OWEP-82-001

IN SITU ACUTE/CHRONIC TOXICOLOGICAL MONITORING OF INDUSTRIAL EFFLUENTS FOR THE NPDES BIOMONITORING PROGRAM USING FISH AND AMPHIBIAN EMBRYO-LARVAL STAGES AS TEST ORGANISMS

SEPTEMBER 1981

WESLEY J. BIRGE

JEFFREY A. BLACK

OFFICE OF WATER ENFORCEMENT AND PERMITS

ENVIRONMENTAL PROTECTION AGENCY

WASHINGTON, D.C. 20460

OWEP-82-001

IN SITU ACUTE/CHRONIC TOXICOLOGICAL MONITORING OF INDUSTRIAL EFFLUENTS FOR THE NPDES BIOMONITORING PROGRAM USING FISH AND AMPHIBIAN EMBRYO-LARVAL STAGES AS TEST ORGANISMS

September 1981

Final Report

EPA Contract No. 68-01-5052
Directive of Work No. 14
JRB Associates Subcontract No. 2800-03-218

Wesley J. Birge

Jeffrey A. Black

University of Kentucky
Lexington, Kentucky 40506

Project Manager, DOW 14
Stephen L. Bugbee (First Year)
William F. Brandes (Second Year)

JRB Representative
Eddy J. Forman

NOTICE

This report has been reviewed through the Office of Water Enforcement and Permits, EPA, and approved. Approval does not signify that the contents necessarily reflect the views and policies of the Environmental Protection Agency, nor does mention of trade names or commercial products constitute indorsement or recommendation for use.

TABLE OF CONTENTS

TABLE OF CONTENTS - continued.

LIST OF TABLES

LIST OF TABLES - continued.

LIST OF FIGURES

ACKNOWLEDGMENTS

We should like to gratefully acknowledge the use of certain facilities and the helpful advice and counsel received from William Peltier, Ron Weldon, and others at the Region IV EPA Laboratory, Athens, Georgia. In addition, we are deeply indebted to personnel and graduate students in our laboratory who contributed to this study or to the preparation of the manuscript, including William E. McDonnell, Albert G. Westerman, Barbara A. Ramey, Stephen Ballard, Donald M. Bruser, Richard Cassidy, William H. Benson, Paul C. Francis, Bobbie Welch, and Pamela Robertson. We should also like to express our gratitude to Eddy Forman, JRB Coordinator, and to William Brandes and Stephen Bugbee, Office of Water Enforcement and Permits, EPA, who served as project managers. We also are appreciative of help received from Robert Logan (Kentucky Division of Water Quality) whose efforts were instrumental in obtaining certain effluent test samples.

ABSTRACT

The Clean Water Act has necessitated further requirements and revisions in the industrial permit program maintained under the National Pollutant Discharge Elimination System (NPDES). It is now evident that in many cases the identification and control of toxic substances cannot be accomplished solely on the basis of an effluent guideline approach, emphasizing the need for reliable and economical procedures with which to quantify directly the net toxicity of complex effluents and estimate acute and chronic effects on aquatic biota. Therefore, the major objective of this investigation was to develop and evaluate fish and amphibian embryo-larval test procedures for the toxicological characterization of municipal and industrial effluents.

In both laboratory and on-site studies, flow-through, static-renewal, and static tests were conducted simultaneously on a broad array of effluents. The principal test organisms included the African clawed frog (Xenopus laevis), bluegill sunfish (Lepomis macrochirus), channel catfish (Ictalurus punctatus), fathead minnow (Pimephales promelas), and rainbow trout (Salmo gairdneri). Except with the trout, exposure was initiated at fertilization and usually maintained through 4 days posthatching, giving exposure periods of 6 to 9 days depending upon the species. In most studies with the trout, exposure was started either at fertilization or at the eyed-egg stage and continued for 9 days. Combining frequencies for mortality and gross teratogenesis, probit analysis was used to calculate LC_{50} and LC_1 values with 95% confidence limits. The LC_1 was defined as the toxicity threshold and was expressed as the effluent concentration (% by volume) required to produce 1% control-adjusted impairment (i.e., mortality, terata) of test populations. Flow-through tests consistently provided the most reliable evaluations of effluent toxicity and usually gave the lowest LC_{50} values. However, LC_{50}'s determined in flow-through and static-renewal tests usually differed only by a factor of 2 or less, and the more economical static-renewal procedure was considered adequate for most routine toxicological screening. Static tests generally proved more variable and less sensitive and were not recommended. Concerning the different animal species, optimum results were obtained with the bluegill sunfish, channel catfish, fathead minnow, and rainbow trout, but numerous other species should prove satisfactory for embryo-larval effluent testing.

The majority of effluents were collected from chemical, rubber, and plastics manufacturing plants, metal plating plants, and sewage treatment plants. Of 19 industrial and municipal effluents or effluent components studied, 18 exhibited moderate to high toxicity in embryo-larval tests. Ten of these were final NPDES effluents which entered receiving waters and, taking the most sensitive test in each case, the LC_{50} values were 0.3%, 6.4%, 6.6%, and 21.6% for 4 major effluents analyzed on site and 0.04%, 9.4%, 29.3%, 39.2%, 43.0%, and 100% for the remaining 6 NPDES effluents

which were tested only in the laboratory. Corresponding LC_1 values ranged from 0.001% to 2.63% for the 6 NPDES effluents analyzed in the laboratory and 0.001% to 0.8% for those analyzed on-site. During these 4 on-site studies, conducted using a mobile laboratory, effluent samples also were collected and transported to the laboratory for simultaneous testing. In all instances, effluent toxicity was substantially less when measured in the laboratory. Also in the 4 major field biomonitoring studies, both fish acute and embryo-larval tests were performed concurrently on the NPDES effluents. The acute toxicity determinations were conducted by the EPA region IV biomonitoring team and consisted of 96-hr flow-through tests with the fathead minnow. Embryo-larval tests gave more reliable detection and better quantification of effluent toxicity. The LC_{50} and LC_1 values for the 4 effluents, determined in flow-through tests with the fathead minnow, ranged from 0.3% to 29.4% and 0.001% to 2.8%, respectively, and narrow 95% confidence intervals were observed in all cases. In the acute tests, it was not possible to determine LC_{50} values for 3 of the 4 effluents. The acute LC_{50} for the most toxic effluent was 8.0% and this was about 27 times the embryo-larval LC_{50} (0.3%), and it differed from the embryo-larval LC_1 value (0.001%) by more than 3 orders of magnitude.

On the basis of these and other results, it was concluded that embryo-larval tests provided a sensitive, reliable, and economical means of quantifying the toxicity of complex effluents. Test results (i.e., LC_1) also can be used to calculate dilution factors required to preclude significant mortality and teratogenesis of sensitive reproductive stages. Such dilution factors, together with transport-fate data and other essential information, should prove useful in assessing the impact of an effluent upon its receiving system. In 3 on-site studies, tests also were conducted on effluent components or effluents at different stages of treatment to determine the utility of embryo-larval biomonitoring for identifying toxic effluent fractions and for determining the effectiveness of waste treatment processes. Results indicated that more accurate and definitive assessments were possible with toxicity data than with chemical parameters.

In view of results of this and earlier studies, it also was proposed that embryo-larval tests can be used to estimate effluent concentrations that produce chronic effects on aquatic biota and which result in long-term ecological degradation. In addition, embryo-larval test procedures developed for effluent biomonitoring appeared equally suitable for direct evaluations on receiving waters. In determinations made under actual environmental conditions, net effects of important variables, including toxic interactions and other factors which affect the toxicity and bioavailability of effluent contaminants, are directly reflected in test responses.

INTRODUCTION AND OBJECTIVES

The Federal Water Pollution Control Act, as amended in 1977 by the Clean Water Act (PL 95-217), provides statutory limitations on toxic discharges which may endanger water quality and environmental health. Under section 101(a)(3), it is stated that the national goal is to prohibit discharges of toxic pollutants in toxic amounts. Compliance with the Clean Water Act has necessitated further requirements and revisions in the industrial permit program maintained under the National Pollutant Discharge Elimination System (NPDES). Recent experiences gained in implementing and enforcing NPDES regulations have reaffirmed the fact that in many cases identification and control of toxic substances cannot be accomplished solely on the basis of an effluent guideline approach, and the Environmental Protection Agency's Office of Water Enforcement and Permits has stressed the need for biomonitoring to provide supplemental toxicity data. This emphasizes the necessity for reliable and scientifically valid procedures with which to obtain direct quantification of the net toxicity of complex effluents and to estimate acute and chronic effects on aquatic biota of receiving waters. Accordingly, this project was undertaken to design test systems and develop methods for in situ (on-site) embryo-larval toxicological monitoring of industrial wastes, and to evaluate such test systems for toxicity screening as applied in the NPDES program.

The principal objectives of this study included 1) development of embryo-larval test systems using fish and amphibian species for on-site

toxicological evaluations of complex effluents; 2) application of embryo-larval test systems to on-site biomonitoring of municipal and industrial effluents; 3) comparisons of sensitivity and reliability of embryo-larval and acute effluent biomonitoring; 4) evaluation of embryo-larval toxicity tests for use under the NPDES program; and 5) a description of effluent biomonitoring procedures using sensitive life-cycle stages of fish and amphibians. Further work involved determining the reliability of laboratory testing of composite effluent samples, using direct on-site biomonitoring as a basis for comparison.

RATIONALE AND SCIENTIFIC BACKGROUND

Present effluent guidelines are based largely on general water quality characteristics (e.g., pH, BOD, suspended solids) and criteria for specific elements and compounds. However, many chemical, physical, and biological variables can affect the net toxicity of a complex effluent as well as its impact upon receiving waters and aquatic biota. In addition, toxicant criteria and other guidelines developed largely from laboratory tests are prone to some imprecision when applied under actual field conditions. This problem is further compounded by the fact that many municipal and industrial effluents contain large suites of contaminants which simultaneously enter receiving waters. Thus, using the present guideline approach, it is difficult 1) to quantify effluent toxicity accurately, 2) to assess the extent to which effluent toxicity varies with ambient conditions, and 3) to provide reliable estimates of environmental impact.

On-site biomonitoring has shown promise as a reliable means of quantifying biological effects of complex effluents (1, 2). Such technology, if properly applied, can provide direct toxicological readout (e.g., mortality, teratogenesis) for combined effects of full suites of toxicants contained in effluents and receiving waters. Net effects of toxic interactions and other variables are directly reflected in test responses. As the latter are determined under actual field conditions (e.g., ambient toxicant concentrations, pH, hardness, suspended solids), this minimizes the need to extrapolate from laboratory data. In addition to evaluating whole effluents, direct toxicological monitoring may be conducted simultaneously on effluent dilutions, permitting determination of dilution ratios required to reduce or preclude toxic effects. On-site toxicological monitoring also may be applied to effluent treatability, providing a quantitative basis for identifying toxic effluent fractions and/or evaluating effectiveness of waste treatment procedures (3).

Peltier (2), in an in-depth study, established the basis for applying acute toxicity testing to effluent biomonitoring, particularly under the NPDES program. In addition to developing a detailed test protocol for acute tests with fish and certain invertebrates, he clearly demonstrated the advantages of and indeed the necessity for direct toxicological characterization of complex effluents. He stated:

> Since it is not economically feasible to determine the toxicity of each of the thousands of potentially toxic substances in complex effluents or to conduct exhaustive chemical analyses of effluents, the most direct and cost-effective approach to the measurement of the toxicity of effluents is to conduct a bioassay with aquatic organisms representative of indigenous organisms in receiving waters.

As noted above, the purpose of this study was to investigate the use of embryo-larval testing to provide improved detection and quantification of effluent toxicity and to consider the use of such testing to estimate chronic toxicity. Several recent investigations lend support to this prospect. Using data from a substantial number of studies conducted with a broad selection of organic and inorganic toxicants, McKim (4) estimated maximum acceptable toxicant concentrations (MATC's) from embryo-larval responses observed through 30 to 90 days posthatching. A high correlation was established between these estimated MATC's and those determined in life-cycle tests. However, 30- to 90-day tests still exceed cost limitations required for successful implementation of on-site effluent biomonitoring under the NPDES program. More recently, Birge, et al. (5-7) conducted short-term embryo-larval tests on numerous inorganic and organic toxicants. Exposure was initiated at or soon after fertilization and continued through 4 to 8 days posthatching. Dose-response data were subjected to log probit analysis (8), and LC_1 values, defined as toxicant concentrations which produced 1% control-adjusted impairment in test populations, compared favorably with MATC's estimated or determined in 30- to 90-day embryo-larval and life-cycle tests (Tables 23, 24). As a number of fish and amphibian species suitable for testing have egg hatching times in the range of 2 to 5 days (e.g., bluegill sunfish, fathead minnow, Xenopus laevis), it appears plausible that embryo-larval tests of 6 to 9 days may constitute a sensitive, reliable, and cost-efficient means of screening effluents for toxicity. Despite the short duration of such tests, fertilized eggs, all embryonic

stages, and early larvae are subjected to exposure. Due to the great complexity of animal development, which involves gene expression and many synthetic processes regulated by sensitive enzyme systems, embryos and larvae are subject to a broad spectrum of toxicant-induced responses. For example, aquatic toxicants may affect biochemical, physiological, and other phenomena associated with 1) fertilization, 2) gene expression and cellular differentiation, 3) proliferation and growth, 4) systemic functions, and 5) the initial accommodation to a free-living existence. Due to their sensitivity and simple culture requirements, eggs and larvae of aquatic organisms are particularly suitable for use in toxicity testing (9, 10).

GENERAL WORK PLAN

This two-year investigation was initiated June 19, 1979, and the various studies conducted are summarized in Table 1. The initial task was to adapt embryo-larval procedures used in toxicity testing for application to effluent biomonitoring. Using a reference toxicant (i.e., phenol), coal-ash effluents, and effluents or process waters from selected industries, laboratory studies were undertaken to compare economy of operation and reliability of static, static-renewal, and flow-through test procedures and to modify conventional test systems for optimum use with the broad array of complex effluents likely to be encountered under the NPDES program. Specific tasks during the first year of the project were as follows.

- Perform laboratory embryo-larval tests on effluents (e.g., 24-hr composites) and a reference toxicant (i.e., phenol).

- o Compare applicability and reliability of embryo-larval static, static-renewal, and flow-through procedures for testing complex effluents.
- o Modify test systems for practical application to field testing.
- o Consider effects of experimental conditions (e.g., pH, DO) on toxicological monitoring and define test parameters which insure reliable and reproducible results.
- o Evaluate and compare sensitivity of alternative test animals (e.g., fish, amphibians) and select species suitable for use in effluent testing.
- o Design a mobile laboratory for on-site biomonitoring, including installation of embryo-larval test systems and necessary supporting facilities.

During the second year of the project, five on-site studies were conducted. The first involved a waste treatment plant in Lexington, Kentucky. Seven-day tests were performed simultaneously on two different sewage effluents. One was taken after primary treatment and the other was collected after secondary treatment but prior to chlorination. This initial field study was conducted 1) to evaluate and perfect set-up and testing procedures, 2) to develop adequate standards for quality assurance, and 3) to determine work loads, staff assignments, and management policies. In addition, selection of this monitoring site was intended to demonstrate the use of embryo-larval testing as a means of evaluating the effectiveness of waste treatment procedures.

During the remainder of the project, four principal on-site studies were conducted outside the Lexington area. These involved major industries selected in consultation with JRB Associates and EPA, and included the following:

1. Chemical Manufacturing Plant
2. Synthetic Rubber Plant
3. Metal Plating Plant
4. Tannery-Secondary Sewage Treatment Plant Complex

Under conditions agreed upon for this investigation, identification of specific industries was not to be disclosed. In each of the four cases, project requirements involved testing the final NPDES effluent. However, at two sites, tests also were conducted on various effluent components or on effluents at various levels of treatment. This concluding phase of the investigation involved several major tasks, including 1) final design and evaluation of on-site embryo-larval biomonitoring procedures for detecting and quantifying effluent toxicity, 2) use of on-site embryo-larval testing to determine effectiveness of waste treatment, 3) comparison of acute and embryo-larval biomonitoring as applied to complex effluents, and 4) comparison of on-site biomonitoring with laboratory testing of effluent samples.

For each NPDES effluent, embryo-larval testing was performed with static, static-renewal, and flow-through procedures, using the same animal species (e.g., fathead minnow, Xenopus laevis). In addition, four test organisms were compared for sensitivity using one or more test systems (e.g., static-renewal, flow-through). During on-site biomonitoring, effluent samples also were collected and transported to the laboratory (University of Kentucky) for simultaneous testing. Furthermore, studies at each of the last four sites were planned to coincide with visits by

the EPA Region IV biomonitoring unit. Using the same effluent and dilution water sources, the EPA team performed on-site acute toxicity tests with the fathead minnow, and this formed the basis of comparison between acute and embryo-larval biomonitoring.

Table 1. Toxicity tests and biomonitoring studies performed.

Test[1]	Study Site	Test System	Test Organism
Initial Laboratory Studies (First Year)			
Phenol	U.K. Lab[2]	Flow-through	*Xenopus laevis*
	U.K. Lab	Static-renewal	*Xenopus laevis*
	U.K. Lab	Static	*Xenopus laevis*
Coal-Ash Effluent	U.K. Lab	Flow-through	Rainbow Trout, Bluegill Sunfish, Bullfrog
	U.K. Lab	Static-renewal	Rainbow Trout, Bluegill Sunfish, Bullfrog
	U.K. Lab	Static	Rainbow Trout, Bluegill Sunfish, Bullfrog
Chemical Manufacturing Plant #1 Undiluted process water	U.K. Lab	Static-renewal	Bluegill Sunfish
Chemical Manufacturing Plant #1 Diluted process water (final effluent)	U.K. Lab	Static-renewal	Bluegill Sunfish
Chemical Manufacturing Plant #2 Cooling water and storm runoff (final effluent)	U.K. Lab	Static-renewal	Channel Catfish
Chemical Manufacturing Plant #3 Undiluted process water (final effluent)	U.K. Lab	Static-renewal	Channel Catfish
Synthetic Rubber Plant #1 Undiluted process water[3] (final effluent)	U.K. Lab	Static-renewal	Bluegill Sunfish
Synthetic Rubber Plant #2 Cooling water and storm runoff (final effluent)	U.K. Lab	Static-renewal	Channel Catfish
Plastics Manufacturing Plant Cooling water (final effluent)	U.K. Lab	Static-renewal	Channel Catfish

Table 1 - continued.

Test[1]	Study Site	Test System	Test Organism
Biomonitoring Studies (Second Year)			
Lexington Sewage Treatment Plant			
Primary effluent	On Site	Static-renewal	Channel Catfish
Unchlorinated secondary effluent	On Site	Static-renewal	Channel Catfish
	On Site	Flow-through	Channel Catfish
Chemical Manufacturing Plant			
Diluted process water (final effluent)	On Site	Flow-through	*Xenopus laevis*, Fathead Minnow
	On Site	Static-renewal	*Xenopus laevis*, Fathead Minnow, Rainbow Trout
	On Site	Static	*Xenopus laevis*, Fathead Minnow
	U.K. Lab	Static-renewal	*Xenopus laevis*
Synthetic Rubber Plant			
Undiluted process water[3] (final effluent)	On Site	Flow-through	*Xenopus laevis*, Fathead Minnow
	On Site	Static-renewal	*Xenopus laevis*, Fathead Minnow, Rainbow Trout
	On Site	Static	*Xenopus laevis*
	U.K. Lab	Static-renewal	Rainbow Trout
Tannery-Sewage Treatment Plant			
Chlorinated secondary effluent (final effluent)	On Site	Flow-through	*Xenopus laevis*, Fathead Minnow
	On Site	Static-renewal	*Xenopus laevis*, Fathead Minnow, Rainbow Trout
	On Site	Static	*Xenopus laevis*
	U.K. Lab	Static-renewal	Rainbow Trout

Table 1 - continued.

Test[1]	Study Site	Test System	Test Organism
Tannery-Sewage Treatment Plant (cont.)			
Unchlorinated secondary effluent	On Site	Static-renewal	*Xenopus laevis*, Fathead Minnow, Rainbow Trout
Tannery effluent	On Site	Static-renewal	*Xenopus laevis*, Fathead Minnow, Rainbow Trout
Metal Plating Plant			
Mixed waste (final effluent)	On Site	Flow-through	*Xenopus laevis*, Fathead Minnow
	On Site	Static-renewal	*Xenopus laevis*, Fathead Minnow
	On Site	Static	*Xenopus laevis*, Fathead Minnow
	U.K. Lab	Static-renewal	*Xenopus laevis*
Component 1 (sludge-bed filtrate)	On Site	Static-renewal	Fathead Minnow
Component 2 (process water)	On Site	Static-renewal	Fathead Minnow
Component 3 (brazing water)	On Site	Static-renewal	Fathead Minnow
Component 4 (cooling water)	On Site	Static-renewal	Fathead Minnow

[1]Final effluents include only those discharges which were subject to NPDES regulations.

[2]Laboratory of aquatic toxicology and ecology (W.J. Birge), School of Biological Sciences, University of Kentucky.

[3]The NPDES discharge contained undiluted process water mixed with certain other wastes (e.g., sanitary discharge).

