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GEO-CENTERS and Orange County Water District
Test Report: Final Report

TEST TITLE: Fish Biomonitoring Component of the Biomonitoring Demonstration Project

DATA REQUIREMENT: Animal care procedures were followed as per Aquatic Toxicology: Best Practices for the Culture and Testing of Selected Fish and Frog Species published by GEO-CENTERS, INC., 2000.

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EXECUTIVE SUMMARY

The Santa Ana River is the primary source of recharge water to the Orange County groundwater basin. Water quality within the watershed area is impacted by upstream water uses, including wastewater discharges, urban and agricultural run-off, and climatic variables. Traditionally, chemical contaminant analyses have been used to gauge water quality. In this Biomonitoring Demonstration Project, we tested the feasibility of fish biomonitoring as a supplement to chemical monitoring for nine months at the Off-River Forebay Recharge System site in Orange County, CA.

Fish biomonitoring has been used as a water evaluation tool by federal, academic, and industrial researchers in the U.S. and abroad. Previous applications tested point source discharges in the environment, typically with discrete samples, or single chemical exposures in the laboratory. In this application, however, fish biomonitoring was used to test the feasibility of using fish as sentinel models under continuous flow-through conditions to compare source water for groundwater recharge to a comparison water. The purpose of this demonstration project was to determine if fish biomonitoring could be meaningfully applied to source water under actual field conditions and to determine if fish biomonitoring could provide toxicity information that would complement traditional chemical single compound analyses.

A mobile laboratory was located adjacent to the Santa Ana River on March 8, 2000. Japanese medaka fish (*Oryzias latipes)*, zebrafish (*Brachydanio rerio)*, and bluegill sunfish (*Leopmis macrochirus*) were housed in glass aquaria with continuous flow-through exposures to either test water from on-site shallow groundwater or to dechlorinated tap water (comparison water) that had been filtered through granular activated carbon. Water quality parameters, fish growth, and fish survival were closely monitored for the test durations of nine months for medaka and zebrafish, six months for bluegill.

The test water was not available to the mobile lab throughout the test. Early in the summer, the test water collection system went dry due to maintenance on the recharge facilities. The decision was made to continue the test as the juvenile medaka had received continuous exposure during their most sensitive rapid growth phase. From June 18, 2000 through October 23, 2000, the test water aquaria received comparison water. After October 23, test water supply to test aquaria was reinstated through test completion in December 2000.

Temperature control, dissolved oxygen levels, and intermittent water delivery were engineering challenges during the project. Elevated summertime water temperatures were dampened by cooler ambient air temperatures within the mobile laboratory, while dissolved oxygen levels were increased through in-line aeration and supplemental aquarium aeration. Water delivery issues that occurred during the project were resolved, and portions of the water delivery system were simplified.

Other water quality parameters, such as pH, conductivity, alkalinity, hardness, and ammonia remained at healthful levels throughout the test. Test water and comparison water monitoring results for these parameters were similar in that the range of values measured for each parameter (mean or median ± standard deviation) strongly overlapped for both types of water.

Fish survival varied among species. The highest percent survival levels were seen in medaka, followed by bluegill, then zebrafish. Among medaka exposed to comparison water, 665/720 (92.4%) survived, while medaka exposed to test water had an even higher survival rate of 683/720 (94.9%). Among bluegills, 10/16 (62.5%) survived comparison water exposure, while 13/16 (81.3%) survived test water exposure. Zebrafish survival was 69/240 (28.8%) in comparison water and 137/240 (57.1%) in test water. The reason for the lower survival rate of the zebrafish is unknown, but it may have been related to purchasing a mixed age class from a commercial vendor and to a suspected microbial bloom that affected three aquaria. Bluegill survival rates of 100% for the first three months suggest that future biomonitoring of test water with bluegill will be feasible.

Fish growth for medaka was evaluated through comparison of length and weights. Average length for comparison water medaka was 29±1.9 millimeter (mm). A similar average length was observed for test water medaka (29±1.8 mm). The comparison water medaka had an average weight of 389±88.5 milligram (mg), while the test water medaka had an average weight of 407±84.3 mg. Medaka fish growth was not impaired by test water as both lengths and weights for test water fish were the same or more than comparison water fish lengths and weights. Both zebrafish and bluegill demonstrated similar overlapping ranges (mean ± standard deviation) for fish lengths and weights when fish growth was compared by water type, which is suggestive of the toxicological similarity of test and comparison water.

Fish biomonitoring demonstrated that overt fish toxicity mechanisms were not present in the test water. Future medaka fish histopathology will be beneficial for optimizing the design of future experiments in that a determination could be made as to whether cellular mechanisms were adversely affected by test water exposure. The Biomonitoring Demonstration Project showed fish biomonitoring to be both a feasible complement to traditional chemical analyses of water quality and a valuable public relations asset.

PROJECT DESCRIPTION

The Biomonitoring Demonstration Project was performed in a mobile laboratory facility adjacent to the Off-River Forebay Recharge System in Orange County, CA. The site is located in the City of Anaheim. The objectives of this project were three-fold: test the feasibility of using fish as a water quality monitoring tool, evaluate the site-specific environmental factors that may affect fish husbandry, and test if fish can be maintained for nine months to evaluate chronic

health outcomes under varying water conditions, including baseflow and stormflow. The project co-sponsor, Orange County Water District (OCWD), was responsible for monitoring of water samples for chemical contaminants during the nine-month exposure. Fish survival and growth were used to evaluate the above objectives.

INTRODUCTION

The Santa Ana River is the primary source of recharge for the Orange County groundwater basin that provides about 75% of the drinking water supply for over two million people. During the summer months, a majority of the Santa Ana River flow is tertiary-treated reclaimed water discharged from upstream municipal facilities in San Bernardino and Riverside counties. Santa Ana River water quality is also influenced by input from non-point sources, such as urban and agricultural runoff. The Santa Ana River watershed contains approximately 382,000 cows located on about 25,000 acres upstream of OCWD's recharge system.

OCWD initiated the Santa Ana River Water Quality and Health (SARWQH) Study to address concerns about the quality of the Santa Ana River and verify that current groundwater practices are safe. The Biomonitoring Demonstration Project is one component of the multi-disciplinary SARWQH Study that is evaluating the quality of the Santa Ana River and the impact of recharging the Santa Ana River in the groundwater basin. Concern has been raised over human health issues related to using reclaimed water of as source water (National Research Council, 1998). Conventional chemical monitoring takes into account measurable levels of single compounds. Many compounds are present in reclaimed water in trace amounts, which may or may not be detected by chemical contaminant analyses. Within complex mixtures, toxicity may result from various combination mechanisms such as synergism, potentiation, and antagonism (Eaton and Klassen, 1996).

