (St.)	52.63	8

$\underline{\xi_4\ \xi_2\ \xi_3\ \xi_1}$

3.6 Series VI, NaCl

Table 3.11. ANOVA Table for Series VI, NaCl

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	2,486.42	3	828.807	10.3588**
Within	1,600.20	20	80.0102	---
Total	4,086.63	23	---	---

**Significant at the 1% level.

Table 3.12. Experimental Mean LC_{50} Values for Series VI, NaCl

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. Tests (n_i)
1 (R.T.)	80.00	5
2 (G.S.)	73.50	4
3 (F.M.)	74.25	8
4 (St.)	97.57	7

$$\underline{\xi_2\ \xi_3\ \xi_1}\ \underline{\xi_4}$$

The estimated common LC_{50} for species 1, 2 and 3 combined is $\hat{\xi}_{123} = 75.76$.

3.7 Series I, PCP

Table 3.13. ANOVA Table for Series I, PCP

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	9,321.86	3	3,107.29	119.772**
Within	570.75	22	25.9434	---
Total	9,892.61	25	---	---

**Significant at the 1% level.

Table 3.14. Experimental Mean LC_{50} Values for Series I, PCP

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. Tests (n_i)
1 (R.T.)	20.88	8
2 (G.S.)	51.67	3
3 (F.M.)	66.25	8
4 (St.)	31.43	7

$$\underline{\xi_1\ \xi_4\ \xi_2\ \xi_3}$$

Note that in this case each species is distinct from all remaining species.

3.8 Series II, PCP

Table 3.15. ANOVA Table for Series II, PCP

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	9,321.86	3	3,107.29	119.772**
Within	570.754	22	25.9434	---
Total	9,892.62	25	---	---

**Significant at the 1% level.

Table 3.16. Experimental Mean LC_{50} Values for Series II, PCP

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. Tests (n_i)
1 (R.T.)	21.50	2
2 (G.S.)	71.67	3
3 (F.M.)	67.25	8
4 (St.)	31.00	6

$$\xi_1 \; \xi_4 \; \xi_3 \; \xi_2$$

The estimated common LC_{50} values for species (1,4) and (2,3) are $\hat{\xi}_{14} = 28.63$, $\hat{\xi}_{23} = 68.45$.

3.9 Series III, PCP

Table 3.17. ANOVA Table for Series III, PCP

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	9,140.44	3	3,046.81	23.5008**
Within	2,463.30	19	129.647	---
Total	11,603.74	22	---	---

**Significant at the 1% level.

Table 3.18. Experimental Mean LC_{50} Values for Series III, PCP

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. Tests (n_i)
1 (R.T.)	23.83	6
2 (G.S.)	72.75	4
3 (F.M.)	61.00	6
4 (St.)	28.57	7

$$\underline{\xi_1\ \xi_4\ \xi_3\ \xi_2}$$

The estimated common LC_{50} value for species 1 and 4 combined is $\hat{\xi}_{14} = 26.38$.

3.10 Series IV, PCP

Table 3.19. ANOVA Table for Series IV, PCP

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	12,797.8	3	4,265.94	171.469**
Within	671.727	27	24.8788	---
Total	13,469.5	30	---	---

**Significant at the 1% level.

Table 3.20. Experimental Mean LC_{50} Values for Series IV, PCP

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. tests (η_i)
1 (R.T.)	11.00	8
2 (G.S.)	62.14	7
3 (F.M.)	58.13	8
4 (St.)	37.00	8

$$\underline{\xi_1\ \xi_4\ \xi_3\ \xi_2}$$

The estimated common LC_{50} value for species 2 and 3 combined is $\hat{\xi}_{23} = 60.00$.