EMBRYO-LARVAL BIOMONITORING SYSTEMS AND TEST PROCEDURES

Static, static-renewal, and flow-through systems. As noted above, procedures for effluent biomonitoring using fish and amphibian embryo-larval stages were modified from those previously developed for conventional toxicity testing (1, 5, 7, 9, 11). Deep petri dishes served as exposure chambers in all tests. These covered Pyrex dishes were modified for use in static-renewal and flow-through systems. In static-renewal tests, effluent and dilution water were changed at regular 12- or 24-hr intervals. A combination inlet-outlet tube was attached to the lower wall of the exposure chamber, approximately 8 mm above the bottom of the dish, to permit solution changes without disturbing test organisms. Generally 12 exposure chambers were used in each static-renewal test, permitting duplicate dishes for controls and each of five effluent concentrations (e.g., 100%, 50%, 10%, 1%, 0.1%). The static-renewal exposure chamber and the system used for effluent changes were described in an earlier publication (12) and are illustrated further in Figures 1 and 2. The chamber holds 400 mL of test solution, and approximately 50 mL remain when emptied to the level of the combination inlet-outlet. The renewal interval was 12 hrs, except in tests with phenol (reference toxicant) and effluents from the last four biomonitoring sites (Table 1). During these studies, solutions were changed at regular 24-hr intervals. Control populations were maintained with the same dilution water source and renewal interval as specified for the effluents. Static tests were performed using the same procedures

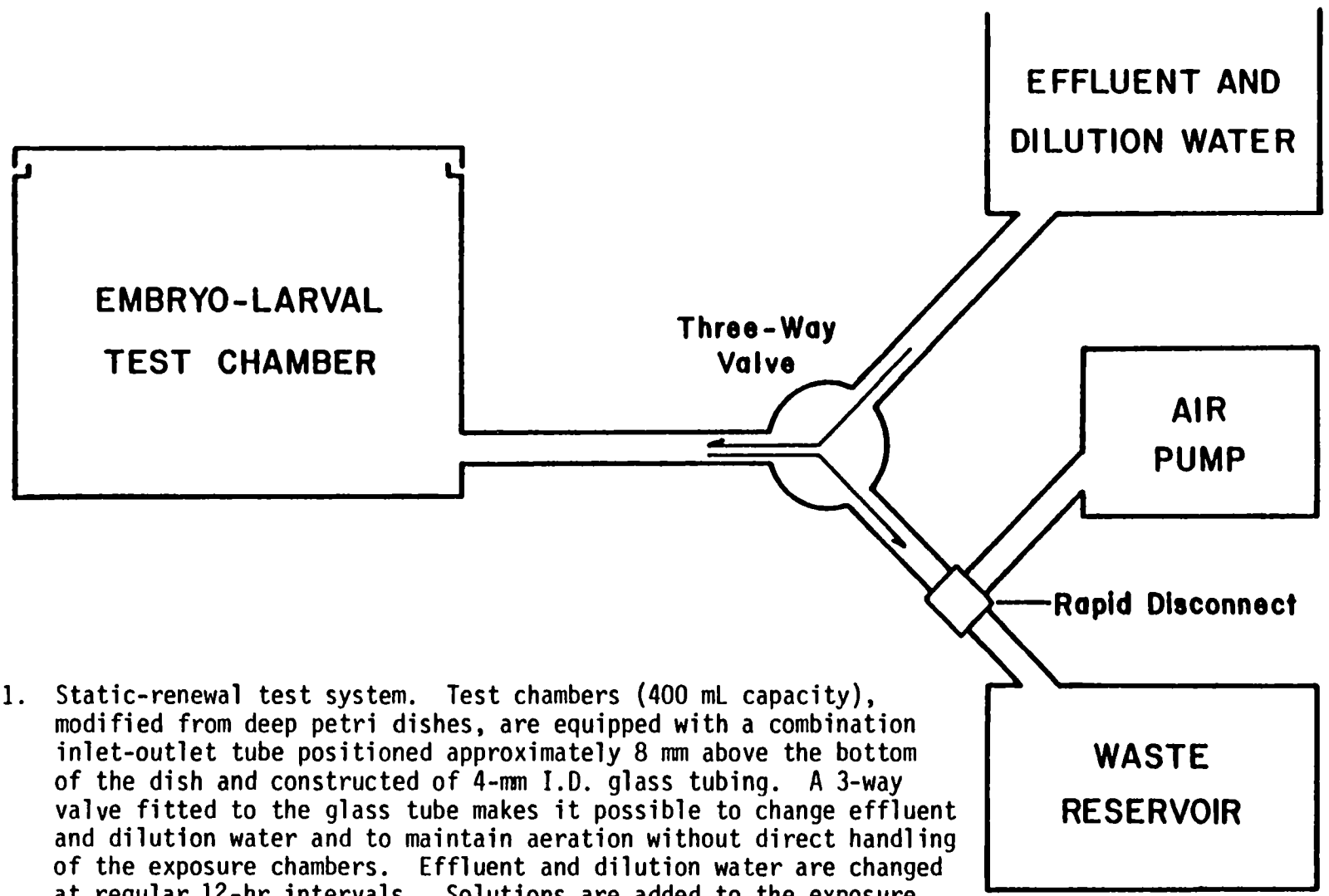


Figure 1. Static-renewal test system. Test chambers (400 mL capacity), modified from deep petri dishes, are equipped with a combination inlet-outlet tube positioned approximately 8 mm above the bottom of the dish and constructed of 4-mm I.D. glass tubing. A 3-way valve fitted to the glass tube makes it possible to change effluent and dilution water and to maintain aeration without direct handling of the exposure chambers. Effluent and dilution water are changed at regular 12-hr intervals. Solutions are added to the exposure chamber, using a 3" Pyrex funnel attached to the 3-way valve by a short section of tubing. Exposure chambers are emptied to the level of the combination inlet-outlet using an additional line attached to the 3-way valve. Between solution changes, this outlet line is used to provide continuous, moderate aeration to the exposure chambers if required to maintain dissolved oxygen at a minimum of 60% saturation.

as described for static-renewal testing, except that standard deep petri dishes served as exposure chambers and solution changes were omitted.

Inlet and outlet tubes (10 mm I.D. Pyrex) were annealed to petri dishes used as exposure chambers in the flow-through tests. The inlet was positioned approximately 7 mm above the bottom of the dish, and the outlet was attached on the opposite side, just below the shoulder. Volume to the level of the outlet was approximately 300 mL, and flow rate was maintained at 200 mL/hr, giving a retention time of 1.5 hr. Flow rate was monitored using either timed volumetric measurements or Gilmont no. 12 flow meters. Control water, full-strength effluent, and 50% effluent were delivered to respective exposure chambers using variable speed, multiple-channel peristaltic pumps. Effluent concentrations below 50% were obtained using a serial diluter system previously designed by Freeman and Birge (unpublished observations). The diluter was provided with standpipes which were adjusted as necessary to maintain constant dilution ratios. The latter were determined daily, using timed volumetric measurements on effluents and dilution water. Overall fluctuation in exposure concentration usually was within 5% and seldom exceeded 10%. Test chamber solutions were monitored daily for pH, alkalinity, hardness, conductivity, dissolved oxygen, and temperature. Test procedures and the diluter system are further described in the Appendix. In the studies that follow, only those toxic discharges which were subject to NPDES regulations are designated as final effluents.

Animal species and duration of exposure. During the first year of the project, all testing was conducted in the laboratory using a reference

toxicant (i.e., phenol), coal-ash effluents, and various industrial effluents (composite and grab samples). Animal species used in these embryo-larval investigations included the African clawed frog (Xenopus laevis), bluegill sunfish (Lepomis macrochirus), channel catfish (Ictalurus punctatus), and rainbow trout (Salmo gairdneri). Exposure was initiated within 12 hrs of fertilization and continued through 4 to 8 days posthatching. Average hatching times for the warmwater species (20-22°C) were 2 days for Xenopus, 3 days for bluegill, and 5 days for catfish. For the rainbow trout, the hatching period was 23 days (12.5°C). In addition, a limited number of 96-hr tests were initiated using 1-day-old larvae of the bullfrog (Rana catesbeiana).

During the second year, the initial on-site study was conducted at the Lexington Sewage Treatment Plant, and effluents from primary and secondary treatment were subjected to 7-day tests initiated with 1-day-old larvae of the channel catfish. During the four additional on-site biomonitoring investigations conducted in the second year, including the correlated laboratory studies, effluent toxicity tests were performed using embryos and larvae of Xenopus, fathead minnow, and rainbow trout. Egg exposure for warmwater species was initiated 8 to 12 hrs after fertilization, except during the last two on-site biomonitoring studies (i.e., Tannery-Sewage Treatment Plant Complex, Metal Plating Plant) in which exposure of fathead minnow eggs was delayed up to 24 hrs after spawning. However, as eggs were transported in an ice-packed container, thereby slowing developmental time, the exposure period to hatching did not vary significantly

from that observed in other tests with this species. In addition, transport conditions did not appear to affect egg viability, as the survival of control organisms ranged from 80% to 92% in flow-through and static-renewal tests. Exposure to effluents usually was maintained through 4 days post-hatching, except in a few on-site tests which were delayed due to the unavailability of eggs. In such cases, treatment was curtailed 3 days after hatching due to time constraints imposed on field investigations. In embryo-larval tests conducted with the warmwater species, temperature was maintained at 20-22°C and average hatching times were 2 days for Xenopus and 4.5 days for fathead minnows. In studies with the rainbow trout, temperature was maintained at 11.5-12.5°C and both fresh eggs and eyed eggs were used as test organisms. In the case of fresh eggs, exposure was initiated 30 min to 2 hr after fertilization and maintained for 9 days. Eyed eggs were exposed through 4 days posthatching and average hatching time was 5 days, giving an exposure period of 9 days.

Test responses and expression of data. Test responses included frequencies of egg hatchability, embryo-larval survival, and teratogenesis. Determinations of teratic organisms were limited to gross defects considered likely to preclude survival, as discussed previously by Birge, et al. (11, 13). Defects most commonly encountered were acute kyphosis, lordosis, scoliosis, and other gross anomalies of the vertebral column. Percent hatchability was based on all organisms, normal and aberrant, which lived to complete the hatching process and frequencies of teratic organisms observed among experimental and control animals were expressed as percentages

of newly hatched populations. Depending upon test conditions and the supply of eggs, sample size (organisms per dish) usually ranged from 50 to 100 and was never less than 25. In determining percent survival, teratic organisms were counted as lethals, except when tests were terminated prior to hatching (i.e., fresh trout eggs). Taking accumulative dose-response data at the end of the exposure period, log probit analysis (8) was used to determine LC_{50} values (percent effluent by volume) with 95% confidence limits. In addition, LC_1 values, defined as concentrations producing 1% control-adjusted impairment of test populations, were calculated for most effluents. Mortality consistently was the predominant test response. Moreover, as teratogenesis was based solely upon severe defects which preclude survival or normal reproduction and as it is particularly important to quantify effluent toxicity in terms of organismal impairments that affect population density and viability, it was deemed more appropriate to express combined test responses as lethal concentrations (LC) rather than as effective concentrations (EC).

INITIAL PERFORMANCE EVALUATIONS

Tests with reference toxicant. Phenol initially was selected as a reference toxicant with which to compare reliability and sensitivity of flow-through, static-renewal, and static test procedures. Embryo-larval stages of Xenopus laevis were used as test organisms and exposure was maintained from fertilization through 8 days posthatching, giving a treatment time of 10 days. In the flow-through study, good regulation

of phenol concentrations was obtained and the LC_{50} was 7.5 mg/L (Table 2). Though the duration of exposure was somewhat longer, this value was within the range of LC_{50}'s reported in a previous investigation with six other amphibian species (14). When embryo-larval responses were analyzed 4 days after hatching in phenol tests conducted at a water hardness of 100 mg/L as $CaCO_3$, LC_{50}'s varied from 0.04 to 0.23 mg/L when determined with more sensitive amphibian species (e.g., Rana pipiens, Rana catesbeiana) and LC_{50} values ranged from 2.45 to 9.87 mg/L in tests with more tolerant species (e.g., Bufo fowleri, Rana palustris). In toxicity tests conducted through 8 days posthatching at a water hardness of 50 mg/L, LC_{50} values were 0.54 and 1.19 mg/L when determined with embryo-larval stages of the rainbow trout and goldfish, respectively (9). This is in general agreement with the on-site effluent studies reported below, in which Xenopus embryos and early larvae usually were more tolerant than similar stages of the fathead minnow and rainbow trout.

Compared to results obtained in the flow-through test, there was approximately a two-fold reduction in toxicity when phenol was administered using static-renewal procedures, and no significant effects on test organisms were observed in the static test (Table 2). Though accurate mean phenol exposures could not be determined in static and static-renewal tests, analyses performed on water samples from the exposure chambers revealed substantial reductions in phenol concentration with time. Analyses performed during the static-renewal study indicated that actual phenol concentrations were within 5% of nominal concentrations when water samples

were collected at the beginning of the 24-hr renewal intervals. However, phenol concentrations usually dropped to 50% or less of nominal values when determinations were performed at the end of the 24-hr renewal intervals. By comparison, phenol retention at the conclusion of the static test generally was less than 10%. As biomass volume was not excessive (< 0.5 cc per exposure chamber), tissue uptake likely was not the principal factor affecting phenol exposure concentrations.

Tests with coal-ash effluent. The three embryo-larval test procedures were further evaluated in studies with a complex coal-ash effluent. The latter was produced in the laboratory, using procedures previously described by Birge (1). This involved flow-through washing of a 50-kg sample of precipitator-collected fly ash from a coal-fired power plant. The rate of flow was 1 L/hr and retention time in the fly-ash leaching chamber was 42 hrs. Effluent toxicity was evaluated using flow-through, static-renewal, and static tests with embryonic and larval stages of each of three organisms (i.e., rainbow trout, bluegill sunfish, bullfrog). The results are presented in Tables 3-5. Selected chemical characteristics of the influent water and the resulting effluent are given in Table 6. The same influent water source also was used to perform effluent dilutions and to maintain control organisms. Coal-ash effluents are known to contain detectable concentrations of many toxic metals, and trace metal concentrations decrease with leaching time (1). Due to their predictable leaching patterns and high concentrations in ash effluents, aluminum and zinc were selected as reference toxicants with which to compare exposures in the three test systems.

The LC_{50} values did not differ significantly when determined using flow-through or static-renewal procedures with bluegill embryo-larval stages or early bullfrog larvae (Tables 3, 4), and the median lethal concentrations varied by only a factor of two in tests with the trout (Table 5). However, toxicity of the coal-ash effluent was appreciably greater in static tests and this presumably was due to higher concentrations of toxic metals, as reflected by the values obtained for aluminum and zinc (Table 6). Several factors undoubtedly contributed to this situation. For example, concentrations of coal-ash metals (i.e., Al, Zn) were much more stable in aqueous test systems than was phenol. In addition, as noted above, metal concentrations decreased with leaching time and, therefore, the effluent became progressively less toxic during the course of testing. While this diminution in toxicity was reflected in the flow-through and static-renewal tests, mean toxicity over the duration of the exposure period was overestimated in the static system due to the one-time sampling of the ash effluent.

Initial tests with industrial effluents. The first application of embryo-larval toxicity testing to actual industrial effluents was undertaken in the laboratory. Composite and grab samples of seven different effluents from six major industries, including the final NPDES effluent from each, were evaluated using static-renewal procedures with developmental stages of the bluegill sunfish and channel catfish (Table 7). In all cases, tests were performed with undiluted effluent, as well as with dilutions ranging down to 0.01% effluent. Good delineation of dose-response data was observed by 4 days posthatching in all but one case, and LC_{50} values ranged from

0.04% for process water from Chemical Manufacturing Plant No. 3 to 43% for process water from Synthetic Rubber Plant No. 1. Lowest toxicity was observed for cooling water/storm runoff from Chemical Manufacturing Plant No. 2. Though the median lethal concentration could not be determined, it was estimated to be about 100% effluent.

The LC_1 values determined for the seven effluent sources ranged from 0.001% to 2.63%. The LC_1 was defined as the percent effluent concentration which produced 1% control-adjusted impairment in test populations, and this value can be used to estimate the effluent dilution required to preclude appreciable toxicity to embryos and early larvae. The LC_{50} and LC_1 values are summarized in Table 8 and general effluent characteristics are given in Table 9. No unusual variations in effluent characteristics were noted except for high conductivity readings obtained for four of the seven effluents. An extremely high conductivity of 75,000 µmhos/cm was observed for the most toxic effluent (process water, Chemical Manufacturing Plant No. 3). However, the next highest conductivity (4,420 µmhos/cm) was recorded for the second least toxic effluent (Synthetic Rubber Plant No. 1), indicating that there was no consistent correlation between conductivity and toxicity.

Table 2. Effects of phenol on embryo-larval stages of *Xenopus laevis* as determined in flow-through, static-renewal, and static tests.

Test System	Phenol Concentration (mg/L)		Percent Survival at 8 Days Posthatching[1]
	Nominal	Actual (mean ± S.E.)	
Flow-through	10.0	12.6 ± 0.6	42
	1.00	1.30 ± 0.06	73
	0.100	0.112 ± 0.012	95
	0.010	0.0096 ± 0.0011	98
	0.0010	0.0014 ± 0.0005	98
LC_{50} (mg/L) (95% confidence limits)			7.5 (4.7 - 14.2)
Static-renewal	10.0	-	56
	1.0	-	80
	0.1	-	86
	0.01	-	98
	0.001	-	100
LC_{50} (mg/L) (95% confidence limits)			18.0 (8.1 - 57.9)
Static	10.0	-	93
	1.0	-	97
	0.1	-	97
	0.01	-	99
	0.001	-	94
LC_{50} (mg/L)			ND[2]

[1]Average hatching time was 2 days, giving an exposure period of 10 days. Survival values were control-adjusted in this initial test.

[2]Not determined.

Table 3. Effects of coal-ash effluent on embryo-larval stages of the bluegill sunfish as determined in flow-through, static-renewal, and static tests.

Test System	Ash Effluent Concentration (Percent)	Percent Hatchability[1]	Percent Survival at 4 Days Posthatching
Flow-through	100	39(11)	33
	10	76(1)	73
	1	94	94
	0.1	95	94
	0.01	98	97
	Control	99	98
LC_{50} (% Effluent) (95% confidence limits)			43.2 (30.4 - 66.2)
Static-renewal	100	46(10)	42
	10	70(1)	68
	1	86	82
	0.1	94	94
	0.01	98	97
	Control	97	97
LC_{50} (% Effluent) (95% confidence limits)			52.3 (29.1 - 100)
Static	100	34(14)	24
	10	64(3)	59
	1	82	78
	0.1	91	88
	0.01	95	92
	Control	96	94
LC_{50} (% Effluent) (95% confidence limits)			19.4 (11.7 - 33.2)

[1]Egg hatchability was based on all animals, normal and aberrant, which completed hatching. Frequencies of teratic survivors appearing in hatched populations were expressed parenthetically.

Table 4. Effects of coal-ash effluent on newly hatched larvae of the bullfrog as determined in flow-through, static-renewal, and static tests.

Test System	Ash Effluent Concentration (Percent)	Percent Survival after 96 Hours
Flow-through	100	43
	10	75
	1	85
	0.1	91
	0.01	98
	Control	98
LC_{50} (% Effluent) (95% confidence limits)		83.5 (45.8 - 100)
Static-renewal	100	49
	10	70
	1	86
	0.1	95
	0.01	98
	Control	98
LC_{50} (% Effluent) (95% confidence limits)		95.1 (51.2 - 100)
Static	100	33
	10	65
	1	84
	0.1	94
	0.01	97
	Control	96
LC_{50} (% Effluent) (95% confidence limits)		31.0 (20.1 - 50.5)

Table 5. Effects of coal-ash effluent on embryo-larval stages of the rainbow trout as determined in flow-through, static-renewal, and static tests.

Test System	Ash Effluent Concentration (Percent)	Percent Hatchability[1]	Percent Survival at 4 Days Posthatching
Flow-through	100	23(8)	20
	10	69(2)	67
	1	84(1)	83
	0.1	92	92
	0.01	93	93
	Control	94(1)	93
LC_{50} (% Effluent) (95% confidence limits)			25.2 (20.3 - 31.1)
Static-renewal	100	41(15)	35
	10	83(5)	78
	1	91(3)	87
	0.1	94(1)	93
	0.01	95(1)	94
	Control	97	97
LC_{50} (% Effluent) (95% confidence limits)			53.4 (40.9 - 72.3)
Static	100	18(40)	11
	10	58(10)	52
	1	85(5)	81
	0.1	92(2)	90
	0.01	94(1)	93
	Control	97	97
LC_{50} (% Effluent) (95% confidence limits)			10.1 (8.0 - 12.6)

[1]Egg hatchability was based on all animals, normal and aberrant, which completed hatching. Frequencies of teratic survivors appearing in hatched populations were expressed parenthetically. Exposure was initiated at fertilization and continued through 4 days posthatching.

Table 6. Selected chemical characteristics of coal-ash effluent used in flow-through, static-renewal, and static embryo-larval tests.

Effluent Characteristics[1,2]	Test System			Dilution Water
	Flow-through	Static-renewal	Static	
pH	7.7 ± 0.1	7.9 ± 0.1	7.8 ± 0.1	7.8 ± 0.1
Alkalinity (mg/L as $CaCO_3$)	58 ± 2	61 ± 2	60 ± 1	65 ± 2
Hardness (mg/L as $CaCO_3$)	75 ± 7	70 ± 6	75 ± 3	74 ± 6
Conductivity (µmhos/cm)	145 ± 4	144 ± 5	141 ± 2	138 ± 3
Aluminum (µg/L)	20 ± 10	20 ± 10	120 ± 50	10 ± 10
Zinc (µg/L)	11.0 ± 1.9	14.0 ± 2.9	36.0 ± 10.8	5.0 ± 1.8

[1]Chemical characteristics expressed as mean ± standard error in tests with bluegill sunfish.

[2]Aluminum and zinc were selected as reference toxicants.

Table 7. Initial 12-hour static-renewal embryo-larval tests on composite and grab samples of industrial effluents analyzed in the laboratory.

Effluent Source	Test Species	Effluent Concentration (Percent)	Percent Hatchability[1]	Percent Survival	
				Hatching	4 Days Posthatching
Chemical Manufacturing Plant #1 Undiluted process water	Bluegill Sunfish	100	20(59)	8	0
		50	55(12)	48	22
		10	79(2)	77	63
		5	90	90	82
		1	95	95	91
		0.1	98	98	97
		0.01	98	98	97
		Control	99	99	98
LC_{50} (% Effluent) (95% confidence limits)					16.0 (13.1 - 19.2)
Chemical Manufacturing Plant #1 Diluted process water (final effluent)	Bluegill Sunfish	100	53(14)	45	0
		50	74(5)	71	47
		10	90(1)	89	79
		5	96	96	89
		1	98(1)	97	95
		0.1	98	98	95
		0.01	99	99	98
		Control	99	99	98
LC_{50} (% Effluent) (95% confidence limits)					29.3 (24.1 - 35.1)

Table 7 - continued.