The incorporation of biomonitoring with traditional chemical monitoring improves the certainty of detection of uncharacterized contaminants that may be biologically active in the source water that may not be detected with chemical monitoring alone. Biomonitoring is the use of living organisms as "sensors" in water quality surveillance to detect changes in an effluent or water and to indicate whether aquatic life may be endangered (Rand and Petrocelli, 1985). The use of alternative species for biomonitoring fills a need in the scientific database by clarifying dose-response at environmentally relevant doses (Stone, 1995). Problems in assessing source water are more easily addressed with test systems that reside in water, such as aquatic species. Fish have been studied and found useful for evaluation of complex toxic mixtures for growth, development, and carcinogenicity (Teh and Hinton, 1997; Hawkins *et al,* 1985; Twerdok *et al,* 1997). Fish are advantageous as test systems in that many of the same cellular mechanisms and developmental processes present in mammals are conserved phylogenetically (Powers, 1989).

The US Army Center for Environmental Health Research (USACEHR) has developed fast, relatively inexpensive, non-mammalian toxicity assessment techniques that can be used at field sites (Gardner *et al*, 1990). Through a cooperative research agreement with USACEHR, GEO-CENTERS, INC., was able to provide USACEHR fish biomonitoring techniques and skilled technical expertise to the Orange County Water District.

Alternative animal models such as fish are a natural choice for testing aqueous solutions. Fish have been shown to be sensitive to trace levels of contaminants in aquatic media (Gardner *et al*, 1998). As water-dwelling organisms, fish are immersed in the exposure solution, with dosing occurring through dermal, oral, and respiratory routes. Japanese medaka (*Oryzias latipes*) are an attractive test model because they are hardy, small in size, easy to culture, and have a relatively short time-to-tumor response. In chronic testing with drinking water disinfection by-products, medaka were shown to have statistically significant findings in the gallbladder and bile ducts of fish treated with 1.4 milligram per liter (mg/L) chloroform (Toussaint *et al.*, 2001) or 1.4 mg/L bromodichloromethane (Toussaint *et al.*, in press). Continuous biological monitoring with fish provides an immediate indication of abnormal organism response and is appropriate for monitoring acute toxicity (van der Schalie, 1986). Measurements of ventilatory depth, cough rate and whole body movement of bluegill fish has been shown to be comparable to no effect levels in early life stage tests with other fish (van der Schalie *et al.*, 1988).

The water from the Santa Ana River is recharged into the groundwater basin through the riverbed and through several off-channel recharge basins, as shown in Figure 1. One of these off-channel basins is the Off-River System, which parallels the Santa Ana River, as shown in Figure 2. Test water for the Biomonitoring Demonstration Project was collected from six-inch diameter perforated pipe about four feet beneath the Off-River Recharge Basin. The test water is estimated to have a subsurface residence time of less than one week, and has undergone minimal soil-aquifer treatment, a process that typically improves water quality (National Research Council, 1998)

Whole animal response was evaluated through fish growth and fish survival. The timeline for the project is shown in Figure 3.

MATERIALS AND METHODS

Model Selection. Two types of small aquarium fish and one native American fish species used in many testing protocols were selected for use in this project. Zebrafish (*Brachydanio rerio*) grow well in aquaria. They reach sexual maturity early and spawn year round. The adult size of a zebrafish is about 1 inch in length. Japanese medaka (*Oryzias latipes*) are another small aquarium fish that are popular for testing purposes. They are a small, hardy, non- native fish that are easy to culture. In addition to spawning year round, medaka have a low spontaneous neoplasm rate. The adult size of a mature medaka is approximately 1 inch in length.

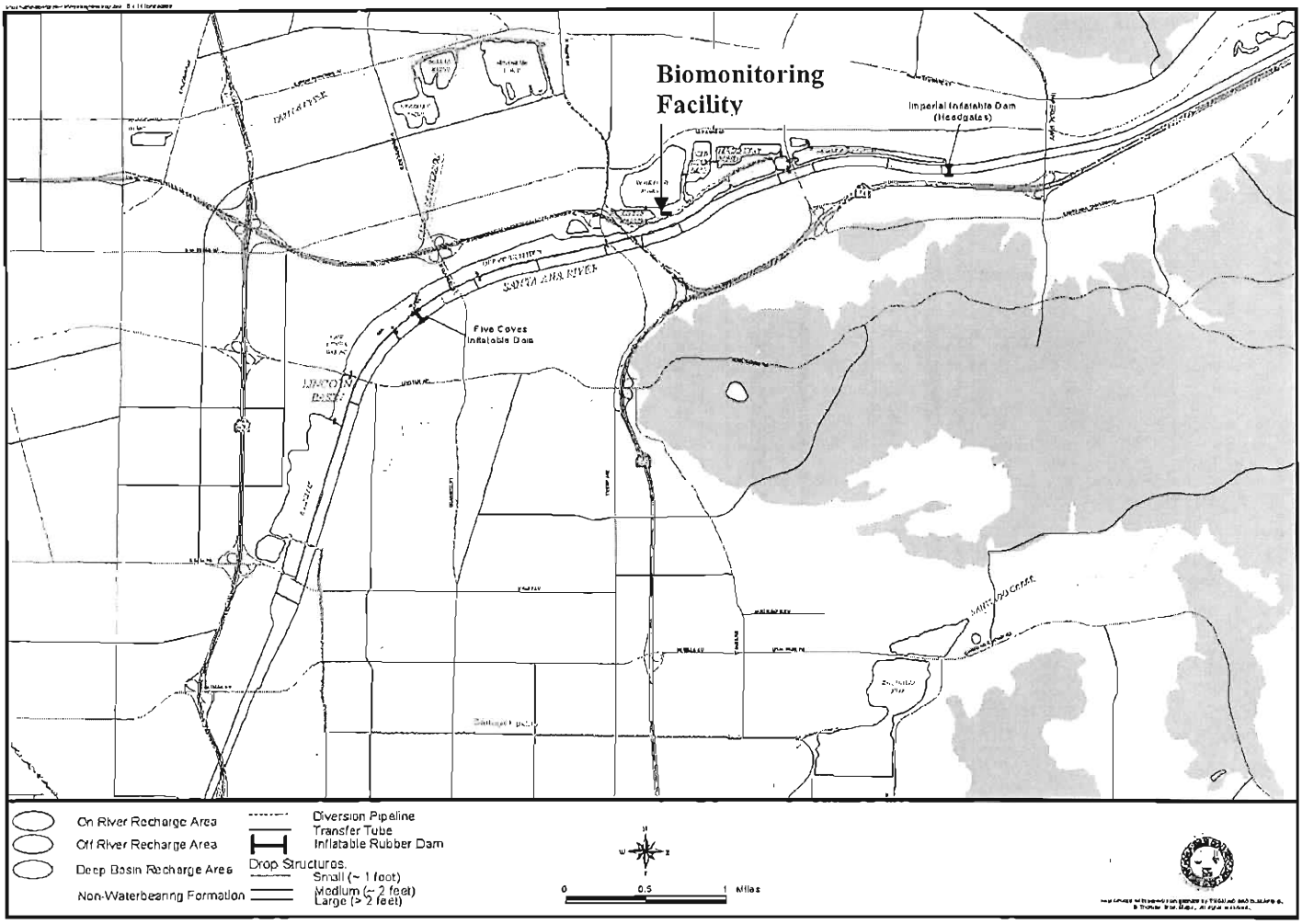

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Figure 1. OCWD Recharge Facilities