3.11 Series V, PCP

Table 3.21. ANOVA Table for Series V, PCP

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	5,058.01	3	1,686.00	113.873**
Within	384.957	26	14.806	---
Total	5,442.97	29	---	---

**Significant at the 1% level.

Table 3.22. Experimental Mean LC_{50} Values for Series V, PCP

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. Tests (η_i)
1 (R.T.)	23.33	6
2 (G.S.)	49.63	8
3 (F.M.)	56.38	8
4 (St.)	31.63	8

$\xi_1 \; \xi_4 \; \xi_2 \; \xi_3$

3.12 Series VI, PCP

Table 3.23. ANOVA Table for Series VI, PCP

Source	Sum of Squares	D.F.	Mean Square	F-Ratio
Among	3,104.41	3	1,034.80	59.2614**
Within	471.465	27	17.4617	---
Total	3,575.87	30	---	---

**Significant at the 1% level.

Table 3.24. Experimental Mean LC_{50} Values for Series VI, PCP

Species (i)	LC_{50}(%) ($\hat{\xi}_i$)	No. Tests (n_i)
1 (R.T.)	27.57	7
2 (G.S.)	43.63	8
3 (F.M.)	52.50	8
4 (St.)	30.63	8

$$\underline{\xi_1\ \xi_4\ \xi_2\ \xi_3}$$

The estimated common LC_{50} value for species 1 and 4 combined is $\hat{\xi}_{14} = 29.20$.

Two points are worthy of comment here. First, throughout Series I through VI, NaCl, there is no significant difference between golden shiner and fathead minnow. Second, with the exception of NaCl, Series V, in all those series in which there are statistically significant differences, stickleback is the least sensitive species. In Series V, NaCl, stickleback is the most sensitive species. This is consistent with the large variability, over time, which was noted in Section 2.4.

With respect to PCP, two things should be noted. First, rainbow trout, either alone or in concert with stickleback, is consistently significantly more sensitive than the remaining species. Second, with the exception of Series III, fathead minnow, either alone or in concert with golden shiner, is consistently significantly less sensitive than the remaining species. In Series III it is golden shiner alone, followed by fathead minnow alone, which is significantly less sensitive than the remaining species.

4. Variability Among Species over Time

The question"Is the variability among species homogeneous through time?" is a natural and interesting one. The proper vehicle for answering this question is the series of ANOVA tables of Section 3. Specifically, for a given toxicant and a given series, the variability among species is found in the "Among" (species) line in the "Mean Square" column of the ANOVA table. Let σ_i^2 denote the population variance of the LC_{50} values of the i^{th} series, where i ranges from I to VI. The

"Mean Square Among" (species) (or MS_A) is an unbiased estimate of σ_i^2. The null hypothesis, then, is

$$H_0 : \sigma_i^2 = \sigma^2, \quad i=1,\ldots,6,$$

where σ^2 is the common, but unknown, population variance. Let k denote the number of variances to be tested, ν_i the number of degrees of freedom for the i^{th} variance, or group, and let $N = \sum_{i=1}^{k} \nu_i$. In the present case we have $k=6$ (series) and $\nu_i = 3$ (the number of degrees of freedom is one less than the number of species tested). Then $N = \sum_{i=1}^{k} \nu_i = 18$. The test criterion is

$$M = N \ln\{\frac{1}{N} \sum_{i=1}^{k} \nu_i (MS_{Ai})\} - \sum_{i=1}^{k} \nu_i \ln(MS_{Ai}),$$

where MS_{Ai} is the "mean square, among" for the i^{th} test series. Under H_0, M is distributed approximately as χ^2 with $k-1=5$ degrees of freedom. Straightforward substitution into the equation above yields, for NaCl, $M = 10.043$. The critical value of chi square with five degrees of freedom at the 5% level of statistical significance is $\chi^2_{5;.95} = 11.070$. For PCP the value of M is $M = 1.732$. Since $M < 11.070 = \chi^2_{5;.95}$ for both NaCl and PCP we accept H_0 in both cases. That is, we conclude that there is insufficient evidence to reject H_0 at the 5% level of statistical significance, and behave as though we believe the variation among species is homogeneous through time.