Effluent Source	Test Species	Effluent Concentration (Percent)	Percent Hatchability[1]	Percent Survival	
				Hatching	4 Days Posthatching
Chemical Manufacturing Plant #2 Cooling water and storm runoff (final effluent)	Channel Catfish	100	59(7)	55	45
		50	84(2)	82	78
		10	93	93	91
		5	97	97	94
		1	96	96	93
		0.5	97	97	96
		0.1	100	100	98
		0.01	100	100	98
		Control	98	98	97
LC_{50} (% Effluent)					~100
Chemical Manufacturing Plant #3 Undiluted process water (final effluent)	Channel Catfish	100	0	0	0
		50	0	0	0
		10	0	0	0
		5	0	0	0
		1	0	0	0
		0.5	23	23	0
		0.1	76	76	33
		0.01	94	94	78
		Control	98	98	98
LC_{50} (% Effluent) (95% confidence limits)					0.04 (0.03 - 0.05)
Synthetic Rubber Plant #1 Undiluted process water (final effluent)	Bluegill Sunfish	100	45(6)	42	20
		50	65(3)	63	54
		10	83(1)	82	79
		5	91(1)	90	90
		1	93	93	92
		0.1	97	97	96
		0.01	100	100	99
		Control	98	98	97
LC_{50} (% Effluent) (95% confidence limits)					43.0 (34.1 - 55.

Table 7 - continued.

Effluent Source	Test Species	Effluent Concentration (Percent)	Percent Hatchability[1]	Percent Survival	
				Hatching	4 Days Posthatching
Synthetic Rubber Plant #2 Cooling water and storm runoff (final effluent)	Channel Catfish	100	34(14)	29	0
		50	57	57	17
		10	70	70	46
		5	86	86	72
		1	94	94	88
		0.5	98	98	96
		0.1	96	96	94
		0.01	97	97	95
		Control	98	98	97
LC_{50} (% Effluent) (95% confidence limits)					9.4 (6.7 - 13.0)
Plastics Manufacturing Plant Cooling water (final effluent)	Channel Catfish	100	40(10)	36	19
		50	68	68	53
		10	86	86	76
		5	98	98	92
		1	99	99	97
		0.5	98	98	96
		0.1	100	100	98
		0.01	98	98	98
		Control	99	99	99
LC_{50} (% Effluent) (95% confidence limits)					39.2 (28.6 - 55.4)

[1]Egg hatchability was based on all animals, normal and aberrant, which completed hatching. Frequencies of teratic survivors appearing in hatched populations were expressed parenthetically.

Table 8. LC_{50} and LC_1 values for composite and grab samples of industrial effluents analyzed in laboratory embryo-larval tests.

Effluent Source	Test Species	LC_{50} (% Effluent)	95% Confidence Limits	LC_1 (% Effluent)	95% Confidence Limits
Chemical Manufacturing Plant #3 Undiluted process water (final effluent)	Channel Catfish	0.04	0.03 - 0.05	0.001	0.0006 - 0.003
Synthetic Rubber Plant #2 Cooling water and storm runoff (final effluent)	Channel Catfish	9.4	6.7 - 13.0	0.34	0.12 - 0.67
Chemical Manufacturing Plant #1 Undiluted process water	Bluegill Sunfish	16.0	13.1 - 19.2	1.17	0.65 - 1.80
Chemical Manufacturing Plant #1 Diluted process water (final effluent)	Bluegill Sunfish	29.3	24.1 - 35.1	2.63	1.44 - 4.03
Plastics Manufacturing Plant Cooling water (final effluent)	Channel Catfish	39.2	28.5 - 55.4	1.27	0.36 - 2.67
Synthetic Rubber Plant #1 Undiluted process water (final effluent)	Bluegill Sunfish	43.0	34.1 - 55.0	1.43	0.56 - 2.65
Chemical Manufacturing Plant #2 Cooling water and storm runoff (final effluent)	Channel Catfish	~100	-	2.53	0.33 - 6.20

Table 9. General characteristics of composite and grab samples of industrial effluent analyzed in 12-hour static-renewal embryo-larval tests conducted in the laboratory.[1]

Effluent Source	Test Species	pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)	Dissolved Oxygen (mg/L)
Dilution Water	-	7.4 ± 0.2	66 ± 3	82 ± 7	183 ± 3	7.6 ± 0.2
Chemical Manufacturing Plant #1 Undiluted process water	Bluegill Sunfish	8.1 ± 0.2	218 ± 12	234 ± 25	1440 ± 25	6.7 ± 0.2
Chemical Manufacturing Plant #1 Diluted process water (final effluent)	Bluegill Sunfish	8.1 ± 0.2	250 ± 30	211 ± 19	360 ± 12	7.0 ± 0.2
Chemical Manufacturing Plant #2 Cooling water & storm runoff (final effluent)	Channel Catfish	7.7 ± 0.04	178 ± 2	109 ± 10	196 ± 6	7.7 ± 0.2
Chemical Manufacturing Plant #3 Undiluted process water (final effluent)	Channel Catfish	8.0 ± 0.5	-	287 ± 13	75000 ± 557	7.9 ± 0.2
Synthetic Rubber Plant #1 Undiluted process water (final effluent)	Bluegill Sunfish	7.9 ± 0.2	114 ± 4	251 ± 26	4420 ± 58	7.1 ± 0.2
Synthetic Rubber Plant #2 Cooling water & storm runoff (final effluent)	Channel Catfish	8.7 ± 0.04	197 ± 1	227 ± 41	1760 ± 10	7.5 ± 0.3
Plastics Manufacturing Plant Cooling water (final effluent)	Channel Catfish	7.7 ± 0.04	77 ± 1	144 ± 26	166 ± 4	7.6 ± 0.1

[1]Chemical characteristics expressed as mean ± standard error.

RESULTS OF ON-SITE BIOMONITORING OF MUNICIPAL AND INDUSTRIAL EFFLUENTS

During the field investigations, on-site embryo-larval biomonitoring experiments were performed at five different test locations, including 1) Lexington Sewage Treatment Plant, 2) Chemical Manufacturing Plant, 3) Synthetic Rubber Plant, 4) Tannery-Secondary Sewage Treatment Plant Complex, and 5) Metal Plating Plant. Biomonitoring was conducted in a mobile laboratory designed to accommodate flow-through, static-renewal, and static test systems, as described in the Appendix. Personnel from the EPA Region IV Laboratory were on site at the last four locations to perform acute effluent toxicity tests. These joint investigations provided an opportunity for direct comparisons of acute and embryo-larval biomonitoring data. The on-site biomonitoring experiments were performed using animal species and test procedures given above. Specific tests performed at the different field sites are summarized in Table 1. Though our principal responsibility was to evaluate final effluents designated under the NPDES program, additional tests were performed on individual effluent components (i.e., Metal Plating Plant) and effluents at different stages of treatment (i.e., Tannery-Secondary Sewage Treatment Plant Complex). Specifications on effluent production and treatment were obtained through the EPA Region IV Laboratory and were provided by plant officials.

The mobile laboratory was moved to each biomonitoring site 2 to 3 days prior to the actual onset of testing to allow adequate time for the assembly of exposure systems and stabilization of test conditions (e.g., flow rate,

temperature). In analyzing dose-response data, log probit analysis (8) was applied to combined frequencies of embryo-larval mortality and teratogenesis to determine control-adjusted LC_{50} values. These values represented exposure concentrations, expressed as percent effluent by volume, that produced 50% impairment of test populations.

During the period of August 1-12, 1980, the first on-site biomonitoring experiment was conducted at a Lexington sewage treatment plant. The plant received domestic sewage which was passed through a grit chamber to primary settling tanks with sludge collectors. Secondary treatment was achieved in an aerated activated sludge system, followed by clarification in secondary settling tanks. Effluent then received tertiary treatment in a lagoon system prior to chlorination and discharge. The mean wastewater flow at the time of study was approximately 7.61 million gallons per day. Toxicity tests on sewage effluents were conducted using larvae of the channel catfish. Treatment was initiated 1 day after hatching and continued for 7 days. Secondary sewage effluent (unchlorinated) was administered to test organisms in both the flow-through and static-renewal systems. In addition, primary sewage effluent was tested using static-renewal procedures. Carbon-filtered tap water (11), transported from the University of Kentucky laboratory to the biomonitoring site, was used for effluent dilutions and maintenance of control animals.

Solutions in all test chambers were monitored daily for general test parameters, using procedures described above. Temperature was regulated at 22.0 ± 0.5°C, and dissolved oxygen, as observed in other tests, was

near saturation. Other water quality characteristics are summarized in Table 19, and dose-response data are given in Table 10. The secondary effluent was not highly toxic in either flow-through or static-renewal tests, producing only 21% to 40% mortality at the 100% concentration. However, toxicity was substantially greater for sewage effluent which received only primary treatment. For example, full-strength and half-strength primary effluent concentrations produced 100% and 53% mortality, respectively, and the LC_{50} was 50.2%. Based on these data and earlier observations (3), it appears that biomonitoring with fish developmental stages affords a useful means of evaluating the effectiveness of waste treatment. Results of this investigation also were used to revise and perfect field monitoring procedures.

The second on-site biomonitoring experiment was performed at a major chemical manufacturing plant in north-central Kentucky during the period of September 6-17, 1980. The plant produced formaldehyde, urea-formaldehyde resins, and phenol-formaldehyde resins. Wastewater was routed through two equalization ponds, two anaerobic ponds, and a clarifier. Prior to discharge, the effluent reportedly was diluted 50:1 with well water. The mean wastewater flow at the time of study was approximately 2.8 million gallons per day.

Biomonitoring experiments performed on site included flow-through tests with the fathead minnow and Xenopus, static-renewal tests with the fathead minnow, Xenopus, and the rainbow trout, and static tests with the fathead minnow and Xenopus (Table 1). The effluent was pumped continuously

from the NPDES sampling station to the mobile laboratory. Dilution water for the embryo-larval tests was collected periodically from a deep well located on the premises. Solutions in all flow-through and static-renewal test chambers were monitored daily for alkalinity, conductivity, dissolved oxygen, hardness, pH, and temperature. Measurements for the static system were recorded daily, except for alkalinity and hardness which were monitored at the beginning and end of the exposure period. Temperature was regulated at 20.5 ± 0.5°C in tests with the fathead minnow and Xenopus and at 12.5 ± 0.5°C with the trout. Other test conditions are summarized in Table 19.

Dose-response data and LC_{50} values for these tests are summarized in Table 11. Undiluted effluent proved to be highly toxic to embryo-larval stages. For example, in flow-through tests with the fathead minnow, exposure to 100% effluent reduced survival to 4% at 3 days posthatching, compared to 93% survival in control populations. Full-strength effluent was somewhat less toxic to Xenopus stages, as survival was 35% when determined in the flow system. In static-renewal tests with the trout, early embryos were highly sensitive to the effluent, as survival frequencies after 9 days of exposure were 64%, 44%, 16%, and 0% at treatment levels of 1%, 10%, 50%, and 100%, respectively. When exposure was initiated at the eyed-egg stage, the trout was substantially more tolerant.

As determined in flow-through tests, effluent LC_{50} values were 29.4% with the fathead minnow and 63.8% with Xenopus (Table 11). In static-renewal tests with these species, LC_{50}'s were 48.9% and ~100%, respectively. The lowest LC_{50} value was 6.6%, calculated in static-renewal tests with early

trout embryos. Due to high survival frequencies at most effluent exposure concentrations, LC_{50}'s could not be determined in other tests.

The third on-site biomonitoring experiment was conducted at a major synthetic rubber plant in north-central Kentucky during the period of September 19 through October 2, 1980. The plant produced three polymers, including polybutadiene rubber, styrene-butadiene rubber, and polybutadiene-acrylic acid-acrylonitrile rubber. The first two polymers were used primarily in the tire industry and the last was a proprietary product. Wastewater from the plant was from four sources, including sanitary effluents, surface runoff, lime slurry from the well water softening process, and process water. The latter contained latex solids, rubber particles, unreacted monomers, sodium chloride, and toluene. In the treatment procedure, the pH of process wastewater was adjusted to 7.5, and the lime slurry and an anionic polyelectrolyte polymer were added just before primary clarification. The clarified wastewater was then mixed with the sanitary discharge, and the resulting effluent entered an extended aeration pond. After a 30-hr retention time, the final wastewater was discharged from the plant, and the mean flow was approximately 1.13 million gallons per day.

Biomonitoring experiments performed on site included flow-through tests with the fathead minnow and Xenopus, static-renewal tests with the fathead minnow, Xenopus, and the rainbow trout, and static tests with Xenopus (Table 1). The effluent was continuously pumped from the NPDES sampling station to the mobile laboratory. Dilution water for the embryo-larval tests was collected periodically from a deep well located on the premises. General

test parameters were monitored as described above and results are summarized in Table 19. Temperature was maintained at 20.5 ± 0.5°C in tests with the fathead minnow and Xenopus and at 12.5 ± 0.5°C with the trout.

Test responses are summarized in Table 12. Undiluted effluent produced substantial frequencies of mortality. In flow-through tests, exposure to 100% effluent through 3-4 days posthatching reduced survival to 13% and 21% for fathead minnows and Xenopus, respectively, compared to 90% to 93% survival for control organisms. As administered in static-renewal tests, undiluted effluent reduced survival of early trout embryos to 0% after 9 days. The LC_{50} values determined in flow-through tests with fathead minnows and Xenopus were 8.3% and 22.9%, respectively (Table 12). Using static-renewal procedures with these species, the effluent LC_{50}'s were 15.6% and ~100%. By comparison, the LC_{50} value was 6.4% when early trout embryos were exposed in the static-renewal system. The survival of trout eyed eggs was considerably higher, with an LC_{50} of 48.4%.

The fourth on-site biomonitoring experiment was performed at a secondary sewage treatment plant in southeastern Kentucky during the period of November 12-24, 1980. The plant received approximately 25% of its waste-load from an adjacent tannery, and the designated NPDES effluent was the chlorinated outflow from secondary treatment. Domestic sewage passed through a grit chamber into a primary clarifier, at which point settleable solids were removed. Tannery waste entered a second primary clarifier and was mixed with the clarified domestic wastewater. Subsequently, the combined wastewater was subjected to treatment in an aerated activated sludge

system, followed by secondary clarification and chlorination. Approximately 1.1 million gallons of outfall per day entered the receiving water.

Biomonitoring experiments on the final chlorinated effluent included flow-through tests with the fathead minnow and Xenopus, static-renewal tests with the fathead minnow, Xenopus, and the rainbow trout, and a static test with Xenopus. Final chlorinated effluent was continuously pumped from the NPDES sampling station to the mobile laboratory. Dilution water for all tests was taken 5 miles upstream from the treatment plant discharge. General test parameters were monitored as described above, and results are summarized in Table 19. In tests with the fathead minnow and Xenopus, temperature was maintained at 22.0 ± 1.0°C. For the trout experiments, temperature was regulated at 11.5 ± 0.5°C.

Results of the toxicity tests are summarized in Table 13. Except for 6% survival observed in the static test with Xenopus, 50% effluent always produced complete mortality. The fathead minnow and Xenopus were about equally sensitive to the final effluent, as reflected by LC_{50} values of 0.3% and 0.4% derived in flow-through tests. The LC_{50} values were 0.5%, 0.7%, and 0.9% when determined in static-renewal tests with early trout embryos, fathead minnow embryo-larval stages, and Xenopus embryo-larval stages, respectively.

The Tannery-Secondary Sewage Treatment Plant Complex afforded an unusual opportunity to monitor simultaneously a highly toxic untreated effluent, as well as the final treated NPDES effluent. Raw tannery wastes and unchlorinated and chlorinated effluents from the secondary sewage

plant were tested using static-renewal procedures to determine the applicability of on-site biomonitoring for evaluating the effectiveness of waste treatment. These tests were repeated with three animal species, and the results are presented in Table 14 and summarized in Table 15. As the tannery wastes were reported to contain substantial concentrations of metallic toxicants, the different effluent sources and the upstream dilution water were analyzed for cadmium, chromium, copper, iron, and zinc, and these data are reported in Table 15, together with general water quality parameters.

Taking data for all three animal species, LC_{50}'s ranged from 0.08% to 0.4% for untreated tannery wastes, 0.9% to 1.6% for unchlorinated secondary effluent, and 0.5% to 0.9% for the final chlorinated secondary effluent (Tables 14, 15). Chlorination resulted in a moderate increase in toxicity. However, it was apparent from these data that the overloaded sewage treatment plant provided little improvement in effluent quality. As a matter of fact, the slight reduction in toxicity, as well as the decreases observed for certain metals and general physicochemical parameters (e.g., conductivity, hardness, alkalinity) may have resulted as much from dilution by domestic waste input as from the treatment process. As noted above, the tannery effluent comprised approximately one-fourth of the treatment plant waste-load. Despite reductions in the concentrations of certain metals (e.g., Cd, Cr, Cu), significant metal residues remained in the final effluent, and this undoubtedly contributed to the high toxicity. The impacted receiving water was a third-order stream of moderate size.

The last on-site biomonitoring experiment was performed at a metal plating plant in northeastern Kentucky during the period of April 7-18, 1981. The final NPDES effluent from this plant was comprised of five separate components, including wastewater from the brazing-quencher operation, sludge-bed filtrate, cooling water, chemically-treated wastewater from the plating process, and surface runoff. Flows from these discharges combined to form a tributary on which the NPDES sampling site was situated. At the time of testing, wastewater flow in the tributary was estimated to be approximately 155,000 gallons per day. This was somewhat above the average flow of about 108,000 gallons per day, due to local precipitation and increased surface runoff. Of this total tributary flow, approximately 43% was cooling water, 24% was plating process water, 3% was sludge-bed filtrate, less than 1% was brazing water, and about 30% was surface runoff.

On-site biomonitoring experiments conducted on the final effluent included flow-through, static-renewal, and static tests with the fathead minnow and Xenopus. The final effluent was continuously pumped from the NPDES sampling station to the mobile laboratory. Dilution water for all tests was taken one-half mile upstream from the outfall, and the receiving water was a second-order stream which entered a major river approximately one-half mile below the test site. All tests were monitored as described above. Temperature averaged 22.0 ± 0.5°C, and other physicochemical parameters of the test water are summarized in Table 19.

Results of toxicity tests conducted on the final effluent are given

in Table 16. As determined in the flow-through system, undiluted effluent reduced survival frequencies to 6% and 46% for the fathead minnow and Xenopus, respectively, compared to control survival of 77% to 80%. Effluent LC_{50} values were 21.6% for the fathead minnow and ~100% for Xenopus. Toxicity was significantly less when measured in static-renewal tests. For example, the effluent LC_{50} was 44.7% when the fathead minnow was used. In static-renewal and static tests with Xenopus, LC_{50}'s could not be calculated.

As noted above, the final effluent was a mixture of four separate discharges, supplemented by surface runoff. Additional tests were performed in order to evaluate the comparative toxicity of the individual effluent components (Table 17). In Table 18, these data are summarized together with analyses of selected metals (i.e., Cd, Cr, Cu, Fe, Zn) and general physicochemical characteristics. Components 1 and 2 were the most toxic, with LC_{50} values of 0.01% and 0.05%, respectively, and they comprised about 27% of the final effluent. The LC_{50}'s for Components 3 and 4 were 23.6% and 25.4% and these collectively comprised about 44% of the final effluent, with the remainder coming from surface runoff. The latter was above normal due to local rainfall at the time of testing, and this likely accounted for the lower toxicity of the final effluent, for which the LC_{50} was 44.7%. With some exceptions, overall metal concentrations and conductivity were higher in the more toxic components. However, judging from data summarized in Table 18, it would be difficult to estimate the toxicity of the effluent components accurately on the basis of selected chemical criteria, whereas on-site biomonitoring provided a direct means of measuring toxicity.

Table 10. On-site toxicity tests on primary and secondary sewage treatment plant effluents using newly hatched larvae of the channel catfish.[1,2]

Effluent Source	Test System	Effluent Concentration (Percent)	Percent Survival after 7 Days
After Primary Treatment	Static-renewal	100	0
		50	47
		10	100
		1	100
		0.1	100
		Control	100
LC_{50} (% Effluent) (95% confidence limits)			50.2 (46.5 - 53.5)
After Secondary Treatment, Unchlorinated	Static-renewal	100	60
		50	100
		10	99
		1	100
		0.1	100
		Control	99
LC_{50} (% Effluent)			ND[3]
After Secondary Treatment, Unchlorinated	Flow-through	100	79
		50	97
		10	100
		1	100
		0.1	96
		Control	97
LC_{50} (% Effluent)			ND

[1]Initial on-site biomonitoring test conducted at Lexington, Kentucky.

[2]Tests were initiated with 1-day-old larvae and continued through 8 days posthatching.

[3]Not determined.

Table 11. On-site embryo-larval biomonitoring of a chemical manufacturing plant final effluent.

Test Species	Test System	Effluent Concentration (Percent)	Percent Hatchability or Embryonic Survival[1,2]	Percent Survival	
				Hatching	4 Days Posthatching
Xenopus laevis	Flow-through	100	63(8)	58	35
		50	71(9)	66	53
		10	88(4)	85	78
		1	92(4)	88	83
		0.1	98(1)	97	91
		Control	98	98	96
LC_{50} (% Effluent) (95% confidence limits)					63.8 (39.8 - 100)
Xenopus laevis	Static-renewal	100	79(11)	70	46
		50	86(6)	82	73
		10	94(4)	90	81
		1	97(1)	96	87
		0.1	95	95	93
		Control	97	97	97
LC_{50} (% Effluent)					~100
Xenopus laevis	Static	100	83(12)	73	71
		50	87(7)	81	79
		10	92(4)	88	86
		1	96(2)	94	92
		0.1	98	98	98
		Control	96(2)	94	94
LC_{50} (% Effluent)					ND[3]

Table 11 - continued.