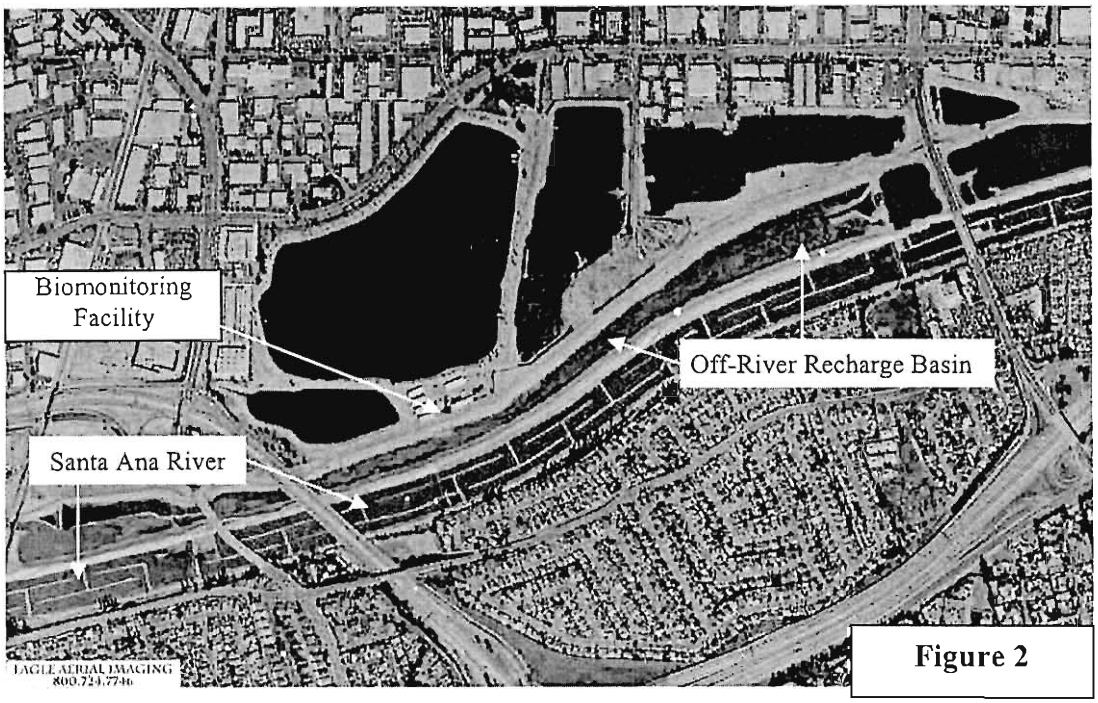

Figure 2

March	April	May	June	July	August
3/8 Mobile lab to CA 3/16 Medaka on 3/21 Zebra on			6/8 Bluegill on 6/18 Test water off 6/26 Static	7/26 Flow through	

Sept	Oct	Nov	Dec	Jan	Feb
	10/23 Test Water on		12/11 End of exposure	Data analysis Trailer prep	Mobile lab to MD

Figure 3. Project Timeline

Bluegill sunfish (*Lepomis macrochirus*) are a native American species that are easily maintained in a laboratory setting. Bluegill adapt well to testing protocols and have been used successfully for years in an acute toxicity monitoring program which monitors fish respiratory response to waterborne substances. The length of juvenile bluegills used for this project was about 3-4".

Animal Care - Medaka. Japanese medaka fish were supplied from USACEHR in-house cultures and were reared according to Aquatic Toxicology, Best Practices for the Culture and Testing of Selected Fish and Frog Species (GEO-CENTERS, INC., 2000). Fish were shipped overnight from USACEHR directly to the mobile biomonitoring laboratory, arriving at the mobile facility on 3/14/00. Fish were then held in comparison water (dechlorinated tap water) until 3/16/00 when they were fourteen days of age. Medaka were randomized in groups of ten fish each to twelve centrally divided ten-gallon aquaria, with 60 medaka in each aquarium section, with an overall total of 1440 medaka used. Juvenile fish were fed nematodes (microworms) *ad libitum* three feedings per day and approximately 24 hour old brine shrimp nauplii (Argentemia® Gold Label Brine Shrimp Cysts, Argent Chemical laboratories, Redmond, WA) two times a day, transitioning to two feedings of microworms and two feedings of live brine shrimp nauplii at 15 days. The fish were transitioned to the adult fish regimen described below at 23 days, with the amount of food provided continuously adjusted upward during the fish life span to ensure adequate nutrition. Excess food was siphoned from test aquaria as required. Adult fish were given two feedings of contaminant-analyzed flake food (Aquatox Certified Diet, Ziegler Brothers, Gardners, PA) on weekdays, and one feeding per day of the same flake food on the weekends. The adults also received one daily feeding of live brine shrimp nauplii approximately 24 hour old. Fish were fasted 24 hours prior to the nine-month histopathology sacrifice. Durotest Optima Choice fluorescent bulbs with a color rendering index of 91 provided the light for the 16 h light/8 h dark light cycle at USACEHR and at the mobile biomonitoring laboratory. At the mobile lab, fish were housed in ten-gallon fish aquaria, each modified with a silicone glued glass partition at the aquaria midpoint. Animal identification numbers were issued at euthanasia or early death. All tanks were siphoned of excess food and debris on an as-needed basis.

Animal Care – Zebrafish. Zebrafish of unknown age class were purchased from Carolina Biological, Burlington, NC, prior to study onset. All fish husbandry procedures used for medaka care were used for zebrafish. Upon arrival at the mobile lab on 3/21/00, the zebrafish were randomized to four divided ten-gallon aquaria, to a density of 60 fish per aquaria section, with an overall total of 480 zebrafish used.

Animal Care – Bluegill. Bluegill sunfish were obtained from a protected pond in Middletown, MD. Bluegill were overnight shipped from the USACEHR aquaculture facility. Upon arrival at the mobile laboratory on 6/8/00, bluegill were randomized to two divided ten-gallon aquaria, so that six bluegills were in each aquarium section and 32 bluegill were used. Bluegill received contaminant-analyzed Salmon Starter #3 (Zeigler Brothers, Gardners, PA) *ad libitum* twice a day. Although bluegill are normally held at lower temperatures than 25±2°C in

USACEHR testing protocols, they were held at the same temperature as the other fish for this project due to the limited amount of waterbath space.