5. Additional Suggestions in Connection with the Present Data or Future Toxicity Trials

5.1 Allocation of Fish to Concentrations

Based on a fixed number of concentrations and a fixed total number of fish, what allocation of fish to dosage levels will yield a minimum variance in the estimation of the LC_{50}? The recommendation of the DFG is to employ twenty to thirty fish at each concentration as a way of reducing the confidence interval for the LC_{50}. This is a sound suggestion, but preliminary statistical calculations show that one can expect even greater reductions in the length of the confidence interval by some other allocation than that of equal numbers at all dosage levels. If one is committed to a parametric model of a sigmoid dosage-response curve, then it seems clear that differing amounts of information are present along different parts of the curve. Specifically, it would appear that the maximum amount of information is to be found in the region in which the curve is changing most rapidly. This, of course, occurs in the vicinity of the LC_{50}. Thus, one fish placed at a concentration near the LC_{50} could be expected to yield more information about the LC_{50} than could the same fish placed at a concentration farther removed in either direction. One could thus expect an improvement in precision of estimation of the LC_{50} by moving some, but possibly not all, fish from the tails of the tolerance dosage curve and reallocating them toward the center of the distribution. This presupposes some prior experimentation in order to locate, at least approximately, the region in which the LC_{50} is expected

to lie. Thus the statistical problem is one of determining the optimal allocation of a fixed total number of fish to some preassigned number of levels of concentration.

Obviously, the optimum allocation problem described above is a subject only for future similar toxicity trials.

5.2 Multivariate Statistical Analysis

The present statistical analysis, together with the DFG-generated estimates of the various LC_{50} values on which the analysis is based, fixes attention on only a single parameter of the sigmoid-shaped dosage-response curve. This parameter, the more important of the (usually) two parameters in any such curve, is, of course, the LC_{50}, which can also be described as the "location" parameter of any such family of curves. Ignored, but not forgotten in the statistical analysis has been the second parameter, the "shape" parameter of the family of dosage-response curves. This parameter determines how steeply the curve climbs from near zero at low dosages (or concentrations, in the present case) to near one at high dosages. It thus determines the "shape" of a dosage-response curve, whereas the LC_{50} determines its "location". A more nearly complete analysis would continue to assess the statistical significance of the LC_{50}, while simultaneously taking account of the behavior and influence of the estimate of the shape parameter upon the estimate of the location parameter (LC_{50}). The enlarged statistical problem, then, no longer remains a univariate statistical problem, but rather becomes a bivariate statistical problem, accessible by a substantial body of multivariate statistical methods. Such methods can be applied to the present

study, which contains adequate experimental data for the estimation of both parameters.

5.3 Recognition of the Time Domain

Finally, it should be noted that the present series of experiments conducted by the DFG has yielded a wealth of experimental data, only a fraction of which has been used in the computation of LC_{50}'s and, perforce, in the subsequent statistical analysis. Specifically, for each level of concentration, there are fish-kill counts at 24, 48, 72 and 96 hours, of which only the 96-hour counts are used. There are, in the statistical literature, methods which make effective use of the time-domain as well as of the concentration domain. Specifically, although there are, in the present case, only four time intervals, one could make use of life-table methods, of which there are many.

The use of such methods could enlarge the scope of scientific questions which could be asked of, and answered by the experimental data. For example, for a given toxicant at a reference concentration, one could ask "What is the median lethal time (LT_{50}) for a given species?"

In addition, one might hope to achieve certain economies by shortening the total length of time for a typical test to something significantly less than 96 hours. Specifically, if one could devise a suitable mathematical model for a two-dimensional dosage-response surface (an obvious extension of the present widely-used one-dimensional dosage-response curves), there would automatically follow methods of estimating the parameters of such a model. If this estimation could be done satisfactorily

at a sufficiently early time in the test, the test could be safely discontinued and what is now the estimated LC_{50} at 96 hours could become the predicted LC_{50} at 96 hours. Criteria for early discontinuance of a test could be based on the size of confidence regions for the parameters of the model, a straightforward statistical procedure.

If the foregoing were deemed desirable, one might then wish to consider modifications of the experimental protocol aimed at improving the precision of the parameter estimates. One such technique might be increased surveillance of each fish tank in order to fix more precisely the exact time of death of each fish therein. If this could be automated, so much the better. For example, if there existed a device which could detect and record as little as a five percent drop in the metabolic activity of a total biomass, then one could automate the monitoring of a tank of twenty fish. This, of course, is a suggestion for future consideration.