Test Species	Test System	Effluent Concentration (Percent)	Percent Hatchability or Embryonic Survival[1,2]	Percent Survival	
				Hatching	3 Days Posthatching
Fathead Minnow	Flow-through	100	18(41)	10	4
		50	51(13)	45	38
		10	76(9)	70	69
		1	82(4)	79	79
		0.1	90	90	90
		Control	93	93	93
LC_{50} (% Effluent) (95% confidence limits)					29.4 (22.1 - 36.4)
Fathead Minnow	Static-renewal	100	26(32)	18	5
		50	60(7)	56	54
		10	82(1)	81	79
		1	88	88	86
		0.1	93	93	93
		Control	96	96	96
LC_{50} (% Effluent) (95% confidence limits)					48.9 (38.3 - 60.2)
Fathead Minnow	Static	100	72(9)	66	66
		50	77(5)	73	73
		10	80	80	80
		1	92	92	92
		0.1	97(3)	94	94
		Control	89	89	89
LC_{50} (% Effluent)					ND[3]

Table 11 - continued.

Test Species	Test System	Effluent Concentration (Percent)	Percent Hatchability or Embryonic Survival[1,2]	Percent Survival	
				Hatching	4 Days Posthatching
Rainbow Trout (eyed)	Static-renewal	100	88	88	81
		50	88(2)	87	84
		10	91(3)	88	88
		1	92(3)	89	88
		0.1	99(1)	97	96
		Control	99(1)	98	96
LC_{50} (% Effluent)					ND[3]
Rainbow Trout	Static-renewal	100	0	-	-
		50	16	-	-
		10	44	-	-
		1	64	-	-
		0.1	80	-	-
		Control	86	-	-
LC_{50} (% Effluent) (95% confidence limits)			6.6 (1.9 - 13.5)		

[1]Egg hatchability was based on all animals, normal and aberrant, which completed hatching. Frequencies of teratic survivors appearing in hatched populations were expressed parenthetically.

[2]Trout tests were conducted using fresh eggs, unless specified otherwise.

[3]Not determined.

Table 12. On-site embryo-larval biomonitoring of a synthetic rubber plant final effluent.

Test Species	Test System	Effluent Concentration (Percent)	Percent Hatchability or Embryonic Survival[1,2]	Percent Survival	
				Hatching	3 Days Posthatching
Xenopus laevis	Flow-through	100	77(24)	58	21
		50	85(20)	67	45
		10	90(15)	76	57
		1	93(9)	85	78
		0.1	99(6)	93	89
		Control	100(3)	97	93
LC_{50} (% Effluent) (95% confidence limits)					22.9 (13.4 - 36.9)
Xenopus laevis	Static-renewal	100	85(31)	58	48
		50	90(23)	70	67
		10	95(17)	78	78
		1	96(7)	89	85
		0.1	97(5)	93	91
		Control	100(3)	97	93
LC_{50} (% Effluent)					≈100
Xenopus laevis	Static[3]	100	88(24)	67	9
		50	87(22)	67	58
		10	94(12)	83	77
		1	96(4)	93	89
		0.1	98(2)	96	95
		Control	98(2)	97	97
LC_{50} (% Effluent) (95% confidence limits)					39.3 (27.9 - 50.6)

Table 12 - continued.

Test Species	Test System	Effluent Concentration (Percent)	Percent Hatchability or Embryonic Survival[1,2]	Percent Survival	
				Hatching	3-4 Days Posthatching[4]
Fathead Minnow	Flow-through	100	35(22)	27	13
		50	52(12)	46	20
		10	65(5)	61	50
		1	77(4)	74	67
		0.1	85(2)	83	79
		Control	94(1)	92	90
LC_{50} (% Effluent) (95% confidence limits)					8.3 (4.6 - 13.2)
Fathead Minnow	Static-renewal	100	49(15)	42	12
		50	57(15)	48	34
		10	70(4)	67	59
		1	77(1)	76	70
		0.1	85	85	83
		Control	94	94	91
LC_{50} (% Effluent) (95% confidence limits)					15.6 (8.5 - 24.7)
Rainbow Trout (eyed)	Static-renewal	100	85(11)	75	37
		50	92(4)	88	53
		10	96(3)	93	75
		1	99(3)	96	89
		0.1	99	99	93
		Control	99	99	97
LC_{50} (% Effluent) (95% confidence limits)					48.4 (25.5 - 100)

Table 12 - continued.

Test Species	Test System	Effluent Concentration (Percent)	Percent Hatchability or Embryonic Survival[1,2]	Percent Survival	
				Hatching	3 Days Posthatching
Rainbow Trout	Static-Renewal	100	0	-	-
		50	14	-	-
		10	56	-	-
		1	62	-	-
		0.1	85	-	-
		Control	93	-	-
LC_{50} (% Effluent) (95% confidence limits)			6.4 (3.3 - 10.3)		

[1]Egg hatchability was based on all animals, normal and aberrant, which completed hatching. Frequencies of teratic survivors appearing in hatched populations were expressed parenthetically.

[2]Trout tests were conducted using fresh eggs, unless otherwise specified.

[3]Fungal contamination was detected at the 100% effluent exposure concentration and probably contributed to mortality.

[4]Tests with fathead minnows and eyed trout stages were continued through 3 and 4 days posthatching, respectively.

Table 13. On-site embryo-larval biomonitoring of the chlorinated final effluent from a secondary sewage treatment plant receiving tannery waste.

Test Species	Test System	Effluent Concentration (Percent)	Percent Hatchability or Embryonic Survival[1,2]	Percent Survival	
				Hatching	4 Days Posthatching
Xenopus laevis	Flow-through	100	0	0	0
		50	0	0	0
		10	24(12)	21	9
		1	35(2)	34	24
		0.1	80	80	78
		Control	95	95	94
LC_{50} (% Effluent) (95% confidence limits)					0.4 (0.3 - 0.6)
Xenopus laevis	Static-renewal	100	0	0	0
		50	2(100)	0	0
		10	23(20)	18	15
		1	48(17)	40	39
		0.1	85	85	80
		Control	91	91	91
LC_{50} (% Effluent) (95% confidence limits)					0.9 (0.5 - 1.5)
Xenopus laevis	Static	100	0	0	0
		50	35(83)	6	6
		10	39(12)	35	27
		1	49	49	46
		0.1	81	81	81
		Control	96	96	96
LC_{50} (% Effluent) (95% confidence limits)					1.2 (0.7 - 2.0)

Table 13 - continued.

Test Species	Test System	Effluent Concentration (Percent)	Percent Hatchability or Embryonic Survival[1,2]	Percent Survival	
				Hatching	4 Days Posthatching
Fathead Minnow	Flow-through	100	-	-	-
		50	0	0	0
		10	30(39)	18	7
		1	62(11)	55	33
		0.1	77(6)	72	61
		0.01	83	83	81
		Control	91	91	91
LC_{50} (% Effluent) (95% confidence limits)					0.3 (0.2 - 0.6)
Fathead Minnow	Static-renewal	100	0	0	0
		50	24(100)	0	0
		10	28(29)	20	16
		1	52	52	44
		0.1	80	80	76
		Control	96	96	96
LC_{50} (% Effluent) (95% confidence limits)					0.7 (0.3 - 1.5)
Rainbow Trout	Static-renewal	100	0	-	-
		50	0	-	-
		10	4	-	-
		1	47	-	-
		0.1	67	-	-
		Control	97	-	-
LC_{50} (% Effluent) (95% confidence limits)			0.5 (0.3 - 0.8)		

Table 13 - continued.

---

[1]Egg hatchability was based on all animals, normal and aberrant, which completed hatching. Frequencies of teratic survivors appearing in hatched populations were expressed parenthetically.

[2]Trout tests were conducted using fresh eggs.

Table 14. On-site study at a tannery-secondary sewage treatment plant complex involving embryo-larval biomonitoring of raw tannery waste, unchlorinated effluent from secondary treatment, and chlorinated final effluent from secondary treatment.[1,2]

Effluent Source	Test Species	Effluent Concentration (Percent)	Percent Hatchability or Embryonic Survival[3,4]	Percent Survival	
				Hatching	4 Days Posthatching
Tannery	*Xenopus laevis*	100	0	0	0
		50	0	0	0
		10	0	0	0
		1	42	42	34
		0.1	73	73	63
		Control	90	90	90
LC_{50} (% Effluent) (95% confidence limits)					0.4 (0.2 - 0.7)
Tannery	Fathead Minnow	100	0	0	0
		50	0	0	0
		10	8(100)	0	0
		1	44(18)	36	20
		0.1	92	92	68
		Control	92	92	92
LC_{50} (% Effluent) (95% confidence limits)					0.3 (0.1 - 0.5)
Tannery	Rainbow Trout	100	0	-	-
		50	0	-	-
		10	0	-	-
		1	7	-	-
		0.1	43	-	-
		Control	97	-	-
LC_{50} (% Effluent) (95% confidence limits)			0.08 (0.03 - 0.13)		

Table 14 - continued.

Effluent Source	Test Species	Effluent Concentration (Percent)	Percent Hatchability or Embryonic Survival [3,4]	Percent Survival	
				Hatching	4 Days Posthatching
After Secondary Treatment, Unchlorinated	*Xenopus laevis*	100	0	0	0
		50	20(38)	13	0
		10	34(13)	29	21
		1	58(13)	51	51
		0.1	80	80	78
		Control	90	90	90
LC_{50} (% Effluent) (95% confidence limits)					1.5 (0.7 - 2.6)
After Secondary Treatment, Unchlorinated	Fathead Minnow	100	0	0	0
		50	24(100)	0	0
		10	56(14)	48	28
		1	72	72	52
		0.1	92	92	76
		Control	92	92	92
LC_{50} (% Effluent) (95% confidence limits)					1.6 (0.4 - 3.5)
After Secondary Treatment, Unchlorinated	Rainbow Trout	100	0	-	-
		50	0	-	-
		10	24	-	-
		1	50	-	-
		0.1	73	-	-
		Control	97	-	-
LC_{50} (% Effluent) (95% confidence limits)			0.9 (0.5 - 1.4)		

Table 14 - continued.

Effluent Source	Test Species	Effluent Concentration (Percent)	Percent Hatchability or Embryonic Survival [3,4]	Percent Survival	
				Hatching	4 Days Posthatching
After Secondary Treatment, Chlorinated (final effluent)	*Xenopus laevis*	100	0	0	0
		50	2(100)	0	0
		10	23(20)	18	15
		1	48(17)	40	39
		0.1	85	85	80
		Control	91	91	91
LC_{50} (% Effluent) (95% confidence limits)					0.9 (0.5 - 1.5)
After Secondary Treatment, Chlorinated (final effluent)	Fathead Minnow	100	0	0	0
		50	24(100)	0	0
		10	28(29)	20	16
		1	52	52	44
		0.1	80	80	76
		Control	96	96	96
LC_{50} (% Effluent) (95% confidence limits)					0.7 (0.3 - 1.5)
After Secondary Treatment, Chlorinated (final effluent)	Rainbow Trout	100	0	-	-
		50	0	-	-
		10	4	-	-
		1	47	-	-
		0.1	67	-	-
		Control	97	-	-
LC_{50} (% Effluent) (95% confidence limits)			0.5 (0.3 - 0.8)		

Table 14 - continued.

---

[1]Untreated tannery effluent constituted approximately 25% of the wasteload of the sewage treatment plant.

[2]Tests were performed using static-renewal procedures.

[3]Egg hatchability was based on all animals, normal and aberrant, which completed hatching. Frequencies of teratic survivors appearing in hatched populations were expressed parenthetically.

[4]Trout tests were conducted using fresh eggs.

Table 15. Chemical and toxicological characteristics of three effluents from a tannery-secondary sewage treatment plant complex.

Effluent Characteristics[1,2]	Effluent Source			Dilution Water
	Tannery[3]	STP (unchlorinated)	Final STP (chlorinated)	
pH	8.5 ± 0.1	7.8 ± 0.1	7.5 ± 0.1	6.9 ± 0.1
Alkalinity (mg/L as $CaCO_3$)	473 ± 31	207 ± 6	203 ± 4	13 ± 1
Hardness (mg/L as $CaCO_3$)	1317 ± 58	406 ± 23	385 ± 19	16 ± 1
Conductivity (µmhos/cm)	5829 ± 99	1744 ± 97	1746 ± 111	18 ± 1
Cadmium (µg/L)	21 ± 9	-	5 ± 1	n.d.[4]
Chromium (µg/L)	856 ± 134	-	118 ± 3	n.d.
Copper (µg/L)	29 ± 2	-	16 ± 1	6 ± 5
Iron (µg/L)	66 ± 7	-	80 ± 3	n.d.
Zinc (µg/L)	53 ± 8	-	122 ± 16	2 ± 2
LC_{50} (Rainbow trout)	0.08%	0.9%	0.5%	-
LC_{50} (Fathead minnow)	0.3%	1.6%	0.7%	-
LC_{50} (*Xenopus laevis*)	0.4%	1.5%	0.9%	-

[1]Chemical characteristics expressed as mean ± standard error.

[2]LC_{50} values were determined during on-site biomonitoring, using embryo-larval static-renewal procedures.

[3]Untreated tannery effluent constituted approximately 25% of the wasteload of the secondary sewage treatment plant.

[4]Not detected.

Table 16. On-site embryo-larval biomonitoring of a metal plating plant final effluent.

Test Species	Test System	Effluent Concentration (Percent)	Percent Hatchability[1]	Percent Survival	
				Hatching	4 Days Posthatching
Xenopus laevis	Flow-through[2]	100	62(2)	61	46
		50	82(9)	75	68
		10	86(3)	83	76
		1	98	98	89
		0.1	91(2)	89	72
		Control	93	93	77
LC_{50} (% Effluent)					~100
Xenopus laevis	Static-renewal	100	74(14)	64	64
		50	85(5)	78	78
		10	86(6)	80	70
		1	87(1)	86	86
		0.1	93	93	92
		Control	98(1)	97	94
LC_{50} (% Effluent)					ND[3]
Xenopus laevis	Static	100	84(14)	73	70
		50	87	87	80
		10	88	88	81
		1	93	93	88
		0.1	94	94	89
		Control	98	98	89
LC_{50} (% Effluent)					ND[3]

Table 16 - continued.

Test Species	Test System	Effluent Concentration (Percent)	Percent Hatchability[1]	Percent Survival	
				Hatching	4 Days Posthatching
Fathead Minnow	Flow-through	100	10(2)	8	6
		50	37(17)	31	31
		10	60(3)	58	53
		1	73(1)	72	72
		0.1	82	82	82
		Control	80	80	80
LC_{50} (% Effluent) (95% confidence limits)					21.6 (13.9 - 29.5)
Fathead Minnow	Static-renewal	100	21	21	13
		50	53	53	43
		10	73	73	72
		1	81	81	79
		0.1	87	87	83
		Control	87	87	86
LC_{50} (% Effluent) (95% confidence limits)					44.7 (32.5 - 54.6)
Fathead Minnow	Static[2]	100	23(33)	15	15
		50	37(11)	33	33
		10	40(9)	36	36
		1	-	-	-
		0.1	87	87	85
		Control	75(3)	73	71
LC_{50} (% Effluent) (95% confidence limits)					10.6 (0.00 - 29.3)

Table 16 - continued.

---

[1]Egg hatchability was based on all animals, normal and aberrant, which completed hatching. Frequencies of teratic survivors appearing in hatched populations were expressed parenthetically.

[2]Fungal contamination was detected in two flow-through chambers used for *Xenopus* (*i.e.*, 0.1%, control) and in most static chambers used for the fathead minnow, and this probably contributed to mortality.

[3]Not determined.

Table 17. On-site embryo-larval biomonitoring of final effluent and effluent components from a metal plating plant using embryo-larval stages of the fathead minnow.[1]

Final Effluent and Effluent Components	Effluent Concentration (Percent)	Percent Hatchability[2]	Percent Survival	
			Hatching	4 Days Posthatching
Component 1 (Sludge-bed filtrate)	10	1(100)	0	0
	5	17(16)	14	14
	1	34	34	34
	0.1	-	-	-
	0.01	50	50	50
	Control	87	87	86
LC_{50} (% Effluent) (95% confidence limits)				0.01 (0.0005 - 0.04)
Component 2 (Process Water)	10	3	3	0
	5	11	11	5
	1	16(23)	13	11
	0.1	34(7)	32	32
	0.01	67	67	63
	Control	87	87	86
LC_{50} (% Effluent) (95% confidence limits)				0.05 (0.02 - 0.10)
Component 3 (Brazing Water)	100	51(14)	43	4
	50	44(12)	39	37
	10	57	57	57
	1	84	84	80
	0.1	85	85	83
	Control	86	86	85
LC_{50} (% Effluent) (95% confidence limits)				23.6 (14.1 - 33.2)

Table 17 - continued.

Final Effluent and Effluent Components	Effluent Concentration (Percent)	Percent Hatchability[2]	Percent Survival	
			Hatching	4 Days Posthatching
Component 4 (Cooling Water)	100	47	47	31
	50	30	30	27
	10	62	62	58
	1	73	73	73
	0.1	76	76	73
	Control	87	87	86
LC_{50} (% Effluent) (95% confidence limits)				25.4 (9.0 - 63.0)
Final Effluent	100	21	21	13
	50	53	53	43
	10	73	73	72
	1	81	81	79
	0.1	87	87	83
	Control	87	87	86
LC_{50} (% Effluent) (95% confidence limits)				44.7 (32.5 - 54.6)

[1]Tests were conducted using static-renewal procedures.

[2]Egg hatchability was based on all animals, normal and aberrant, which completed hatching. Frequencies of teratic survivors appearing in hatched populations were expressed parenthetically.

Table 18. Chemical and toxicological characteristics of effluent components and final effluent from a metal plating plant.

Effluent Characteristics[1,2]	Effluent Components[3]				Final Effluent	Dilution Water
	1	2	3	4		
Percent Final Effluent[4]	3	24	<1	43	-	-
pH	9.2 ± 0.1	9.2 ± 0.2	7.8 ± 0.1	7.2 ± 0.1	8.1 ± 0.1	8.1 ± 0.1
Alkalinity (mg/L as $CaCO_3$)	1844 ± 204	157 ± 9	86 ± 5	310 ± 26	273 ± 10	260 ± 7
Hardness (mg/L as $CaCO_3$)	148 ± 13	86 ± 6	163 ± 3	438 ± 7	281 ± 11	272 ± 5
Conductivity (µmhos/cm)	12209 ± 1966	866 ± 37	189 ± 3	509 ± 58	716 ± 42	229 ± 5
Cadmium (µg/L)	31 ± 5	9 ± 2	8 ± 1	58 ± 46	6 ± 1	n.d.[5]
Chromium (µg/L)	741 ± 282	484 ± 205	6 ± 3	585 ± 48	296 ± 135	13
Copper (µg/L)	239 ± 44	57 ± 12	14 ± 2	11 ± 1	23 ± 5	n.d.
Iron (µg/L)	377 ± 66	425 ± 157	44 ± 21	36 ± 5	292 ± 146	31
Zinc (µg/L)	2488 ± 585	893 ± 223	1173 ± 139	74 ± 8	379 ± 115	n.d.
LC_{50} (Fathead minnow)	0.01%	0.05%	23.6%	25.4%	44.7%	-

[1]Chemical characteristics expressed as mean ± standard error.

[2]LC_{50} values were determined during on-site biomonitoring, using embryo-larval static-renewal procedures.

[3]The final NPDES effluent was comprised of four separate components, including sludge-bed filtrate (1), process water (2), brazing process water (3), and cooling water (4). The final effluent was further diluted with surface runoff at time of testing.

[4]Remaining component of final effluent was from surface runoff.

[5]Not detected.

Table 19. General characteristics of final effluent, effluent components, and dilution water used in embryo-larval biomonitoring experiments.[1]

Test Site	Test System	Test Species	pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)	Dissolved Oxygen (mg/L)
Lexington Sewage Treatment Plant							
Dilution water	-	-	7.1 ± 0.1	61 ± 2	105 ± 8	186 ± 1	8.3 ± 0.1
Primary effluent	Static-renewal (on site)	Catfish	7.6 ± 0.1	124 ± 64	102 ± 10	-	8.0 ± 0.4
Unchlorinated secondary effluent	Static-renewal (on site)	Catfish	7.6 ± 0.04	173 ± 15	130 ± 8	308 ± 9	8.0 ± 0.2
	Flow-through (on site)	Catfish	7.6 ± 0.03	181 ± 3	133 ± 9	312 ± 3	8.4 ± 0.2
Chemical Manufacturing Plant							
Dilution water	-	-	7.6 ± 0.1	276 ± 14	327 ± 6	308 ± 3	8.0 ± 0.1
Diluted process water (final effluent)	Flow-through (on site)	*Xenopus* & F. minnow	7.6 ± 0.1	304 ± 6	387 ± 6	402 ± 7	7.8 ± 0.1
	Static-renewal (on site)	*Xenopus* & F. minnow	7.5 ± 0.1	252 ± 11	338 ± 11	359 ± 7	8.1 ± 0.1
	Static-renewal (on site)	Trout	7.5 ± 0.2	288 ± 13	358 ± 14	337 ± 11	10.0 ± 0.2
	Static (on site)	*Xenopus* & F. minnow	7.9 ± 0.1	312 ± 2	352 ± 28	419 ± 3	8.1 ± 0.4
	Static-renewal (laboratory)	*Xenopus*	7.7 ± 0.3	233 ± 34	368 ± 7	373 ± 32	7.9 ± 0.2

Table 19 - continued.