Water Distribution. Both test water and comparison water were supplied to the mobile biomonitoring laboratory from OCWD-derived sources. Inside the mobile lab, each water type was diverted to a unique set of water processing equipment. The comparison water was particle filtered (20 micron filter), carbon filtered, then aerated in large, tandem polypropylene vats prior to distribution to fish aquaria. The test water was particle filtered (80 micron filter) and passed over Venturi in-line aerators before distribution to the fish aquaria. During the test period, test water dissolved oxygen levels began to drop in response to the greater dissolved oxygen requirement of larger fish, so in tank air stones were added to all test aquaria in April. At the project onset, water distribution to fish aquaria was via proportional diluter. Neither test water nor comparison water was diluted, so the existing solenoid diluter system in the mobile lab was used solely to partition water into equivalent amounts in this project. Two proportional diluters delivered the required test water volumes to the test aquaria. The diluters were set to deliver 300 $\pm$15 mL to each aquarium every 3 min $\pm$15 seconds, yielding 9-10 tank volumes per day through July when use of the diluters was discontinued. During the test, continuous water distribution became problematic and a simpler water distribution system was designed and installed. A nylon manifold system with two sizes of ports (1/8-inch and 1/16-inch) was installed in July 2000. This manifold system did not have an electrical component and greatly simplified water distribution trouble-shooting. Water flow rates in medaka and zebrafish aquaria when water was distributed by the manifold system were 100-300 mL/min, while in bluegill aquaria manifold-distributed flow rates were 200-400 mL/min.

Test Design. One pair of divided glass aquaria was randomly assigned to each treatment, test water or comparison water. The test began when fourteen-day old medaka fry ($\pm$1 day), were randomized to each test aquarium. The fry were released into the test aquaria on Day 1 of the exposure. Each ten-gallon divided glass aquarium held approximately 15 L of water. Aquaria were covered with custom made glass tops. All fish aquaria were placed in water baths, with an equal number of test water and comparison water aquaria (by species) represented in each of the two water baths. Water circulators and water heaters in the water bath optimized water movement and equalized water temperatures within each water bath. The desired test temperature was 25$\pm$2°C. Test water and comparison water were gravity fed from the proportional diluter through Teflon tubing to the test aquaria. Effluent from test water aquaria was collected via an outflow at the opposite end of the aquaria through a sealed collection system that emptied into the Off-River System. Comparison water aquaria discharged directly to the water bath, which subsequently discharged to the Off-River System. Moribund and dead fish were recorded and removed from project aquaria upon observation. All surviving fish were euthanized after nine months of flow-through exposure with an overdose of ethyl 3-aminobenzoate, methanesulfonic salt (MS-222), weighed and measured. Medaka were processed in Bouin's solution for histopathology. A schematic of the mobile laboratory layout is shown in Figure 4.

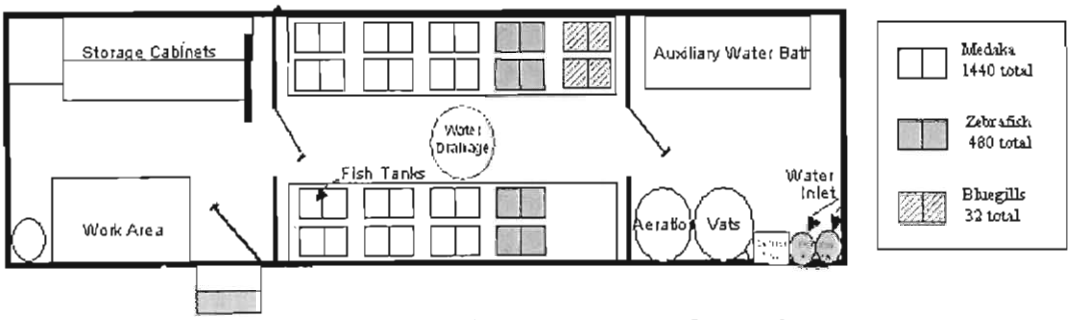

Figure 4. Mobile laboratory schematic

Environmental Conditions. Temperature was monitored daily in representative tanks in each water bath. Once weekly in all project aquaria, the following water quality parameters were measured: pH, dissolved oxygen, and conductivity. Once weekly in representative tanks from each type of project water, alkalinity and hardness were monitored. Once monthly, ammonia levels were measured in all project aquaria. All data were recorded on aquaria-specific forms.

OCWD analyzed water samples for chemical contaminants one to three times during the study. Samples were collected inside the mobile trailer before the water entered the aquaria. Sampling dates were 3/15/00, 10/2/00 (comparison water only), 11/13/00 (test water only for organics, both waters for inorganics and metals), and 12/7/00 (test water only). The following parameters were measured: 1,1,1,2-tetrachloroethane, 1,1,1-trichloroethane, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, 1,1-dichloroethane, 1,2,3-trichlorobenzene, 1,2,3-trichloropropane, 1,2,4-trimethylbenzene, 1,2-dibromo-3-chloropropane, 1,2-dibromoethane, 1,2-dichlorobenzene, 1,2-dichloropropane, 1,3,5-trimethylbenzene, 1,3-dichlorobenzene, 1,4-dichlorobenzene, 1-napthol, 2,2-dichloropropane, 2,4,5-TP (Silvex), 2,4-D, 2-chlorotoluene, 3,hydroxycarbofuran, 4,4'-DDD, D,4'-DDE, 4,4'-DDT, 4-chlorotoluene, 4-isoproplytoluene, acenapthene, acenapthylene, alachlor, aldicarb, aldicarb sulfone, adlrin, alkalinity-phenolphthalein, aluminum, ammonia nitrogen, anthracene, antimony, apparent color (unfiltered), arsenic, atrazine, barium, baygon, bentazon, benzene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,I)perylene, benzo(k)fluroanthene, beryllium, bicarbonate (as $CaCO_3$), bicarbonate (as HCO3), bis(2-chloroethyl) ether, bis(2-ethylhexyl) adipate, boron, bromacil, bromide, bromobenzene, bromochloromethane, bromoform, bromomethane, butachlor, butylbenzyl phthalate, cadmium, caffeine, calcium, carbaryl, carbofuran, carbon tetrachloride, carbonate (as $CaCO_3$), chlordane, chlordane-alpha, chlordane-gamma, chloride, chlorobenzene, chlorobenzilate, chloroethane, chloroform, chloromethane, chloroneb, chlorophyll a, chlorothalonil, chloropyrifos, chromium, chrysene, cis-1,2-dichloroethene, cis-1,3-dichloropropene, copper, cyanide, dalapon, DCPA-Dacthal, diazinon, dibenzo(a,h)anthracene, dibromochloromethane, dicambam dichlorodifluoromethane, dieldrin, diethyl phthalate, disopropyl ether, dimethoate, dimethyl phthalate, di-n-butylphthalate, di-n-octylphthalate, dinoseb, diquat, dissolved organic carbon, dissolved sulfide, diuron, electrical