Test Site	Test System	Test Species	pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)	Dissolved Oxygen (mg/L)
Synthetic Rubber Plant							
Dilution water	-	-	7.6 ± 0.1	409 ± 34	445 ± 19	476 ± 6	8.0 ± 0.1
Undiluted process water (final effluent)	Flow-through (on site)	*Xenopus* & F. minnow	7.7 ± 0.1	134 ± 3	296 ± 9	3962 ± 143	8.3 ± 0.1
	Static-renewal (on site)	*Xenopus* & F. minnow	7.7 ± 0.1	140 ± 3	294 ± 5	4138 ± 193	7.9 ± 0.1
	Static-renewal (on site)	Trout	7.7 ± 0.1	174 ± 17	298 ± 14	3204 ± 290	10.4 ± 0.1
	Static (on site)	*Xenopus*	7.8 ± 0.2	134 ± 4	297 ± 31	4986 ± 60	8.1 ± 0.1
	Static-renewal (laboratory)	Trout	7.2 ± 0.1	154 ± 4	298 ± 4	4323 ± 121	8.8 ± 0.1
Tannery-Sewage Treatment Plant							
Dilution water	-	-	6.9 ± 0.1	13 ± 1	16 ± 1	18 ± 1	8.0 ± 0.1
Chlorinated secondary effluent (final effluent)	Flow-through (on site)	*Xenopus* & F. minnow	7.5 ± 0.1	203 ± 4	385 ± 19	1746 ± 111	7.9 ± 0.1
	Static-renewal (on site)	*Xenopus* & F. minnow	8.1 ± 0.1	203 ± 4	317 ± 19	1490 ± 38	7.4 ± 0.1
	Static-renewal (on site)	Trout	8.0 ± 0.1	211 ± 1	-	1317 ± 93	8.4 ± 0.1
	Static (on site)	*Xenopus*	8.0 ± 0.1	203 ± 1	291 ± 9	1397 ± 3	7.3 ± 0.1
	Static-renewal (laboratory)	Trout	8.2 ± 0.1	248 ± 9	281 ± 16	1210 ± 65	9.0 ± 0.1

Table 19 - continued.

Test Site	Test System	Test Species	pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)	Dissolved Oxygen (mg/L)
Unchlorinated secondary effluent	Static-renewal (on site)	*Xenopus* & F. minnow	7.8 ± 0.1	207 ± 6	406 ± 23	1744 ± 97	7.5 ± 0.1
	Static-renewal (on site)	Trout	8.1 ± 0.1	210 ± 1	-	1333 ± 133	8.7 ± 0.1
Tannery effluent	Static-renewal (on site)	*Xenopus* & F. minnow	8.5 ± 0.1	473 ± 31	1317 ± 58	5829 ± 99	5.7 ± 0.1
	Static-renewal (on site)	Trout	8.4 ± 0.2	503 ± 16	-	5417 ± 60	8.6 ± 0.1
Metal Plating Plant							
Dilution water	-	-	8.1 ± 0.1	260 ± 7	272 ± 5	229 ± 5	8.7 ± 0.03
Mixed waste (final effluent)	Flow-through (on site)	*Xenopus* & F. minnow	8.1 ± 0.1	263 ± 12	265 ± 14	731 ± 58	7.8 ± 0.1
	Static-renewal (on site)	*Xenopus* & F. minnow	8.1 ± 0.1	273 ± 10	281 ± 11	716 ± 42	8.0 ± 0.1
	Static (on site)	*Xenopus* & F. minnow	8.0 ± 0.3	228 ± 36	302 ± 54	553 ± 8	8.3 ± 0.1
	Static-renewal (laboratory)	*Xenopus*	8.0 ± 0.1	240 ± 8	307 ± 4	765 ± 112	8.5 ± 0.4
Component 1 (sludge-bed filtrate)	Static-renewal (on site)	F. minnow	9.2 ± 0.1	1844 ± 204	148 ± 13	12209 ± 1966	7.6 ± 0.2
Component 2 (process water)	Static-renewal (on site)	F. minnow	9.2 ± 0.2	157 ± 9	86 ± 6	866 ± 37	8.0 ± 0.2
Component 3 (brazing water)	Static-renewal (on site)	F. minnow	7.8 ± 0.1	86 ± 5	163 ± 3	189 ± 3	8.0 ± 0.2
Component 4 (cooling water)	Static-renewal (on site)	F. minnow	7.2 ± 0.1	310 ± 26	438 ± 7	509 ± 58	7.7 ± 0.3

[1] Chemical characteristics expressed as mean ± standard error.

ANALYSIS OF RESULTS AND EVALUATION OF EFFLUENT BIOMONITORING PROCEDURES

On the basis of observations presented above, it is apparent that embryo-larval tests afford an effective means of detecting and quantifying effluent toxicity. It is also evident that biomonitoring results may vary significantly with the different test systems and animal species selected for use. Therefore, it is important to standardize test procedures within limits necessary to insure reliable results and yet afford reasonable economy of operation.

Reliability and sensitivity of alternative embryo-larval test systems. Ten comparative evaluations were made in which flow-through, static-renewal, and static procedures were all used to test selected effluents or the reference toxicant (i.e., phenol). In each case, the three tests were conducted using the same exposure period, animal species, dilution water and, in so far as possible, general test parameters (e.g., temperature, dissolved oxygen, water hardness). In most instances, flow-through tests provided the greatest resolution of dose-response data and the lowest LC_{50} values. When toxicity appeared greater in static tests, such results were due to effluent sampling during periods of peak toxicity, as in the coal-ash study, or to extrinsic factors (e.g., fungal contamination). Though such problems were encountered infrequently, static test conditions were more susceptible to deterioration (Tables 12, 16).

In twelve instances, flow-through and static-renewal tests were

conducted simultaneously. Based on 95% confidence intervals, animal responses did not vary significantly for the two different methods in eight of the twelve cases. The LC_{50} values usually were within a factor of about two or less, and seldom differed by more than a factor of 2.5. Considering these data and the greater economy of operation, static-renewal procedures appear applicable for routine toxicological screening of effluents. However, when precise quantification of effluent toxicity is required, the flow-through test is recommended. Based on the above considerations, static procedures appear less suitable for use in effluent biomonitoring with embryo-larval stages. Compared to static-renewal procedures, the small gain in economy does not offset the reduced sensitivity and greater variability of results observed in static tests. Furthermore, as the composition of many effluents varies significantly with time, static tests provide a less representative measure of toxicity.

Test organisms and responses. Of those organisms used in on-site effluent biomonitoring, early trout embryos (fertilization through 9 days) and embryo-larval stages of the fathead minnow (fertilization through 3 to 4 days posthatching) consistently were the most sensitive, based on LC_{50} values. Results obtained with these species gave the most reliable data for detection and quantification of effluent toxicity. Eyed trout eggs carried through 4 days posthatching and embryo-larval stages of Xenopus were appreciably more tolerant. For example, using data obtained in on-site static-renewal tests, the LC_{50} values for the Chemical Manufacturing Plant effluent were 6.6%, 48.9%, and ~100% when determined with early

trout embryos, fathead minnow embryo-larval stages, and Xenopus embryo-larval stages, respectively (Table 11). An LC_{50} could not be determined with eyed trout eggs. Using the same procedures, LC_{50} values for the Synthetic Rubber Plant effluent were 6.4%, 15.6%, 48.4%, and ~100% when determined with early trout embryos, fathead minnow embryo-larval stages, eyed trout eggs, and Xenopus embryo-larval stages, respectively (Table 12). In instances when static-renewal procedures are the method of choice for testing industrial effluents, Xenopus stages and eyed trout eggs probably should not be used as the principal test organisms. However, differences in sensitivity between Xenopus and the fathead minnow decreased somewhat when flow-through procedures were used. For example, taking on-site effluent toxicity data for the Tannery-Sewage Treatment Plant Complex, Synthetic Rubber Plant, Metal Plating Plant, and Chemical Manufacturing Plant, the LC_{50} values were 0.3%, 8.3%, 21.6%, and 29.4% when determined with embryo-larval stages of the fathead minnow and 0.4%, 22.9%, ~100%, and 63.8% when determined with embryo-larval stages of Xenopus (Tables 11, 12, 13, 16).

In the event that embryo-larval toxicity testing is applied to effluent biomonitoring under the NPDES program, it will become necessary to standardize procedures sufficiently to provide reasonable quality assurance. However, some flexibility should be maintained regarding the selection of animal species. An abundant supply of viable eggs is essential for a reliable testing program, and this requirement can be met more effectively and economically by drawing upon a reasonable

complement of species.

In addition to animal species evaluated during field studies, embryo-larval stages of the channel catfish and bluegill sunfish were used in effluent toxicity tests conducted in the laboratory (Table 8), and good results were obtained with both species. Therefore, on the basis of field and laboratory observations reported above, organisms considered suitable for either flow-through or static-renewal effluent testing included embryo-larval stages of the bluegill sunfish, channel catfish, and the fathead minnow, and fertilized eggs of the rainbow trout. Based on previous results obtained in conventional toxicity testing with embryo-larval stages (5, 7, 9, 11, 14), a number of additional warm-water species should prove suitable for use, and a list of preferred and optional species is included in the Appendix.

Despite the greater tolerance of its embryo-larval stages, Xenopus may be useful as an optional test species. The reproductive biology and embryology of this species have been well-documented, due to the fact that it has served as one of the principal experimental organisms in the field of developmental biology. Parental stocks are easily maintained, spawning can be induced with hormonal injections, and a typical spawn contains from 1,000 to 2,000 eggs. Eggs can be taken soon after fertilization and are easily handled in test systems. When testing is required at locations which lack fish culturing facilities, it may prove more practical and economical to maintain a Xenopus colony.

Both embryo-larval mortality and teratogenesis proved to be important

and easily discernible test responses. Though mortality was the predominant response, most effluents evaluated at concentrations of 10% to 100% in on-site studies produced significant frequencies of teratic organisms (Tables 11-14, 16, 17), and for the more toxic effluents or effluent components, teratogenesis often was significant at concentrations ranging down to 0.1% to 1% (Tables 13, 14, 17). Due to the limited duration of exposure, no consideration was given to growth. Though some attention was given to retarded development, delayed hatching, and minor anomalies, no additional responses with clearly identifiable endpoints were observed which could be applied consistently to effluent testing. As embryo-larval mortality and gross teratogenesis are sensitive, irreversible responses, their occurrence can be expected to impact upon reproductive success and population density and, therefore, they constitute the most feasible criteria to use in short-term tests with early life stages of fish and amphibians.

Comparison of on-site and laboratory testing of industrial effluents. One aspect of this investigation was to explore the possibility of using embryo-larval stages in laboratory tests to screen industrial effluents for toxicity. Therefore, during the four major on-site studies, 24-hr composite samples of the final NPDES effluents designated for testing were transported to the laboratory for simultaneous analyses. Two to three 20-liter aliquots of each effluent were collected using a standard composite sampler (ISCO) equipped with an ice bath. Upon collection, each Pyrex container was stoppered, packed in ice, and transported to the laboratory for immediate use. Due to the heavy volume of testing conducted

during the on-site studies, it was necessary to perform this correlated field/laboratory investigation using static-renewal procedures and test organisms for which there was the greatest availability. Consequently, three of the four tests were conducted using either eyed trout eggs or Xenopus embryo-larval stages, and this resulted in less sensitivity than would have been desired. However, it is evident from the dose-response data that effluent toxicity consistently was less when measured in the laboratory (Table 20). Results given for the Tannery-Secondary Sewage Treatment Plant Complex were particularly significant. These tests were performed using fertilized trout eggs, and the effluent LC_{50} values were 0.5% and 32.1% as determined in the field and laboratory, respectively. This difference was between one and two orders of magnitude, considering the narrow 95% confidence intervals. Though LC_{50} values could not be determined for most of the remaining tests, a survey of dose-response data indicated less deviation between on-site and laboratory determinations, but disparities could have been greater had more sensitive test organisms been used. Considering results given in Table 20, it is apparent that effluent toxicity can be quantified more accurately in on-site tests. However, based on these results and those obtained for seven industrial effluents evaluated during the first year of the project (Table 8), tests conducted in the laboratory using embryo-larval stages of sensitive aquatic species could prove useful in preliminary effluent screening.

Comparison of acute and embryo-larval effluent biomonitoring. In each of the four major biomonitoring studies, the EPA Region IV team performed

Table 20. Comparison of effluent toxicity determined in on-site and laboratory static-renewal tests using fish and amphibian embryo-larval stages.

NPDES Effluent Source[1]	Test Species	Effluent Concentration (Percent)	Percent Survival[2]	
			On Site	Laboratory
Chemical Manufacturing Plant	*Xenopus laevis*	100	46	72
		50	73	72
		10	81	78
		1	87	84
		0.1	93	87
		Control	97	85
LC_{50} (% Effluent)			∿100	ND[3]
Synthetic Rubber Plant	Rainbow Trout (eyed)	100	37	93
		50	53	85
		10	75	90
		1	89	94
		0.1	93	96
		Control	97	95
LC_{50} (% Effluent) (95% confidence limits)			48.4 (25.5 - 100)	ND
Metal Plating Plant	*Xenopus laevis*	100	64	83
		50	78	80
		10	70	86
		1	86	90
		0.1	92	83
		Control	94	88
LC_{50} (% Effluent)			ND	ND
Tannery-Sewage Treatment Plant	Rainbow Trout	100	0	0
		50	0	41
		10	4	78
		1	47	90
		0.1	67	92
		Control	97	94
LC_{50} (% Effluent) (95% confidence limits)			0.5 (0.3 - 0.8)	32.1 (25.5 - 38.4)

[1]Final NPDES effluents specified for testing.

[2]Percent survival was determined 4 days after hatching for *Xenopus* and eyed stages of the rainbow trout, and after 9 days of exposure when testing was initiated with freshly fertilized trout eggs.

[3]Not determined.

96-hr acute tests on the designated NPDES effluents, using the fathead minnow in a flow-through system. One objective of this joint investigation was to evaluate effluent toxicity using both acute and embryo-larval test procedures. Precisely the same effluent sources and dilution waters were used in these comparisons and the results are summarized in Table 21. Embryo-larval tests gave more reliable detection and better quantification of effluent toxicity. In addition, it was possible to use dose-response data from embryo-larval tests to calculate LC_1 values. The LC_1 was defined as the toxicity threshold for embryo-larval mortality and/or teratogenesis. This provided an additional reference point with which to evaluate effluent toxicity and, as discussed below (p. 79), such values may be used to estimate effluent concentrations which produce chronic effects on aquatic biota.

In view of data presented in Table 21, it was not possible in the EPA acute tests to determine LC_{50} values in three of the four cases studied, and this precluded an adequate quantitative comparison between fish acute and embryo-larval test responses for the selected effluents. We were able to make such a comparison only in the case of the most toxic effluent, which originated from the Tannery-Sewage Treatment Plant Complex. In this instance, the acute LC_{50} of 8.0% effluent was about 27 times the embryo-larval LC_{50} (0.3%) and differed from the embryo-larval LC_1 value (0.001%) by more than three orders of magnitude. However, it is likely that the quantitative difference between fish acute and embryo-larval test responses will narrow with decreasing effluent toxicity. Considering results for the Metal Plating Plant and most other effluents of equal or lesser toxicity

summarized in Tables 8 and 21, it is estimated that fish acute LC_{50} values probably would not differ by more than one and two orders of magnitude from fish embryo-larval LC_{50} and LC_1 values, respectively, but further study will be required to establish such correlations.

It is obvious from results in Table 21 that fish acute tests cannot be used consistently to quantify the effects of effluents of intermediate or moderate toxicity. This is due to the fact that it is not possible to determine LC_{50} values without extending exposure ranges by concentrating toxic substances beyond levels present in 100% effluent. As it is not practical or plausible to concentrate effluents for such purposes, it appears that fish acute tests, which have been used extensively in assessments of specific toxicants, will prove less useful in the characterization of complex effluents.

In the development of freshwater criteria for specific elements or compounds, principal reliance traditionally has been placed on MATC's developed in chronic life-cycle studies (4, 7). Due to the great time and cost of life-cycle tests, reliable MATC's are available for a relatively small number of priority toxicants. In order to facilitate the use of toxicity data in hazard assessment, application factors have been employed to estimate MATC's or comparable values from acute LC_{50}'s (16). However, it does not appear that application factors will prove as useful in the characterization of complex effluents. This assumption is based on several considerations, including 1) the inability to determine fish acute LC_{50} values for many effluents and 2) the difficulty of establishing a reliable application

factor for a complex toxicant mixture, the composition of which may fluctuate significantly with time. Furthermore, it is evident that MATC's can be estimated more accurately and about as economically by using short-term embryo-larval tests, as discussed below (p. 79). The LC_1 also can be used to calculate the dilution factor required to preclude significant mortality and teratogenesis of sensitive reproductive stages. Such dilution factors, together with transport-fate data and other essential information, should prove useful in assessing the impact of an effluent on its receiving system.

APPLICATIONS OF EFFLUENT BIOMONITORING WITH EMBRYO-LARVAL STAGES

Characterization of effluents. Using LC_{50}'s determined in flow-through and static-renewal tests, moderate to high toxicity was observed with all but one of the 19 industrial and municipal effluents and effluent components studied (Tables 8, 10, 14, 17, 22). Only the secondary treatment effluent from the Lexington Sewage Treatment Plant failed to exert significant toxicity (Table 10). Using the most sensitive test in each case, the LC_{50}'s for the 18 toxic effluents ranged from 0.04% to 0.9% for six; 6.4% to 16.0% for four; 21.6% to 29.3% for four; and 39.2% to 100% for four (Tables 8, 10, 14, 17, 22). Out of these, ten were final NPDES effluents which entered receiving waters and the LC_{50} values were 0.3%, 6.4%, 6.6%, and 21.6% for the four major effluents analyzed on site (Table 22) and 0.04%, 9.4%, 29.3%, 39.2%, 43.0%, and ~100% for the effluent samples tested in the laboratory (Table 8). It is likely that LC_{50}'s for the last six effluents would have been lower if determined in on-site tests.

The LC_1 values ranged from 0.001% to 2.63% for the six final effluents analyzed in the laboratory (Table 8) and, taking data for the most sensitive animal species (i.e., fathead minnow or trout), the LC_1's varied from 0.001% to 0.8% for the four major NPDES effluents analyzed on site (Table 22). Considering data presented above, it is apparent that toxicity tests with embryo-larval stages provided a sensitive and reliable means of evaluating the toxicity of complex effluents.

Table 22. LC_{50} and LC_1 values for final NPDES effluents tested on site.

NPDES Effluent Source	Test System	Test Species[1]	LC_{50} (% Effluent)	95% Confidence Limits	LC_1 (% Effluent)	95% Confidence Limits
Chemical Manufacturing Plant	Flow-through	Fathead minnow	29.4	22.1 - 36.4	2.8	1.1 - 5.1
	Static-renewal	Rainbow trout	6.6	1.9 - 13.5	0.08	0.001 - 0.47
Synthetic Rubber Plant	Flow-through	Fathead minnow	8.3	4.6 - 13.2	0.02	0.002 - 0.08
	Static-renewal	Rainbow trout	6.4	3.3 - 10.3	0.07	0.01 - 0.24
Tannery-Sewage Treatment Plant	Flow-through	Fathead minnow	0.3	0.2 - 0.6	0.001	0.0002 - 0.006
	Static-renewal	Rainbow trout	0.5	0.3 - 0.8	0.004	0.001 - 0.01
Metal Plating Plant	Flow-through	Fathead minnow	21.6	13.9 - 29.5	0.8	0.1 - 2.0

[1]Embryo-larval tests for the fathead minnow were maintained through 3-4 days posthatching; trout were exposed from fertilization through 9 days of development.

Use and legal defensibility of effluent toxicity data. On the basis of the above results, embryo-larval testing appears highly suitable for use under the NPDES program. Permit users could apply on-site testing 1) for periodic monitoring of their effluent discharges, 2) to design and/or evaluate effectiveness of waste treatment systems, and 3) to provide additional documentation in permit applications. Regulatory agencies at State and Federal levels could employ embryo-larval testing, or data obtained by such means, in revising NPDES program requirements, in permit issuance, in granting special dispensations, in determining compliance, and in the documentation of violations considered likely to result in litigation. As the Clean Water Act specifically addresses the discharge of toxic pollutants in toxic amounts, direct toxicological measurements should have greater relevance in legal actions than chemical criteria based on laboratory investigations.

Such effluent toxicity testing is authorized under CWA sections 308 and 402 (35-37). Even in cases where promulgated BAT or BCT effluent guidelines exist, toxicity-based permit limits could be observed on a case-by-case basis under CWA section 402(a)(1). Biological monitoring is defined under CWA section 502 and, as reviewed by Weber (38), some form of biological monitoring is stated or implied in at least 19 sections of the amended Federal Water Pollution Control Act.

While embryo-larval effluent biomonitoring provides the means for sensitive detection and reliable quantification of toxic discharges, such testing also may prove useful in estimating levels of effluent toxicity which produce chronic effects on aquatic biota. In recent studies by Birge, et al. (5, 6), comparisons were made between LC_1 values and maximum acceptable

toxicant concentrations (MATC) determined for a substantial number of organic and inorganic toxicants. The LC_1 values were determined in embryo-larval tests which extended from fertilization through 4 days posthatching, using essentially the same procedures as given above for effluent testing. The MATC's were estimated in 30- to 90-day embryo-larval tests or determined in chronic life-cycle studies, as reported in the literature. The MATC is generally defined as the highest toxicant concentration which has no adverse effect on test organisms (7). However, this value cannot be fixed precisely in most life-cycle studies and, therefore, it usually is taken to fall within the range between the highest no-effect level and the lowest concentration which produces statistically significant responses. Though comparisons for particular toxicants were complicated somewhat by differences in test procedures, water characteristics (e.g., hardness), and the use of different animal species, the LC_1 values generally fell within or near the MATC ranges (Tables 23, 24; ref. 5, 6). Correlations were particularly close in cases where toxicity determinations were made using the same animal species (e.g., Table 24; chromium, lead, silver). When different species were used for the same toxicant, variations between LC_1's and MATC's were no greater than variations among the different MATC's (e.g., Table 24; mercury, zinc). Comparisons of LC_1's with the MATC for 2,4-D were particularly interesting, indicating the rainbow trout to be more sensitive than the fathead minnow, and the latter to be more sensitive than the largemouth bass. This order of species sensitivity was not inconsistent with that frequently observed in tests with other toxicants (7, 11).

These findings further support the premise that probit LC_1 values determined in short-term embryo-larval tests usually approximate the levels of sensitivity which can be statistically verified in chronic life-cycle studies. Limitations involved in long-term investigations frequently curtail use of sufficient replicate exposures and the precise control of test variables required to provide statistical differentiation of low-level test responses. On the other hand, short-term embryo-larval tests can be conducted with greater precision and the dose-response relationship usually can be characterized more adequately, thus permitting reliable probit analysis of test responses. These factors tend to nullify the differences in sensitivity between chronic life-cycle studies and short-term embryo-larval tests. Probit analysis further permits a best-fit determination for the entire dose-response, and discrete lethal concentrations (e.g., LC_{50}, LC_{10}, LC_1) with confidence limits can be determined at any point on the curve. This option should become more important if and when toxicity is integrated more quantitatively with other factors (e.g., transport-fate phenomena, assimilative capacity, reproductive resiliancy) also germane to the development of freshwater criteria and numerical effluent limitations. Many MATC's, particularly if 95% confidence limits are applied to the upper and lower values, reflect rather broad concentration ranges. Such data will prove less useful if the hazard assessment process is further modified to integrate toxicity with important environmental and biological factors which also affect the magnitude of impact on aquatic biota. As chronic studies are not generally applicable to effluent testing, due to time and cost constraints and various technical problems, effluent biomonitoring

Table 23. Life-cycle MATC values compared with embryo-larval LC_1's for organic compounds.[1]

Organic Compound	LC_1 Water Hardness (mg/L as $CaCO_3$) 50	200	Embryo-Larval Test Species[2]	MATC	Water Hardness (mg/L as $CaCO_3$)	Life-Cycle Test Species	Type of Life-Cycle Test[3]
Atrazine (µg/L)	29.0	77.2	Rainbow trout	65 - 120	35.7	Brook trout	plc (15)
2,4-D (mg/L)	0.03	0.02	Rainbow trout	0.3 - 1.5	111 - 192	Fathead minnow	plc (16)
	13.1	3.2	Largemouth bass				
	8.2	8.9	Goldfish				
Malathion (µg/L)	141	440	Goldfish	200 - 580	111 - 192	Fathead minnow	plc (16)
NTA (mg/L)	16.9	20.2	Rainbow trout	54 - 114	34.0 - 45.2	Fathead minnow	plc (17)
	28.5	30.1	Goldfish				
	138	131	Channel catfish				
PCB (µg/L) (Capacitor 21)	0.5	0.9	Largemouth bass	(A1254) 1.8-4.6	44 - 46	Fathead minnow	clc (18,19)
	-	1.0	Rainbow trout	(A1242) 5.4-15.0			
	3.5	1.3	Redear sunfish	(A1248) 1.1-3.0			
				(A1260) 2.1-4.0			

[1]Modified from a previous study by Birge, *et al.* (5).

[2]Tests were conducted using a flow-through system and organisms were exposed from fertilization through 4 days posthatching.

[3]MATC's taken from partial (plc) and complete (clc) life-cycle tests. References are given parenthetically.

Table 24. MATC's compared with LC_1 values determined in static-renewal tests with rainbow trout embryo-larval stages.[1]

Element[2]	LC_1[3] (µg/L)	MATC (µg/L)	Species	Test[4]	Ref.
Cadmium	8.0	1.7 - 3.4	brook trout	clc	20
		3.0 - 6.5	flagfish	el	4
		3.8 - 11.7	brown trout	el	21
		4.1 - 12.5	coho salmon	el	21
		7.4 - 16.9	flagfish	clc	4
		8.1 - 16.0	flagfish	el	22
Chromium	21.5	51 - 105	rainbow trout	el	23
		200 - 350	brook trout	clc	24
Copper	3.4	3.0 - 5.0	brook trout	el	23
		5.0 - 8.0	brook trout	el	23
		9.4 - 17.4	brook trout	clc	25
Lead	10.3	4.1 - 7.6	rainbow trout	plc	26
		7.2 - 14.6	rainbow trout	plc	26
		31.3 - 62.5	flagfish	clc	4
		58 - 119	brook trout	clc	27
		71 - 146	rainbow trout	el	23
Mercury	0.2	0.07 - 0.13	fathead minnow	clc	4
		0.17 - 0.33	flagfish	plc	4
		0.29 - 0.93	brook trout	clc	28
Silver	0.1	0.09 - 0.17	rainbow trout	plc	29
Zinc	216	30 - 180	fathead minnow	plc	30
		139 - 267	flagfish	el	22
		532 - 1368	brook trout	plc	4

[1]Modified from a previous study by Birge, *et al.* (6).

[2]Administered in static-renewal tests from fertilization through 4 days posthatching.

[3]Determined with the probit method of Finney (8), rather than the procedure of Daum (31) used in earlier investigations (1, 3, 10).

[4]MATC's were estimated from 30- to 90-day embryo-larval tests (el) or determined in partial (plc) and complete (clc) life-cycle studies.

with embryo-larval stages should provide an economical and reliable means of estimating chronic effects under the NPDES program.

In determining the LC_1, it is important to achieve an adequate delineation of test responses. Sharp truncations of or internal discontinuites within the dose-response may skew or preclude the calculation of LC_1 values. In this initial investigation, due to the requirements of comparing three test systems and evaluating several alternative animal species, it generally was not possible to repeat tests or to use an extended selection of exposure concentrations. Therefore, it usually was necessary to compromise on five effluent exposure concentrations spaced to cover an extended dose-response range. Despite this limitation, adequate characterization of test responses usually was obtained, particularly with the more sensitive animal species (i.e., fathead minnow, rainbow trout). In future investigations, however, it may be advisable to use an initial "range-finding" test to gauge effluent toxicity, permitting a more precise selection of an exponential series of exposure concentrations for use in final testing (2). While probit analysis is recommended, embryo-larval test responses alternatively can be analyzed using standard statistical procedures.

Characterization of receiving waters. Though it is possible to define effluent toxicity, it is often a complex matter to estimate the impact of toxic outfall upon receiving waters. Dilution, sediment and water characteristics, structure and density of the biomass, and various other factors may grossly alter effects. Toxic properties and pharmacodynamics of the contaminants, their propensity for persistence, and possible interactions may

further complicate hazard assessment. Consequently, when receiving waters are affected by industrial or municipal effluents, which frequently contain complex mixtures of toxicants, reliable impact assessments may be difficult to achieve using present effluent guidelines. One of the most important applications of on-site biomonitoring may be in the direct evaluation of receiving systems. Toxicity tests performed simultaneously on the effluent, the mixing zone, and contiguous water (e.g., upstream, downstream) could provide comparative data useful in estimating acute and chronic effects on aquatic biota. Using a mobile laboratory, such as that described in the Appendix, and taking representative effluent and water samples at 12- to 24-hr intervals, toxicity tests could be performed on six or more sites during a single operation. As such studies would be conducted under actual environmental conditions, net effects of important variables — toxic interactions and other factors which affect the toxicity and bioavailability of effluent contaminants — would be reflected directly in test responses. Such results, together with appropriate chemical monitoring data, should provide more precise evaluations on the environmental impact of industrial and municipal wastes. Correlated effluent/receiving water studies, involving toxicological and chemical monitoring, as well as biotic surveys, should also provide essential baseline information required 1) to quantify the extent to which transport-fate phenomena and other characteristics of the receiving system affect bioavailability and toxicity of effluent contaminants and 2) to determine an accurate means of extrapolating from effluent toxicity determinations to probable effects in receiving waters. It is of interest to note that Ladd (39)

has stressed the need to apply biomonitoring not just to effluents but also to receiving streams, whereby all the interactions affecting toxicity would be considered. In addressing the potential of effluent biomonitoring, Mount (40) recently stressed the fact that the use of an organism is implicit in characterizing toxicity, and that toxicity is a property of wastes and should be regulated within limits necessary to preclude harmful impact upon receiving waters. He further stated (p. 5):

> Some of the advantages of biomonitoring, as opposed to only chemical monitoring, are that it probably more closely approximates the receiving water conditions than nearly anything we can do, and it does consider the interactions that may occur between the components of a waste stream.

In a previous investigation by Birge, et al. (32), embryo-larval tests were conducted on water samples from 11 different streams and rivers which were selected to represent various stages of ecological degradation. Test results correlated closely with independent ecological parameters (e.g., species diversity, density) used to estimate environmental impact. The principal ecological criterion was the retention of fish species diversity over a period of 10 to 20 years, during which time most of the streams were impacted to varying extents by agricultural and industrial development and/or urbanization. At the time of study, two heavily impacted streams had lost all of their original 15 indigenous fish species, and species retention varied from 13% to 100% for the remaining nine streams and rivers. A high correlation ($r = 0.98$) was obtained when percent egg hatchability was compared with fish species retention. Those streams for which retention was 80% or more supported generally healthy and diverse aquatic fauna.

Hatching success also correlated with changes in diversity and density of macroinvertebrate populations in five instances where such data were available. On the basis of these results, it was concluded that biomonitoring studies using early life stages of fish and amphibians provide an accurate means by which to evaluate quality of receiving waters and estimate prospects for long-term ecological degradation. Thus, this study provided significant field validation concerning the use of short-term embryo-larval tests for estimating chronic effects of environmental toxicants on aquatic biota.

Use of embryo-larval toxicity testing in the evaluation of effluent treatability. In three of five on-site studies, consideration was given to effluent components or effluents at different stages of treatment. The objective was to determine the utility of biomonitoring with embryo-larval stages in identifying toxic effluent fractions and in determining effectiveness of waste treatment processes. The initial testing was performed at one of several sewage treatment plants in Lexington, Kentucky. Using newly hatched larvae of the channel catfish as test organisms, an LC_{50} of 50.2% was obtained for the effluent from primary treatment and no appreciable toxicity was observed for secondary effluent (Table 10). This particular plant had established a good performance record, and no apparent impact had been reported for the receiving waters, a fourth-order stream of relatively small size.

During the on-site investigation conducted at the Tannery-Secondary Sewage Treatment Plant Complex in southeastern Kentucky, static-renewal tests using three different animal species were conducted simultaneously on

raw tannery waste, unchlorinated secondary effluent, and the chlorinated final secondary effluent which entered a third-order stream of moderate size. The tannery effluent which provided approximately 25% of the wasteload of the treatment plant was highly toxic, as judged by LC_{50} values of 0.08%, 0.3%, and 0.4% with rainbow trout (fresh eggs), the fathead minnow, and Xenopus, respectively (Table 15). The LC_{50} values ranged from 0.9% to 1.6% and 0.5% to 0.9% for unchlorinated and chlorinated secondary effluents, and the 95% confidence intervals were uniformly small (Table 14). Though values obtained for a number of physicochemical parameters indicated some improvement in water quality after treatment, a more accurate and definitive assessment was possible using toxicity data. It was especially significant that results with the three different animal species were highly consistent concerning differences observed in toxicity among the three effluent sources (Table 15). In this particular case, the receiving water had been heavily impacted.

The results discussed above clearly indicate the value of comparative toxicity data taken before and after effluent treatment. However, in designing and monitoring waste treatment systems, it is also necessary to characterize different effluent components and identify toxic fractions which contribute to the final discharge. During the on-site study conducted at a metal plating plant in northeastern Kentucky (Tables 17, 18), embryo-larval tests were used to differentiate among four separate components. As summarized in Table 18, LC_{50} values determined in static-renewal tests with the fathead minnow were 0.01%, 0.05%, 23.6%, 25.4%, and 44.7% for Components 1, 2, 3, 4, and the final effluent, respectively. In addition to the four

specific plant effluents, surface runoff contributed at least 30% to the final effluent. Due to local precipitation at the time of testing, the contribution from surface runoff was difficult to quantify and could have been greater. This dilution undoubtedly accounted in substantial measure for the lower toxicity of the final effluent. Considering data summarized in Table 18, it appeared that the quality of the different effluent components and the final effluent could be compared more precisely and economically using toxicity data than by exhaustive analyses of multiple physicochemical parameters. Though the latter are important in monitoring waste components, the complexity encountered in the analysis of such data often impedes accurate estimates of toxicity. In this and other investigations, it was possible to obtain direct quantification of toxicity using simple and inexpensive test procedures (i.e., static-renewal).

It was concluded, therefore, that on-site biomonitoring was highly desirable, if not critical, for comparative assessments on effluent fractions and for judging impact potential of untreated and treated effluents. It is also important to note that static-renewal procedures were judged suitable for on-site testing, and that this generally could be accomplished 1) with low to moderate cost, 2) without need for sophisticated instrumentation, 3) with minimum space requirements, and 4) without need for highly technical personnel. The benefits of such testing to industry should far outweigh investments in time and materials.

Recommendations for future work. In view of the potential of toxicological biomonitoring in hazard assessment under the NPDES program, a

number of recommendations should be made concerning further work, as follows:

1) Application of on-site biomonitoring to receiving waters. This should include comparative toxicological and chemical monitoring of the effluent outfall, the mixing zone, and the contiguous waters. In addition, coordinated field studies should be carried out on the impacted waters, involving a) faunistic surveys (e.g., fish, macroinvertebrates, benthic invertebrates), b) toxicant residues in fish tissues, and c) histopathological analyses of fish tissues. Transport-fate phenomena and other important characteristics of the receiving system should be taken into account in correlating results and in assessing the reliability of biomonitoring as a means of quantifying effluent toxicity and estimating impact on receiving waters.

2) Extension of baseline on-site embryo-larval biomonitoring studies to include a larger, representative selection of industrial and municipal effluents and waste treatment systems.

3) Further perfection and standardization of embryo-larval biomonitoring procedures, including computer programs for data analysis and impact assessment.

4) Incorporation of additional test parameters for on-site biomonitoring (e.g., short-term Daphnia test, tissue residue analyses).

5) Establishment on a trial basis of a laboratory screening program for municipal and industrial effluents, working with regulatory agencies and selected industries. Composite effluent samples would be analyzed a) to develop a data base on effluent toxicity, b) to assist in the development of

waste treatment procedures, and c) to assess the effectiveness of laboratory screening.

6) Develop a short-course training program, including laboratory and field experience, to familiarize potential users with biomonitoring techniques.

7) Extend biomonitoring techniques to include the screening of important health-related effects (e.g., teratogenesis, mutagenesis, carcinogenesis).

8) Conduct an international symposium on biomonitoring as applied to aquatic hazard assessment.

SUMMARY

The principal objectives of this study included 1) development of embryo-larval test systems using fish and amphibian species for on-site toxicological evaluations of complex effluents; 2) application of embryo-larval test systems to on-site biomonitoring of selected municipal and industrial effluents; 3) comparisons of sensitivity and reliability of acute and embryo-larval effluent biomonitoring; 4) evaluation of embryo-larval tests for characterization of effluents under the NPDES program; and 5) a description of procedures for effluent testing with fish and amphibian embryo-larval stages. Further work involved determining the reliability of laboratory testing of composite effluent samples, using direct on-site biomonitoring as a basis for comparison.

Development of embryo-larval test systems. Of four test organisms used in on-site effluent biomonitoring, early trout embryos (fertilization through 9 days) and embryo-larval stages of the fathead minnow (fertilization

through 3 to 4 days posthatching) consistently were the most sensitive, based on LC_{50} values (percent effluent). Results obtained with these species gave the most reliable data for on-site detection and quantification of effluent toxicity. Eyed trout eggs carried through 4 days posthatching and embryo-larval stages of Xenopus were appreciably more tolerant. Ten comparative evaluations were made in which flow-through, static-renewal, and static tests were simultaneously used to evaluate selected effluents and a reference toxicant (i.e., phenol). In each case, the three tests were conducted using the same exposure period, animal species, dilution water and, in so far as possible, general test parameters (e.g., temperature, dissolved oxygen, water hardness). In most instances, flow-through tests provided the greatest resolution of dose-response data and the lowest LC_{50} values. Results obtained with flow-through and static-renewal tests often did not vary significantly, and the LC_{50} values usually were within a factor of about two or less, and seldom differed by more than a factor of 2.5. Static procedures proved less suitable for use in effluent biomonitoring with embryo-larval stages.

Application of test systems to effluent biomonitoring. Moderate to high toxicity was observed with all but one of 19 industrial and municipal effluents and effluent components studied. Using the most sensitive test in each case, the LC_{50}'s for the 18 toxic effluents ranged from 0.04% to 0.9% for six; 6.4% to 16.0% for four; 21.6% to 29.3% for four; and 39.2% to ~100% for four. Out of these, ten were final effluents released to receiving waters and the LC_{50} values were 0.3%, 6.4%, 6.6%, and 21.6% for four major effluents analyzed on site and 0.04%, 9.4%, 29.3%, 39.2%, 43.0%,

and ~100% for other effluents tested in the laboratory.

During the major on-site studies, grab and composite samples from NPDES effluents designated for testing were transported to the laboratory for simultaneous analyses. Based on the results, it was concluded that effluent toxicity can be quantified more accurately in on-site tests. However, tests conducted in the laboratory using embryo-larval stages of sensitive aquatic species could prove useful in preliminary effluent screening.

In each of four on-site biomonitoring studies, 96-hr acute tests were performed with the fathead minnow using a flow-through system. By comparison, embryo-larval tests gave more reliable detection and much better quantification of effluent toxicity. For example, in studies with the most toxic effluent, LC_{50} values obtained with the fathead minnow were 8.0% and 0.3% in acute and embryo-larval tests, respectively. In the remaining three tests, effluent LC_{50} values ranged from 8.3% to 29.4% when determined with embryo-larval stages, but fish acute LC_{50} values could not be determined.

In three on-site studies, consideration also was given to effluent components or effluents at different stages of treatment. The objective was to determine the utility of biomonitoring with embryo-larval stages for identifying toxic effluent fractions and for determining the effectiveness of waste treatment processes. Results indicated that more accurate and definitive assessments were possible with toxicity data than with physicochemical parameters. It was concluded, therefore, that on-site biomonitoring was highly desirable, if not critical, for comparative assessments on the impact

potential of untreated and treated effluents and effluent components.

On the basis of results obtained, it was evident that embryo-larval effluent biomonitoring provided sensitive detection and reliable quantification of toxic discharges, and that such testing also may prove useful in estimating levels of effluent toxicity which produce chronic effects on aquatic biota. Permit users could apply on-site testing 1) for periodic monitoring of effluent discharges, 2) to design and/or evaluate effectiveness of waste treatment systems, and 3) to provide additional documentation in permit applications. Regulatory agencies at State and Federal levels could employ embryo-larval testing, or data obtained by such means, in revising NPDES program requirements, in permit issuance, in granting special dispensations, in determining compliance, and in the documentation of violations considered likely to result in litigation.

APPENDIX

TEST PROCEDURES FOR EMBRYO-LARVAL BIOMONITORING

Introduction

The purpose of this Appendix is to provide recommended procedures for the performance of effluent toxicity tests using embryo-larval stages of fish and amphibians. The methods given here were modified from those previously developed for more conventional laboratory studies (1, 5, 7, 9, 11). In addition to procedures discussed below, detailed methods on acute testing and extensive background information pertinent to effluent monitoring have been presented in comprehensive publications by Peltier (2) and Weber and Peltier (33).

Description and Operation of Embryo-Larval Biomonitoring Systems

Two test systems have been found suitable for embryo-larval biomonitoring of municipal and industrial effluents. The flow-through procedure usually is recommended for definitive tests. However, the static-renewal system provides a cost-effective and reliable means for performing preliminary screening of effluent samples. Generally 12 exposure chambers are used in each static-renewal test, permitting duplicate dishes for controls and each of five effluent concentrations. This assembly of 12 test units requires less than 4 square feet of bench space. Solutions usually are renewed every 12 or 24 hrs, but the interval can be modified to accommodate special needs. For example, if an effluent contains highly volatile components, toxicity may be characterized more accurately by using a shorter renewal period (e.g., 6-8 hrs).

The static-renewal test system is illustrated in Figures 1 and 2.3. The exposure chamber is a Pyrex deep petri dish (400 mL), modified by the addition of a glass inlet/outlet tube (4 mm I.D.). This tube, attached to a 3-way valve, allows 1) gradual flow of effluent solution into the exposure chamber, 2) siphoning of spent test water from the chamber, and 3) moderate, continuous aeration of test water if required. Effluent and effluent dilutions are delivered by gravity flow to the exposure chambers using 3" Pyrex funnels and silicone or latex tubing. During the solution renewal step, a small volume of test water (50 mL) is retained in the exposure chamber.

The flow-through system used for embryo-larval biomonitoring is illustrated in Figures 3.2 and 4. Inlet and outlet tubes (10 mm I.D. Pyrex) are annealed to deep petri dishes used as exposure chambers. The inlet is positioned approximately 7 mm above the bottom of the dish and the outlet is attached to the opposite side, just below the shoulder. Teflon or stainless steel screening is placed at the ends of the influent and effluent tubes to prevent loss of test organisms. Flow rate through each exposure chamber is set at 200 mL/hr and monitored by timed volumetric measurements or Gilmont no. 12 flow meters. Retention time for the 300-mL chamber is 1.5 hrs.

Full-strength effluent is continuously pumped from the NPDES sampling station to an overflow-equipped effluent reservoir situated inside the mobile laboratory. Incoming effluent is filtered through glass wool and delivered to a serial diluter by a peristaltic pump (Brinkmann model 131900). Dilution

water, held in a 30-gallon Nalgene tank, is administered to the diluter by gravity flow, which is regulated using a float valve. The purpose of the glass wool filter is to remove only coarse particulates which might produce malfunctioning of the dilution system, and this should not significantly alter effluent toxicity. Considering flow rate, as well as retention time in the effluent reservoir and diluter, elution of soluble toxicants from such particulates should occur prior to the exposure of test organisms.

The diluter, as seen in Figure 3.2, is constructed of 4.7 mm Plexiglas and has overall dimensions of 40 cm (l) by 10 cm (w) by 45 cm (h). The structure is divided into several compartments, including an upper head box (15 cm deep) and four dilution (mixing) chambers which are 10 cm square and 10 cm deep. The length of the box can be increased if more than four dilution chambers are desired. The head box receives dilution water which is distributed to the dilution chambers via adjustable standpipes. Standpipes are fabricated from 15-cm lengths of 3-mm O.D. glass tubing and fitted with size 00 rubber stoppers. Each standpipe is fire-polished to deliver approximately 30 ± 1 mL/min when the intake is positioned at mid-depth in the head box. Vertical adjustment of the standpipes permits precise regulation of dilution water flow.