conductivity, endosulfan I, endosulfan II, endosulfan sulfate, endothall, endrin, endrin aldehyde, endrin ketone, enthl tert-butyl ether, ethylbenzene, etridaizole, field EC, field pH, field temperature, fluoranthrene, fluorine, free chlorine, glyphosate, alpha-BHC, Beta-BHC, Delta-BHC, Gamma-BHC, heptachlor, heptachlor epoxide, hexachlorobenzene, hexachlorobutadiene, hexachlorocyclopentadiene, hydroxide (as $CaCO_3$), hydroxide (as OH), indeno(1,2,3-cd)pyrene, iron, isopropylbenzene, lead, m,p-xylene, magnesium, manganese, mercury, methiocarb, methomyl, methoxychlor, methyl ethyl ketone (MEK), methyl isobutyl ketone (MIBK), methyl tert-butyl ether, methylene chloride, methyl-parathion, metolachlor, metribuzin, molinate, naphthalene, n-butylbenzene, nickel, nitrate, nitrate + nitrite nitrogen, nitrate nitrogen, nitrite, nitrite nitrogen, n-nitrosodimethylamine, norflurazon, organic nitrogen, oxamyl, o-xylene, paraquat, parathion, PCB (1016, 1221, 1232, 1242, 1248, 1254, 1260), pentachlorophenol, permethrin (total of cis/trans), pH, phenathrene, phosphate phosphorus, picloram, potassium, prometon, prometryn, propachlor, propazine, propylbenzene, pyrene, sec-butylbenzene, selenium, settlable solids, silica, simazine, sodium, styrene, sulfate, surfactants, suspended solids, tert-amyl-methyl ether, tert-butyl alcohol, tert-butyl formate, tert-butylbenzene, thallium, thiobenzcarb, title 22 cation-anion balance, title 22 total cations, toluene, total alkalinity (as $CaCO_3$), total anions, total cations, total chlorine, total dissolved solids, total hardness (as $CaCO_3$), total kleldahl nitrogen, total organic carbon (unfiltered), total THMs, toxaphene mixture, trans-1,2 dichloroethene, trans-1,3-dichloropropene, trichloroethene, trichlorofluoromethane, trichlorotrifluoroethane (freon 113), trifluralin, turbidity, ultraviolet absorbance, vinyl chloride, and zinc.

RESULTS AND DISCUSSION

Fish Survival. Of the three species of fish tested in the mobile laboratory, the medaka had the highest survival rates (>92%), as shown in Figure 5. Based on their superior survival rates, medaka appear to be well suited to long-term exposures with both comparison and test water. For all three species, final percent survival was numerically larger in test water than comparison water, so it appears that test water did not impair relative fish survival for the fish tested. Bluegill survival remained at 100% through the first three months on-test for both types of water. This indicated that these fish would do well in an acute flow through system such as the Real-time Environmental Exposure System (REPS), in which the fish are exposed for a duration of less than a month. The zebrafish had the poorest percent survival with about a 10% mortality rate for each of the first three to four months of their exposure to both types of water, as shown in Figure 6. Within some zebrafish aquaria, mortality was 100%. This pattern of mortality is not unusual and it is suspected that a few compromised individuals seeded the aquarium micro-environment with a lethal level of disease agent, such as a bacteria or virus. Secondary opportunistic pathogens, most likely Saprolegnia fungus, colonized the exterior surfaces of the moribund and dead fish. As the zebra fish mortality was across the board, it is believed that this population of zebras was not optimal for testing under these conditions. Of the available zebrafish suppliers, however, this commercial source was the only one that had sufficient stock of zebras available to begin the project, but they were unable to guarantee that

this lot of fish were of the same age cohort. If future testing with zebra is anticipated, it is recommended that same age cohorts be used.