Each dilution chamber is provided with three staggered baffles to insure thorough mixing of effluent and dilution water. Overflow notches cut in the front panel maintain a constant working volume of 850 mL. Given a total flow rate of 33 mL/min (1:10 dilution ratio) for combined effluent and dilution water, retention time in the mixing chamber is approximately

26 min. A peristaltic pump delivers full-strength effluent to the first mixing chamber through 1.6-mm I.D. silicone tubing, which enters the diluter through the rear panel. A second pump channel extracts diluted effluent from the first mixing chamber and delivers it to the next mixing chamber, where further dilution is accomplished by standpipe flow. This process is repeated for all subsequent dilutions. Effluent concentrations (e.g., 10%, 1%, 0.1%, 0.01%) are carried by peristaltic pump channels from each dilution chamber to duplicate embryo-larval exposure chambers. Dilution ratios are determined daily, using timed volumetric measurements of effluents (peristaltic pump flow) and dilution water (standpipe flow). The 100% and 50% effluent concentrations are provided directly to exposure chambers by peristaltic pump flow. Dilution water for control populations is pumped directly from the diluter head box or the dilution water reservoir (Figure 3.2). The flow system requires approximately 800 mL/hr of undiluted effluent and 8 L/hr of dilution water to provide continuous flow to two replicate exposure chambers for control water, full-strength effluent, and four effluent dilutions. This diluter system was developed in a previous study by Freeman and Birge (unpublished observations), and details concerning its design and construction currently are being submitted for publication. In tests reported above, overall errors in flow rates and dilution ratios usually were within 5% and seldom exceeded 10%.

The flow-through system, as described above, provided a reliable means of quantifying the toxicity of the various effluents tested to date. However, should an effluent prove particularly difficult to test, due to

highly volatile or insoluble components, the system can be modified to minimize such problems by employing procedures previously described by Birge, et al. (9).

The test systems described above are housed in a mobile laboratory especially designed for embryo-larval biomonitoring (Figure 2.1). Dimensions of the laboratory are 14 feet in length, 7 feet 10 inches in width, and 7 feet 6 inches in height. The unit is constructed with an aluminum exterior, 3 inches of Styrofoam insulation in the walls and ceiling, and one-half inch plywood interior walls. The two-inch thick wooden floor is overlaid with three-quarter inch marine plywood and heavy-duty vinyl floor covering. Four inches of Styrofoam insulation are laid beneath the floor and retained in place with a 12-gauge galvanized metal subfloor. Due to the heavy insulation, the high reflectivity of the aluminum exterior, and the lack of windows, a 5000-BTU air conditioner is adequate to maintain temperature down to 17°C even when ambient temperature reaches 35°C. A 1500-watt portable heater provides adequate temperature regulation during cold weather. When lower temperatures are required for testing coldwater species (e.g., trout; 12 ± 1°C), up to 14 exposure chambers can be contained in a 4.8 cubic foot table-top refrigerator (Figures 2.2, 2.4).

As shown in Figure 3.1, stand-up-height kitchen cabinets with Formica tops provide ample space for test systems and monitoring equipment. Adjustable shelves are used for additional equipment, peristaltic pumps, reagents, and labware. If a local electrical hookup is not available, power is supplied to the laboratory by a diesel generator. The latter provides current to five 20-amp circuits and approximately 24 outlets.

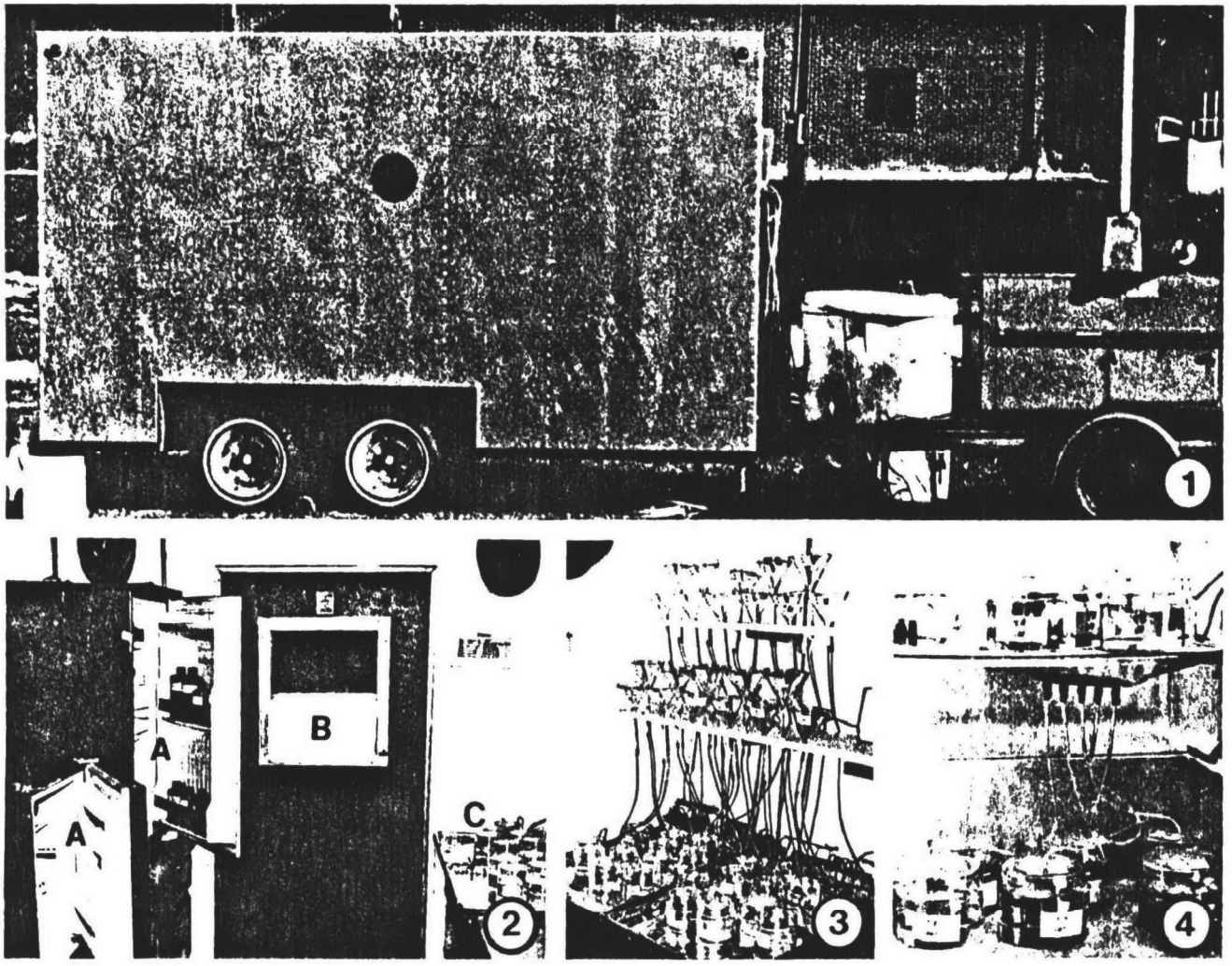
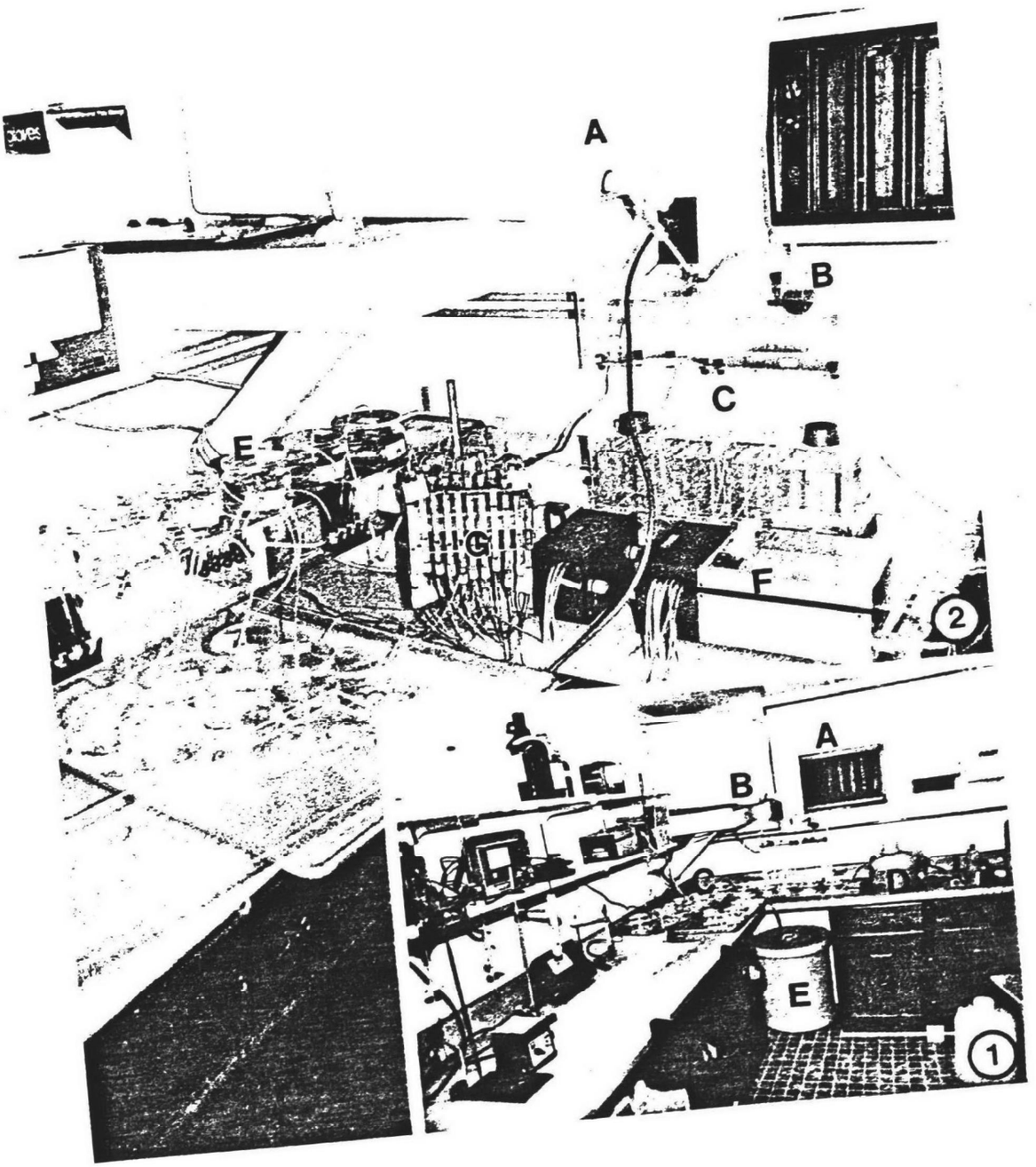

A
A
B
C
1
2
3
4

Figure 3

3.1 Interior view of the front end of the mobile laboratory. Furnishings include adjustable shelving and stand-up-height kitchen cabinets with Formica bench tops. Other facilities include an air conditioner (A), a dilution water reservoir (B), a flow-through effluent test system with embryo-larval exposure chambers and related equipment (C), a flow-through effluent reservoir contained in a stainless steel sink (D), a dilution water storage tank (E), and general laboratory apparatus (*e.g.*, pH meter, dissolved oxygen meter). Due to the heavy insulation of the mobile laboratory, a 5000 BTU air conditioner is adequate to regulate temperature down to 17°C even when ambient temperature reaches 35°C. Dilution water and effluent reservoirs are filled and the flow system is set in operation at least 12 to 24 hrs prior to the onset of testing (introduction of test organisms). During this period, flow rates and dilutions ratios are monitored and adjusted. This time interval also permits effluent and dilution water to reach room temperature which is regulated in the optimum range for selected test organisms. Effluent is pumped into the mobile laboratory from an external source using submersible pumps. Approximately 800 mL/hr of effluent are required to supply a series of 10 exposure chambers (duplicates of five effluent concentrations). Due to the low volume of flow, which can be regulated with a hose clamp, and the standing time in the overflow-equipped effluent reservoir (approximately 45-75 min), undiluted effluent normally reaches the selected test temperature before entering the flow-through system. However, if necessary, further temperature adjustment can be achieved by regulating effluent flow rate, by lengthening the effluent lines, or by varying retention time in the effluent reservoir. After optimum conditions have been reached, test organisms (*e.g.*, fish or amphibian eggs) are placed in the exposure chamber.

3.2 Enlarged view of flow-through effluent test system. A thirty-gallon Nalgene tank serves as the dilution water reservoir (A). The latter is connected by a float valve (B) to a four-stage serial diluter (C) which receives a continuous flow of full-strength effluent provided by a peristaltic pump (D). The same peristaltic pump is used to supply 100% and 50% effluent directly to embryo-larval test chambers (E). Lower dilutions (*e.g.*, 10%, 1%, 0.1%, 0.01%) are conveyed from the diluter to exposure chambers using an additional peristaltic pump (F). Flow rates through peristaltic pump lines are monitored using Gilmont no. 12 flow meters (G).


A
B
C
E
G
F
2
A
B
E
1

Effluent Sampling Procedures, Dilution Water, and Test Conditions

As noted above, effluent used in the flow-through system is pumped continuously from the NPDES sampling station to an effluent reservoir in the mobile laboratory. Approximately 800 mL/hr of effluent are required to supply a series of ten exposure chambers (duplicates of five exposure concentrations). Due to the low volume of flow, which can be regulated with a hose clamp, and the retention time in the effluent reservoir (approximately 45-75 min), undiluted effluent normally reaches the selected test temperature (e.g., 22 ± 1°C for warmwater species) before entering the flow-through system. If necessary, additional temperature control can be achieved by adjusting effluent flow rate, by lengthening effluent reservoir inlet or outlet lines, or by varying retention time in the effluent reservoir. For static-renewal tests, samples are taken from the effluent reservoir every 12 or 24 hrs, diluted to the required effluent concentrations, and administered to the test organisms. A 12-hr renewal interval is recommended. Dilution water is usually collected from an appropriate source near the test site (e.g., deep wells; receiving water upstream from the plant discharge) and stored in 25-gallon tanks in the mobile laboratory. However, when dilution water of acceptable quality cannot be obtained locally, the alternative sources given by Peltier (2) or the reconstituted water described by Birge, et al. (7, 11) are recommended.

When tests are conducted with coldwater species, dilution water and effluent must be cooled prior to testing. For flow-through tests, a

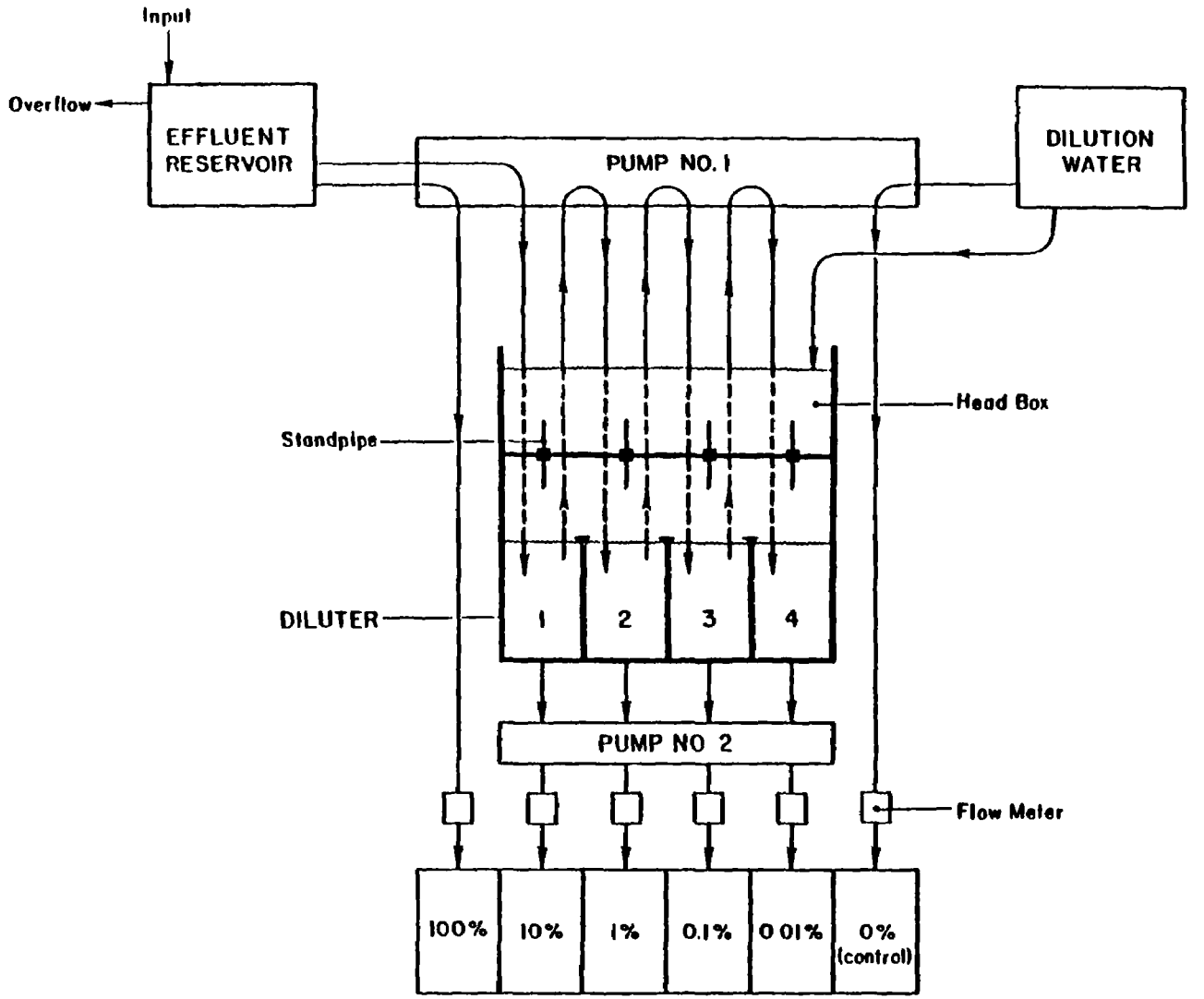


Figure 4. Design of the flow-through effluent test system.

Effluent from a flow-through reservoir is delivered to a four-stage serial diluter by a peristaltic pump. Dilution water, held in a 30-gallon Nalgene reservoir, is conveyed to the diluter head box by gravity flow and distributed to the dilution chambers (i.e., 1-4) via adjustable standpipes. Full-strength effluent is delivered by a peristaltic pump to the first mixing chamber (i.e., 1) and combined with dilution water to effect the desired effluent concentration. A second pump channel extracts diluted effluent from the first mixing chamber and delivers it to the next chamber (i.e., 2) where it is further diluted by standpipe flow, and this process is repeated for all subsequent dilutions. A second peristaltic pump is used to supply effluent dilutions (e.g., 10%, 1%, 0.1%, 0.01%) to corresponding exposure chambers, and 100% effluent is pumped directly from the effluent reservoir. Dilution water for control populations is supplied from either the dilution water reservoir or the diluter head box. Flow rates are monitored using calibrated flow meters or t volumetric measurements. This system will accommodate 12 or more exposure chambers, permitting replicate tests.

refrigerated water bath or immersion cooler may be required. However, static-renewal exposure chambers and a one-day supply of replacement solution (~4 liters) can be maintained in a table-top refrigerator. Continuous aeration should be supplied to the dilution water reservoir and this usually is sufficient to maintain dissolved oxygen at or close to saturation in the exposure chambers. If necessary, aeration should be supplied directly to the exposure chambers to prevent dissolved oxygen from falling below 60% saturation. In the latter case, air flow should be maintained at a low rate to minimize loss of volatile effluent components.

Selection and Handling of Test Organisms

Requirements for the selection of animal test species should include good general health, an uncontaminated native habitat, adequate seasonal availability of viable eggs, ease of transport and handling, and sufficient sensitivity to toxicant stress. Though not essential, it is desirable to select organisms that are indigenous to the effluent receiving stream or similar waters. Biomonitoring experiments can be initiated either with eggs spawned at the test site or with fresh spawns collected from field or laboratory populations. Eggs generally can be transported in ice-packed containers to the test location by air freight or automobile. It is recommended that tests be initiated as soon after fertilization as possible. For warmwater species, tests should be continued through 4 days posthatching. With coldwater fish such as trout, a testing period spanning the first 8 to 9 days of embryonic development has proved

adequate for evaluating effluent toxicity. Fish and amphibian species found suitable for use in short-term embryo-larval toxicity tests are included in Table 25, and characteristics of several of these species are discussed below.

A. Fish

1. Fathead minnow (Pimephales promelas). Eggs and larvae of the fathead minnow are used extensively in toxicity testing and are relatively sensitive to many contaminants. The egg is approximately 1 to 3 mm in diameter, and each female produces 50 to 60 eggs. Eggs can be obtained throughout the year from established laboratory cultures. Although artificial spawning methods can be employed, lower egg viability usually results. The developmental period to hatching is 4 to 5 days at 22°C. Because this organism is somewhat susceptible to fungal contamination, it is recommended that dead embryos be removed from exposure chambers daily.

2. Rainbow trout (Salmo gairdneri). Embryo-larval stages of this species also have been used widely in toxicity testing and usually are highly sensitive to aquatic contaminants. The egg is 6 to 8 mm in diameter, and each female produces between 1,000 and 3,500 eggs. The adults can be spawned in the laboratory or in the field, permitting initiation of tests immediately after fertilization. Although developmental time to hatching is relatively long (23 days at 12.5°C), an exposure period spanning the first 8 to 9 days of development has proved adequate for reliable assessments of effluent toxicity. Depending upon strain and geographic location, gravid adults are available 9 months of the year (August-April). It should be

noted that eyed-egg stages are considerably more tolerant than early embryos.

3. Channel catfish (Ictalurus punctatus). Embryo-larval stages of the catfish are quite sensitive to many aquatic contaminants. The egg is approximately 4 to 8 mm in diameter, and each female produces from 6,000 to 15,000 eggs. The developmental period to hatching is 5 to 6 days at 22°C. While an ideal test species in many respects, catfish have a short spawning season (June - July) and the eggs are susceptible to fungal contamination.