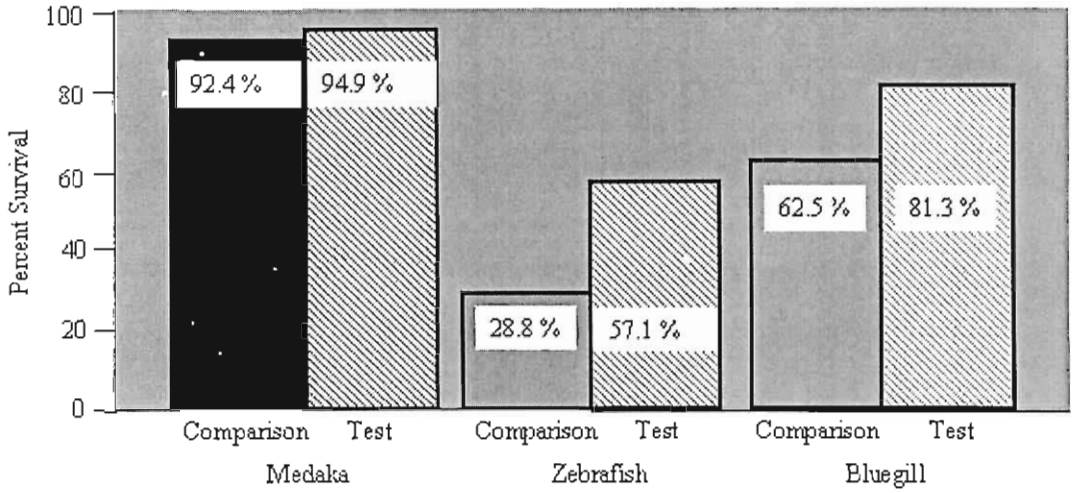

Figure 5. Fish percent survival

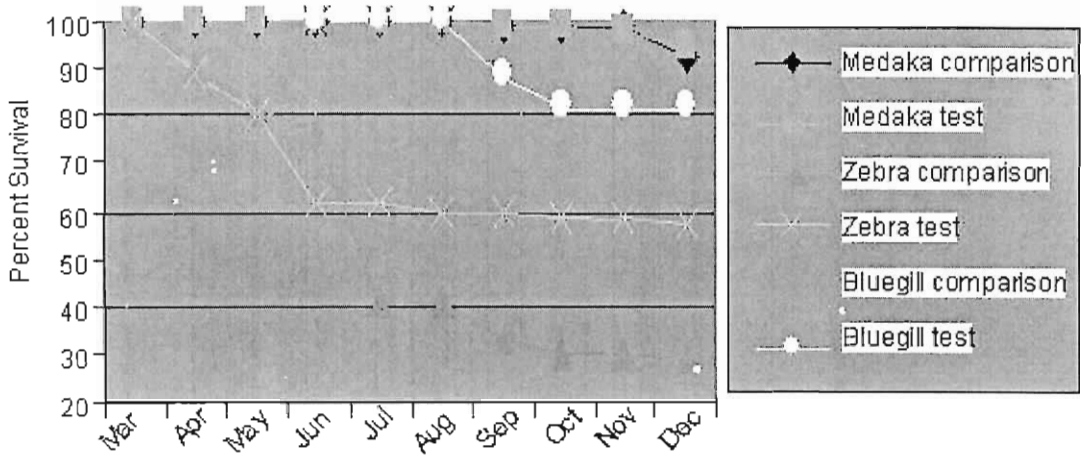

Figure 6. Percent survival through time

Fish Growth. Length and weight measurements were made of all three species of fish at the conclusion of the flow-through exposure in December 2000. However, since the medaka were the only species of the same size and age class at the beginning of the exposure, it is doubtful that much can be learned from the growth measurements of the zebrafish and bluegills. When medaka replicate endpoints were pooled within the same type of source water, the standard deviations of both length and weight measurements for each endpoint (length and weight) overlapped, indicating that the endpoints are similar. Endpoints of average, median,

count, standard deviation, maximum value, minimum value, and standard error are given in Table 1 for medaka length and weight. Raw data and summaries by fish aquaria are given in Appendix A.

Table 1. Medaka final sacrifice lengths and weights, replicates pooled

Parameter	Comparison Water		Parameter	Test Water	
	Length (mm)	Weight (mg)		Length (mm)	Weight (mg)
Average	29	389	Average	29	407
Median	29	378	Median	29	400
Count	667	667	Count	684	684
Standard Dev.	1.9	88.5	Standard Dev.	1.8	84.3
Maximum	38	705	Maximum	36	834
Minimum	23	209	Minimum	19	103
Standard Error	0.07	3.43	Standard Error	0.07	3.23

Fish growth in zebrafish and bluegills showed a similar overlap of standard deviation for both length and weight at test termination. Zebrafish length in comparison water was 34.2±3.4 mm, while length in test water was 33.4±1.4 mm. Zebrafish weight in comparison water was 675.3±109 mg, while weight in test water was 640.0±124 mg. Bluegill length in comparison water was 81.4±24.5 mm, while length in test water was 75.7±18.9 mm. Bluegill weight in comparison water was 12.4±8 grams, while weight in test water was 16.2±13 grams. Although statistical analyses of the growth results were not performed, overlapping ranges (mean ± standard deviation) of fish growth parameters strongly indicated that there is no underlying overt fish toxicity mechanism in the test water.

Water Supply. Comparison water was supplied throughout the nine-month exposure to comparison water aquaria. Test water was discontinued in test water aquaria from June 18 through October 23 due to maintenance on the recharge facilities. During this interruption of test water supply, test water aquaria were supplied with comparison water. This change in source water did not have an adverse impact on fish survival, as there was no corresponding rise in fish mortality within the first month of the change-over. Zebrafish were the only fish type that had notable mortality during this period, but this observed mortality was consistent with previous trends in zebrafish survival in this test system.

Water Distribution. Intermittent pressure and delivery problems were noted with respect to water distribution. Acid cleaning of carbonate deposits from water supply equipment did not entirely resolve the delivery problems. Trouble-shooting was hampered by the complexity of the water distribution system in that special expertise was required to diagnose the electrical/mechanical/plumbing interface. Since the solenoid diluter was not being used to make serial dilutions, it was determined that the diluter would be replaced with a less complex water

distribution system during the test. A prototype of a nylon eight-port manifold distribution system was field-tested successfully by USACEHR during the spring of 2000, and this system was installed and became operational in the mobile lab in July 2000. Intermittent pressure problems were noted in mid-June when all aquaria were receiving comparison water. The water distribution pump was cleaned and later replaced but the pressure issue persisted. After each trouble-shooting session, the pressure appeared to be stable, but water pressure dropped within several days of each type of remediation. This pressure problem extended for almost a month before it was successfully resolved. During that time, all aquaria were changed from flow-through exposure to daily static renewal, with >90% of each aquarium's water volume replaced manually each day. Pressure gauges installed with the manifold system helped to diagnose where the pressure drop was occurring. Close inspection revealed that the jumbo airstones within the second aeration vat were located in close proximity to the discharge point from the vat. With the increased volume of comparison water needed to supply all aquaria during the summer months, air bubbles entrained in the vat effluent stream were randomly lodged within the distribution pump, lowering water pressures. To solve this problem, the airstone in the second aeration vat was disconnected and no further water pressure issues were encountered.

Environmental Conditions. During the first week of the project, it was discovered that the light cycle timer for the mobile laboratory was malfunctioning. The mobile lab received continuous light instead of the desired 18/6h light/dark cycle for the first month of medaka and zebrafish exposure. The timer was repaired on 4/13/00 and the desired light cycle was attained for the remainder of the test. Although the short-term continuous light was not optimal for fish husbandry, both test and comparison fish received the same treatment and any effect (none was observed) would have occurred simultaneously to both sets of fish. Optimum fish survival and growth is directly dependent upon maintaining a specified range of acceptable water quality parameters. Table 2 provides summaries of pooled aquaria water quality measurements within a type of source water by fish species. Temperature and dissolved oxygen were the most critically variable water quality parameters measured (Figures 7 and 8), although alkalinity and conductivity levels rose in the early fall. Temperature stabilization with the fish aquaria was addressed through water flow rate and lowering the ambient air temperature within the mobile lab. By slowing water flows and allowing a longer time for the thermally heated water to cool in the aeration vats, temperature dropped back into the desired range of 25 ±2°C. Lowered dissolved oxygen levels were remediated by supplemental aeration in the aeration vats for comparison water, with Venturi in-line aerators for test water, and by airstones at the aquaria level for all aquaria after April 2000. Aquaria aeration was not implemented at study onset due to the potential for airstripping volatile organics from the test water. However, during the project OCWD-initiated chemical contaminant analyses of the test water demonstrated that volatile organic levels in test water were at or below instrumental detection limits, so no apparent deterrent remained to adding airstones to each fish aquaria.

Table 2. Water quality summaries for comparison and test water[a]

Fish/Water	Parameter	Temp.	pH	D.O.	Cond.	Total NH_3	Un-ion. NH_3	Alkalinity	Hardness
	Average	25.0		7.8	878	0.02	0.002	138	250
Medaka	Median	24.9	8.3	7.9	866	0.02	0.002	116	224
	Count	451	451	451	451	158	158	39	39
Comp.	Stand. Dev.	1.293	0.197	0.786	149	0.000	0.001	43	54
Water	Maximum	28.0	9.0	9.0	1186	0.02	0.003	228	356
	Minimum	21.4	7.6	3.6	81	0.02	0.001	96	180
	Stand. Error	0.061	0.009	0.037	7	0.000	6.3 E-05	7	9

Fish/Water	Parameter	Temp.	pH	D.O.	Cond.	Total NH_3	Un-ion. NH_3	Alkalinity	Hardness
	Average	24.8		7.4	857	0.02	0.002	138	251
Zebrafish	Median	24.6	8.2	7.4	813	0.02	0.002	116	240
	Count	123	123	123	123	53	53	38	38
Comp.	Stand. Dev.	1.143	0.173	0.701	130	0.003	0.001	44	54
Water	Maximum	27.8	8.5	8.9	1181	0.04	0.003	228	348
	Minimum	22.5	7.6	5.4	644	0.02	0.001	92	180
	Stand. Error	0.103	0.016	0.063	12	0.000	8.2 E-05	7	9

Fish/Water	Parameter	Temp.	pH	D.O.	Cond.	Total NH_3	Un-ion. NH_3	Alkalinity	Hardness
	Average	25.0		7.8	936	0.02	0.002	148	260
Bluegill	Median	24.4	8.3	7.9	948	0.02	0.003	128	256
	Count	62	62	62	62	20	20	27	27
Comp.	Stand. Dev.	1.453	0.138	0.717	118	0.000	8.5 E-04	47	70
Water	Maximum	27.9	8.5	8.8	1168	0.02	0.003	228	348
	Minimum	22.9	8.0	5.3	743	0.02	0.001	100	20
	Stand. Error	0.185	0.018	0.091	15	0.000	1.9 E-04	9	13

Fish/Water	Parameter	Temp.	pH	D.O.	Cond.	Total NH_3	Un-ion. NH_3	Alkalinity	Hardness
	Average	24.8		6.8	950	0.02	0.002	167	264
Medaka	Median	24.6	8.1	6.8	970	0.02	0.001	196	280
	Count	490	492	492	492	177	178	23	23
Test Water	Stand. Dev.	1.443	0.268	1.085	122	0.000	8.0 E-04	51	39
	Maximum	28.1	8.7	8.9	1198	0.02	0.003	224	320
All dates	Minimum	21.1	7.3	2.1	110	0.02	0.001	96	200
	Stand. Error	0.065	0.012	0.049	5	0.000	6.0 E-05	11	8

Fish/Water	Parameter	Temp.	pH	D.O.	Cond.	Total NH_3	Un-ion. NH_3	Alkalinity	Hardness
	Average	24.2		6.2	996	0.02	0.001	208	291
Medaka	Median	24.2	7.8	6.2	1028	0.02	0.001	212	296
	Count	250	252	252	252	90	90	13	13
Test Water	Stand. Dev.	1.007	0.235	0.866	103	0.000	2.7 E-04	12	20
Only[b]	Maximum	26.2	8.7	8.4	1100	0.02	0.002	224	320
	Minimum	21.1	7.3	2.1	110	0.02	0 001	176	252
	Stand. Error	0.064	0.015	0.055	7	0.000	2.8 E-05	3	5

Fish/Water	Parameter	Temp.	pH	D.O.	Cond.	Total NH_3	Un-ion. NH_3	Alkalinity	Hardness
	Average	24.6		7.1	936	0.02	0.002	171	267
Zebrafish	Median	24.3	8.1	7.1	968	0.02	0.001	196	272
	Count	136	136	136	136	55	55	38	38
Test Water	Stand. Dev.	1.269	0.211	0.825	127	0.000	6.9 E-04	48	39
	Maximum	27.9	8.5	8.8	1186	0.02	0.003	224	340
All dates	Minimum	22.2	7.6	4.8	658	0.02	0.001	96	200
	Stand. Error	0.109	0.018	0.071	11	0.000	9.3 E-05	8	6

Fish/Water	Parameter	Temp.	pH	D.O.	Cond.	Total NH_3	Un-ion. NH_3	Alkalinity	Hardness
	Average	24.2		6.9	959	0.02	0.001	200	280
Zebrafish	Median	24.1	8.1	6.9	1004	0.02	0.001	208	286
	Count	82	82	82	82	37	37	22	22
Test Water	Stand. Dev.	0.749	0.204	0.752	124	0.000	4.8 E-04	27	26
Only[b]	Maximum	25.7	8.5	8.8	1100	0.02	0.002	224	316
	Minimum	22.4	7.6	5.0	658	0.02	0.001	108	200
	Stand. Error	0.083	0.023	0.083	14	0.000	8.0 E-05	6	6

Fish/Water	Parameter	Temp.	pH	D.O.	Cond.	Total NH_3	Un-ion. NH_3	Alkalinity	Hardness
	Average	25.0		7.4	936	0.02	0.002	157	260
Bluegill	Median	24.6	8.3	7.5	926	0.02	0.003	168	268
	Count	62	62	62	62	20	20	27	27
Test Water	Stand. Dev.	1.544	0.266	0.955	122	0.000	8.9 E-04	49	43
	Maximum	27.9	8.6	8.7	1174	0.02	0.003	220	340
All Dates	Minimum	22.9	7.5	4.1	741	0.02	0.001	100	200
	Stand. Error	0.196	0.034	0.121	15	0.000	2.0 E-04	9	8

Fish/Water	Parameter	Temp.	pH	D.O.	Cond.	Total NH_3	Un-ion. NH_3	Alkalinity	Hardness
	Average	24.1		6.6	1019	0.02	0.001	207	287
Bluegill	Median	24.0	8.0	6.6	1042	0.02	0.001	208	288
	Count	22	22	22	22	5	5	9	9
Test Water	Stand. Dev.	0.869	0.240	0.882	76	0.000	4.5 E-04	10	16
Only[b]	Maximum	25.8	8.2	8.2	1106	0.02	0.002	220	320
	Minimum	23.0	7.5	4.1	866	0.02	0.001	188	268
	Stand. Error	0.185	0.051	0.188	16	0.000	2.0 E-04	3	5

[a]Measurement units = pH in standard units; dissolved oxygen in mg/L; conductivity in ug/cm^3; total and un-ionized ammonia in mg/L NH_3 and N respectively; alkalinity and hardness in mg/L $CaCO_3$.

[b]These data sets do not include the measurements during time period of June 18 through October 23 when comparison water was supplied to test water aquaria.