4. Largemouth bass (Micropterus salmoides). Compared to embryos and larvae of the above three species, developmental stages of the largemouth bass usually are somewhat less sensitive to aquatic toxicants. The egg is 1 to 2 mm in diameter, and each female produces approximately 2,000 to 10,000 eggs. The developmental time to hatching is 3 to 4 days at 22°C and spawning, depending upon temperature, generally occurs from March through June.

5. Bluegill sunfish (Lepomis macrochirus). Embryos and larvae of this species usually exhibit sensitivity similar to that observed with life-stages of the largemouth bass. The egg is small (0.75-1.5 mm in diameter), and each female produces approximately 600 to 2,000 eggs. The developmental period to hatching is 2 to 3 days at 22°C, and the spawning season ranges from April through July.

6. Goldfish (Carassius auratus). Goldfish generally are similar to bass and bluegill in terms of embryo-larval sensitivity to aquatic toxi-

cants. The egg is 1 to 2 mm in diameter, and each female produces between 5,000 and 20,000 eggs. As spawning can be induced by hormonal injection, eggs are available for testing over much of the year (February-October). The developmental period to hatching is approximately 3 days at 22°C.

B. Amphibians

1. African clawed frog (Xenopus laevis). Embryos and larvae of the African clawed frog, a species recently introduced to the United States, appear to exhibit sensitivity somewhat less than that observed for most other amphibian species (e.g., leopard frog). The eggs measure 1.2 to 1.7 mm in diameter, and each female produces between 1,000 and 2,000 eggs. By use of hormonal injections, eggs are available for testing nearly 12 months of the year. The developmental period to hatching is approximately 2 days at 22°C. The reproductive history and embryology of Xenopus laevis is especially well known, as it is one of the major experimental organisms used in developmental biology. Compared to fish species, parental stocks can be maintained with simpler facilities and lower cost.

2. American toad (Bufo americanus). Compared to many other amphibian species, embryos and larvae of the American toad are more tolerant to aquatic toxicants. The eggs are approximately 1.0 to 1.4 mm in diameter, and each female produces from 2,000 to 4,000 eggs. The spawning season spans approximately 5 months (April-August), and developmental time to hatching is 2 to 3 days at 22°C.

3. Bullfrog (Rana catesbeiana). Embryos and larvae of the bullfrog appear to be comparable in sensitivity to developmental stages of

Table 25. Candidate species for use in embryo-larval toxicity tests on industrial and municipal effluents.[1]

PREFERRED SPECIES

Fish

- Bluegill sunfish (*Lepomis macrochirus*)
- Channel catfish (*Ictalurus punctatus*)
- Fathead minnow (*Pimephales promelas*)
- Goldfish (*Carassius auratus*)
- Largemouth bass (*Micropterus salmoides*)
- Rainbow trout (*Salmo gairdneri*)
  - fresh eggs
- Redear sunfish (*Lepomis microlophus*)

Amphibians

- Bullfrog (*Rana catesbeiana*)
- Leopard frog (*Rana pipiens*)

OPTIONAL SPECIES

Fish

- Rainbow trout (*Salmo gairdneri*)
  - eyed eggs

Amphibians

- African clawed frog (*Xenopus laevis*)
- American toad (*Bufo americanus*)
- Fowler's toad (*Bufo fowleri*)
- Narrow-mouthed toad (*Gastrophryne carolinensis*)

[1]Species were restricted to those used in our effluent testing program. Many other aquatic species should prove suitable for this purpose.

the bass, goldfish, and bluegill sunfish. The egg measures 1.2 to 1.7 mm in diameter, and each female produces from 2,000 to 10,000 eggs. The spawning season is from May through August, and developmental time to hatching is 4 to 5 days at 22°C.

4. Leopard frog (Rana pipiens). Embryo-larval stages of the leopard frog have been used extensively in experimental biology and aquatic toxicity tests and are quite sensitive to many contaminants. The egg averages 1.6 mm in diameter, and each female produces from 2,000 to 4,000 eggs. Gravid adults can be obtained from biological supply houses or field populations. Animals can be spawned over a 7-month period (November-May), using hormonal injection. Developmental time to hatching is 5 to 6 days at 22°C. This species is especially applicable to effluent testing due to its broad geographic distribution and other characteristics.

5. Narrow-mouthed toad (Gastrophryne carolinensis). The narrow-mouthed toad consistently has been one of the most sensitive species tested in our laboratory. The egg is 1.0 to 1.2 mm in diameter and each female produces from 100 to 200 eggs. The spawning season is quite long (April-November), and developmental time to hatching is approximately 3 to 4 days at 22°C. Limitations associated with the use of this species involve its rather narrow geographic distribution and low egg production.

Test Responses, Expression of Data, and Statistical Procedures

In the effluent toxicity test systems described above, organisms are exposed to full-strength effluent, several effluent dilutions, and dilution

(control) water. Eggs are examined daily to gauge extent of development and to remove dead animals. Sample size generally ranges from 50 to 100 organisms per exposure chamber. Two species may be tested in the same exposure chamber provided they are separated by a Teflon or stainless steel screen. Only eggs of high viability should be used, and control survival should average about 80% or more.

Test responses include frequencies of egg hatchability, embryo-larval survival, and teratogenesis. Determinations of teratic organisms are limited to gross defects considered likely to preclude survival, as discussed previously by Birge et al. (11, 13). Defects most commonly encountered are acute lordosis, scoliosis, and other gross anomalies of the vertebral column. Percent hatchability is based on all organisms, normal and aberrant, which live to complete the hatching process. In determining percent survival, teratic organisms are counted as lethals, except when tests are terminated prior to hatching (i.e., fresh trout eggs). Taking accumulative dose-response data at the end of the exposure period, log probit analysis (8) is used to determine LC_{50} values (percent effluent by volume) with 95% confidence limits. In addition, LC_1 values, defined as concentrations producing 1% control-adjusted impairment of test populations, are calculated to estimate effluent dilution factors required to preclude toxic effects to embryo-larval stages and to estimate effluent concentrations likely to produce chronic effects. In determining the LC_1, it is important to develop an adequate delineation of test responses. Sharp truncations of or internal discontinuities within the dose-response

may skew or preclude the calculation of LC_1 values. For this reason, it is advisable to use an initial "range-finding" test to estimate effluent toxicity, permitting a more precise selection of an exponential series of exposure concentrations for use in final testing (2).

Performance Evaluation and Personnel Requirements

Reference toxicants may be used to evaluate and standardize performance of embryo-larval effluent biomonitoring systems. Such evaluations usually are required to assess the precision and reproducibility of the flow-through procedure. A known amount of toxicant (e.g., zinc, phenol) is added to the effluent reservoir which supplies the serial diluter, and test solutions from the exposure chambers are analyzed for toxicant concentrations. Accuracy of the test system can be evaluated by comparing calculated nominal concentrations with actual toxicant determinations.

During the performance of effluent toxicity tests, flow rates from the diluter head box and peristaltic pumps should be monitored regularly to determine the accuracy of dilution ratios. Solutions in the exposure chamber should be analyzed daily for general water quality characteristics. Temperature, dissolved oxygen, specific conductivity, and pH may be determined using a YSI telethermometer with thermocouple (model 42SC), YSI oxygen meter (model 54A), YSI conductivity meter (model 33), and a Corning pH meter (model 610). Hardness and alkalinity measurements are accomplished using the EDTA and methyl orange titrimetric procedures described in Standard Methods (34). In addition to the above parameters, it may be desirable to

analyze test solutions for one or more of the principal effluent toxicants. General parameters of test water should be determined either in exposure chambers (e.g., temperature, DO) or soon after sample collection (e.g., alkalinity, hardness). Special attention should be given to procedures used in the collection and preservation of effluents. Effluent samples to be used for toxicity evaluations should be stored without residual air space in tightly capped containers (e.g., Teflon, Pyrex, stainless steel), and tests should be performed as soon as possible to minimize storage time. If tests are not to be initiated soon after collection, effluent samples should be refrigerated. When chemical analyses are to be performed, handling and preservation procedures may vary according to the characteristics of the effluent or the specific toxicants selected for analysis, and reference should be made to Standard Methods (34) or other sources (2, 33).

Embryo-larval tests described above can be performed without the use of highly technical personnel. Though a minimum of two years of study or experience in biology, chemistry, or related disciplines is preferred, individuals of high aptitude but less formal background may prove satisfactory, given adequate training.

Cost Analysis for Effluent Monitoring Using Embryo-Larval Toxicity Tests

Estimates were subject to some imprecision due to the fact that the work performed was largely experimental. The cost of a mobile laboratory as described above ranges from $10,000 to $12,500, depending upon optional

features (e.g., exterior lighting, electrical service, plumbing, construction materials). For extensive field work, a diesel-powered generator also is required. The cost varies with amperage output and different models, ranging from approximately $5,500 to $8,500 for a suitable unit. Minimum equipment necessary to perform flow-through studies, either in the laboratory or on site, totals approximately $8,500 (e.g., diluter, peristaltic pumps, pH meter, telethermometer, dissolved oxygen meter, titration apparatus, exposure chambers, general labware), whereas that required for static-renewal testing costs about $3,000 (e.g., pH meter, titration apparatus, dissolved oxygen meter, telethermometer, and labware). Once obtained, these facilities can be reused extensively with minimal maintenance.

On-site testing, as conducted in this study using a mobile laboratory, requires a two-man crew for a period of 12 to 13 days. Cost of food, lodging, and local travel averages $125 per day. Expendable supplies total about $300 for each flow-through test and $200 when the static-renewal system is used.

Effluent testing conducted by industry personnel, excluding space requirements, electrical supply, and cost of test organisms, would require approximately $200 in expendable supplies and 32 hrs of labor for a series of static-renewal tests performed over an 8- to 10-day period. Labor would more than double with the use of flow-through procedures. Exclusive of permanent equipment and space requirements, a set of static-renewal tests likely could be completed for about $500 (labor and expendable supplies), and the cost probably would increase to $1,000 to $1,200 for

flow-through tests. These are minimal estimates and could increase depending upon wage scale, efficiency of personnel, and conditions at particular testing sites (e.g., access to effluent, availability of test organisms).

Equipment Inventory

A. Test System and Monitoring Equipment

Nalgene rectangular tank (30 gallon)
Nalgene round tank (25 gallon)
Peristaltic pump (Brinkmann model 131900 or model IP-12)
Submersible pump (Little Giant model 2E-NDVR)
Refrigerators (Norcold model DE250, 2.5 cu. ft.; Gerald model GR54, 4.8 cu. ft.)
pH Meter with probe (Corning model 610)
Conductivity meter with probe (YSI model 33)
Oxygen meter with probe (YSI model 54A)
Telethermometer (YSI model 42SC)
Air pumps (Hagen Optima)
Diluter
Pyrex test chambers with covers - flow design
Pyrex test chambers with covers - static and static-renewal design
Long-stem 3" Pyrex funnels with covers
Liquid flow meters (Gilmont no. 12)

B. Test System and Monitoring Supplies

Silicone tubing, assorted diameters
Tubing connectors
Latex rubber tubing
Air valves
Pyrex carboys (5 gallon)
Nalgene carboys (5 gallon)
Pyrex Erlenmeyer flasks
Pyrex graduated cylinders
Pyrex funnels
Burettes with clamps and stands
Disposable beakers (50, 100, 250, 1000 mL)
Stainless steel screening
Pasteur pipettes and bulbs
Pyrex disposable pipettes
Thermometers

C. Chemicals and Reagents

Distilled water
Reagents for hardness determinations
Reagents for alkalinity determinations
Standards for pH meter calibrations
Disinfectant

D. Safety Equipment

First aid kit
Fire extinguishers
Hard hats
Safety glasses
Organic vapor respirators and refill cartridges
Rubber gloves
Latex gloves
Flashlight and batteries

E. Miscellaneous Supplies and Equipment

Nalgene wash bottles
Pyrex disposable screw-cap test tubes
Glass tubing
Hose clamps
Egg spawning supplies
Data collection supplies (stopwatch, calculator)
Tape (masking, electrical, duct)
Aluminum foil
Kimwipes
Towels
Waders

F. Laboratory Maintenance Equipment

Air conditioner (5000 BTU)
Space heater (1500 watts)
3-ton hydraulic jacks and jack stands
Electrical extension cords
Gas and diesel tanks
Tool kit and assorted hand tools
Diesel generator (60 amp)

REFERENCES

1. Birge, W.J. 1978. Aquatic toxicology of trace elements of coal and fly ash. In Energy and Environmental Stress in Aquatic Systems, J.H. Thorp and J.W. Gibbons, eds., DOE Symposium Series (CONF-771114), Washington, D.C. pp. 219-240.

2. Peltier, W. 1978. Methods for Measuring the Acute Toxicity of Effluents to Aquatic Organisms. EPA-600/4-78-012, U.S. Environmental Protection Agency, Cincinnati, Ohio. 52 p.

3. Birge, W.J., J.E. Hudson, J.A. Black, and A.G. Westerman. 1978. Embryo-larval bioassays on inorganic coal elements and in situ biomonitoring of coal-waste effluents. In Surface Mining and Fish/Wildlife Needs in The Eastern United States, D.E. Samuel, J.R. Stauffer, C.H. Hocutt, and W.T. Mason, eds., Publication no. FWS/OBS-78/81, U.S. Department of the Interior, Washington, D.C. pp. 97-104.

4. McKim, J.M. 1977. Evaluation of tests with early life stages of fish for predicting long-term toxicity. J. Fish. Res. Board Can., 34(8): 1148-1154.

5. Birge, W.J., J.A. Black, and D.M. Bruser. 1979. Toxicity of Organic Chemicals to Embryo-larval Stages of Fish. EPA-560/11-79-007, U.S. Environmental Protection Agency, Washington, D.C. 60 p.

6. Birge, W.J., J.A. Black, A.G. Westerman, and J.E. Hudson. 1980. Aquatic toxicity tests on inorganic elements occurring in oil shale. In Oil Shale Symposium: Sampling, Analysis and Quality Assurance, March 1979, C. Gale, ed., EPA-600/9-80-022, U.S. Environmental Protection Agency, Cincinnati, Ohio. pp. 519-534.

7. Birge, W.J., J.A. Black, and B.A. Ramey. 1981. The reproductive toxicology of aquatic contaminants. In Hazard Assessment of Chemicals - Current Developments, J. Saxena and F. Fisher, eds., Academic Press, New York. pp. 59-115.

8. Finney, D.J. 1971. Probit Analysis, Third Edition. Cambridge Press, New York. 333 p.

9. Birge, W.J., J.A. Black, J.E. Hudson, and D.M. Bruser. 1979. Embryo-larval toxicity tests with organic compounds. In Aquatic Toxicology, L.L. Marking and R.A. Kimerle, eds., Special Technical Publication 667, American Society for Testing and Materials, Philadelphia, Pa. pp. 131-147.

10. Birge, W.J., J.A. Black, and A.G. Westerman. 1979. Evaluation of aquatic pollutants using fish and amphibian eggs as bioassay organisms. In Animals as Monitors of Environmental Pollutants, National Academy of Sciences, Washington, D.C. pp. 108-118.

11. Birge, W.J., J.A. Black, A.G. Westerman, and J.E. Hudson. 1979. The effects of mercury on reproduction of fish and amphibians. In Biogeochemistry of Mercury in the Environment, J.O. Nriagu, ed., Elsevier/North Holland Biomedical Press, Amsterdam. pp. 629-655.

12. Birge, W.J. and J.J. Just. 1975. Bioassay Procedures Using Developmental Stages as Test Organisms. Research Report No. 84, U.S. Department of the Interior, Washington, D.C. 36 p.

13. Birge, W.J. and J.A. Black. 1977. Sensitivity of Vertebrate Embryos to Boron Compounds. EPA-560/1-76-008, U.S. Environmental Protection Agency, Washington, D.C. 62 p.

14. Birge, W.J., J.A. Black, and R.A. Kuehne. 1980. Effects of Organic Compounds on Amphibian Reproduction. Research Report No. 121, U.S. Department of the Interior, Washington, D.C. 39 p.

15. Macek, K.J., K.S. Buxton, S. Sauter, S. Gnilka, and J.W. Dean. 1976. Chronic Toxicity of Atrazine to Selected Aquatic Invertebrates and Fishes. EPA-600/3-76-047, U.S. Environmental Protection Agency, Duluth, Minn. 50 p.

16. Mount, D.I. and C.E. Stephan. 1967. A method for establishing acceptable toxicant limits for fish - malathion and the butoxyethanol ester of 2,4-D. Trans. Am. Fish. Soc., 96: 185-193.

17. Arthur, J.W., A.W. Lemke, V.R. Mattson, and B.J. Halligan. 1974. Toxicity of sodium nitrilotriacetate (NTA) to the fathead minnow and an amphipod in soft water. Water Res., 8: 187-193.

18. Nebeker, A.V., F.A. Puglisi, and D.L. DeFoe. 1974. Effect of polychlorinated biphenyl compounds on survival and reproduction of the fathead minnow and flagfish. Trans. Am. Fish. Soc., 103: 562-568.

19. DeFoe, D.L., G.D. Veith, and R.W. Carlson. 1978. Effects of Aroclor 1248 and 1260 on the fathead minnow (Pimephales promelas). J. Res. Board Can., 35(7): 997-1002.

20. Benoit, D.A., E.N. Leonard, G.M. Christensen, and J.T. Fiandt. 1976. Toxic effects of cadmium on three generations of brook trout (Salvelinus fontinalis). Trans. Am. Fish. Soc., 105: 550-560.

21. Eaton, J.G., J.M. McKim, and G.W. Holcombe. 1978. Metal toxicity to embryos and larvae of seven freshwater fish species - I. Cadmium. Bull. Environ. Contam. Toxicol., 19: 95-103.

22. Spehar, R.L. 1976. Cadmium and zinc toxicity to flagfish, Jordanella floridae. J. Fish. Res. Board Can., 33: 1939-1945.

23. Sauter, S., K.S. Buxton, K.J. Macek, and S.R. Petrocelli. 1976. Effects of Exposure to Heavy Metals on Selected Freshwater Fish, Toxicity of Copper, Cadmium, Chromium and Lead to Eggs and Fry of Seven Fish Species. EPA-600/3-76-105, U.S. Environmental Protection Agency, Duluth, Minn. 75 p.

24. Benoit, D.A. 1976. Toxic effects of hexavalent chromium on brook trout (Salvelinus fontinalis) and rainbow trout (Salmo gairdneri). Water Res., 10: 497-500.

25. McKim, J.M. and D.A. Benoit. 1974. Duration of toxicity tests for establishing "no effect" concentrations for copper with brook trout (Salvelinus fontinalis). J. Fish. Res. Board Can., 31: 449-452.

26. Davies, P.H., J.P. Goettl, J.R. Sinley, and N.F. Smith. 1976. Acute and chronic toxicity of lead to rainbow trout Salmo gairdneri, in hard and soft water. Water Res., 10: 199-206.

27. Holcombe, G.W., D.A. Benoit, E.N. Leonard, and J.M. McKim. 1976. Long-term effects of lead exposure on three generations of brook trout (Salvelinus fontinalis). J. Fish. Res. Board Can., 33: 1731-1741.

28. McKim, J.M., G.F. Olson, G.W. Holcombe, and E.P. Hunt. 1976. Long-term effects of methylmercuric chloride on three generations of brook trout (Salvelinus fontinalis): toxicity, accumulation, distribution, and elimination. J. Fish. Res. Board Can., 30: 485-492.

29. Davies, P.H., J.P. Goettl, Jr., and J.R. Sinley. 1978. Toxicity of silver to rainbow trout (Salmo gairdneri). Water Res., 12: 113-117.

30. Brungs, W.A. 1969. Chronic toxicity of zinc to the fathead minnow (Pimephales promelas Rafinesque). Trans. Am. Fish. Soc., 98: 272-279.

31. Daum, R.J. 1969. A revision of two computer programs for probit analysis. Bull. Entom. Soc. Am., 16: 10-15.

32. Birge, W.J., J.A. Black, A.G. Westerman, P.C. Francis, and J.E. Hudson. 1977. Embryopathic Effects of Waterborne and Sediment-Accumulated Cadmium, Mercury, and Zinc on Reproduction and Survival of Fish and Amphibian Populations in Kentucky. Research Report No. 100, U.S. Department of the Interior, Washington, D.C. 28 p.

33. Weber, C.I. and W. Peltier. 1980. Draft - Use of Effluent Toxicity Testing in the Second Round of NPDES Permit Issuance. U.S. Environmental Protection Agency, Washington, D.C.

34. American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 1975. Standard Methods for the Examination of Water and Wastewater, 14th Edition. American Public Health Association, Washington, D.C. 1193 p.

35. Newton, B. and R. Brandes. 1981. Use of effluent toxicity testing in the second round of NPDES permit issuance. Draft policy statement, Industrial Permits Branch, Office of Water Enforcement, U.S. Environmental Protection Agency, Washington, D.C. 46 p.

36. Environmental Protection Agency. 1979. Interim NPDES Compliance Biomonitoring Inspection Manual. MCD-62, U.S. Environmental Protection Agency, Washington, D.C.

37. Environmental Protection Agency. 1979. Draft Application Forms for EPA Programs. Fed. Reg., 44(116): 34346-34416.

38. Weber, C.I. 1980. Federal and state biomonitoring programs. In Biological Monitoring for Environmental Effects, D.L. Worf, ed., Lexington Books, Lexington, Mass. pp. 25-52.

39. Ladd, E.C. 1977. An industrial view of biological monitoring. In Biological Monitoring of Water and Effluent Quality, J. Cairns, Jr., K.L. Dickson, and G.F. Westlake, eds., Special Technical Publication 607, American Society for Testing and Materials, Philadelphia, Pa. pp. 24-29.

40. Mount, D.I. 1980. Biomonitoring of effluents in perspective. In Proceedings of the Seminar on Biological Monitoring and Its Use in the NPDES Permit Program, EPA-600/9-80-026, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio. pp. 3-7.