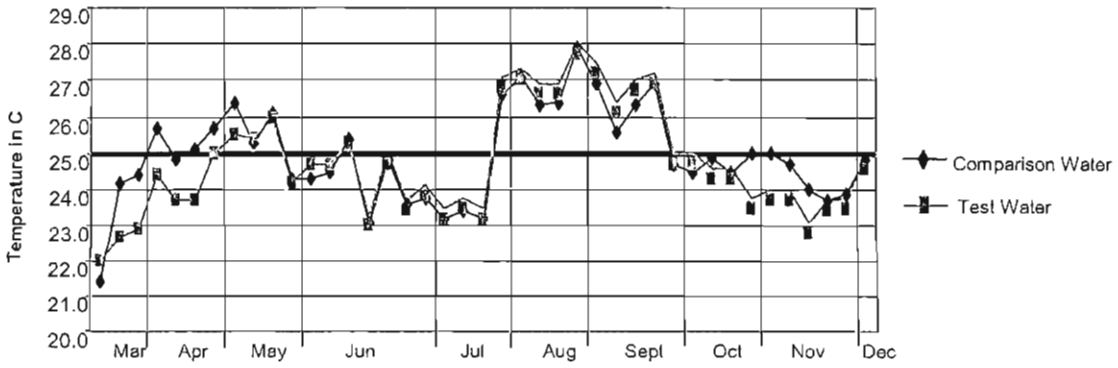

Figure 7. Temperature of comparison and test water in representative fish aquaria

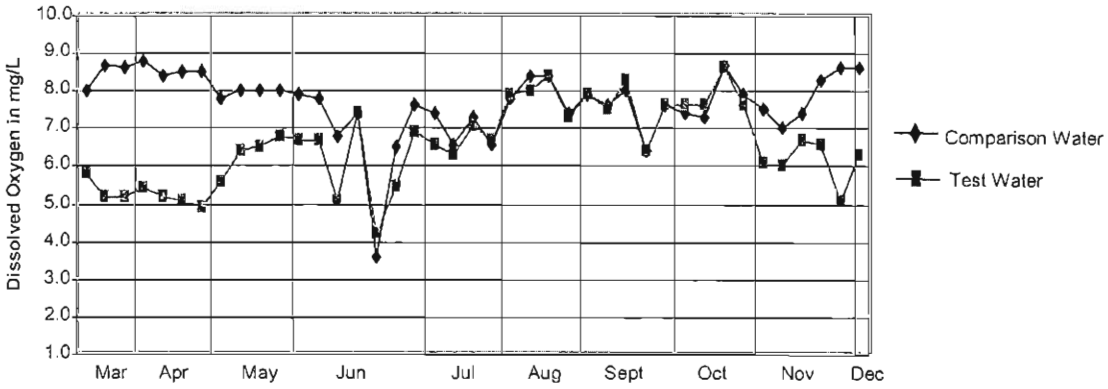

Figure 8. Dissolved oxygen levels of comparison and test water in representative fish aquaria

Tables 3 through 5 provide summaries of the organic chemical water quality for the surface water entering the off-river recharge basin (before percolation), the test water, and the control water. The test water was sampled in the mobile laboratory at the water inlet shown in Figure 4. The control water was sampled in the mobile laboratory directly downgradient of the carbon filtration system. Of the 181 compounds tested, only 4 were detected in the water entering the recharge basin or the test water and the detections were at concentrations ranging from 0.1 to 2.1 micrograms per liter. In the control water, the 181 organic compounds were below the detection limit except for chloroform, bromodichloromethane, and dibromochloromethane and total trihalomethanes at a maximum concentration of 6 micrograms per liter.

Dissolved oxygen levels were observed to decline immediately after carbon change-out (approximately every two months) in the comparison water. This problem was solved by requiring that pre-washed carbon be used and by requiring a longer flush rate once the carbon in the filter was exchanged. Ammonia did not build up to harmful levels in any aquaria during the nine-month flow-through exposure. Both comparison and test water were of high hardness levels and calcium carbonate precipitation was frequently observed in aquaria and water supply equipment.

Periodic cleaning of pumps, solenoids, strainers, and manifold orifices was necessary to ensure consistent water supply. Fish survival rates did not appear to vary dependent on the monitored water quality parameters. Raw data and summaries by fish aquaria for water quality are given in Appendix B. Raw data from chemical contaminant analyses of the test and comparison water are given in Appendix C.

Medaka Carcinogen Evaluation. This unfunded endpoint of the Biomonitoring Project remains to be accomplished. While medaka survival and growth did not appear to be affected by

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Table 3
Chemical Analyses Summary of Organic Compounds for Water Entering the Off-River Recharge Basin
(Santa Ana River at Imperial Highway)

Table 3a Summary of Analyses

			Number of Analytes Analyzed by Sample Date			
EPA Method	Description of Analytes	Test Results[a]	5/2/2000	8/8/2000	8/22/2000	10/31/2000
504	ethylene dibromide (EDB) & 1,2-dibromo-3-chloropropane (DBCP)	None Detected	2	2	-	2
506	Phthalates & Adipates	None Detected	7	7	-	7
507	Nitrogen & Phosphorus containing Pesticides	None Detected	20	20	-	20
508	Chlorinated Pesticides	None Detected	41	41	-	41
515	Chlorinated Acids	None Detected	8	8	-	8
524	Volatile Organic Compounds (VOCs)	Detections (see Table 3b)	2	-	-	2
524	Volatile Organic Compounds (VOCs)	None Detected	69	70	71	68
531	Carbamates	None Detected	11	11	-	11
547	Glyphosate	None Detected	1	1	-	1
548	Endothall	None Detected	1	1	-	1
549	Diquat & Paraquat	None Detected	2	2	-	2
550	Polyaromatic Hydrocarbons	None Detected	16	16	-	16
632	Diuron	None Detected	1	1	-	1

Note: [a] Detection limits are listed in Appendix C

Table 3b Summary of Detections of Organic Compounds

EPA Method	Description of Analytes	Analyte Name	Minimum Concentration	Maximum Concentration	Units	Reportable Detection Limit
524	Volatile Organic Compounds (VOCs)	Chloroform	ND	0.50	ug/L	0.50
524	Volatile Organic Compounds (VOCs)	Total THMs	ND	0.50	ug/L	0.50

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Table 4
Chemical Analyses Summary of Organic Compounds for Test Water

Table 4a Summary of Analyses

			Number of Analytes Analyzed by Sample Date		
EPA Method	Description of Analytes	Test Results[a]	3/15/2000	11/13/2000	12/7/2000
504	EDB & DBCP	No Detections	2	2	2
506	Phthalates & Adipates	No Detections	7	7	7
507	Nitrogen & Phosphorus containing Pesticides	Detection(s) (see Table 4b)	1		
507	Nitrogen & Phosphorus containing Pesticides	No Detections	19	20	20
508	Chlorinated Pesticides	No Detections	41	41	41
515	Chlorinated Acides	No Detections	8	8	8
524	Volatile Organic Compunds (VOCs)	Detection(s) (see Table 4b)	2		2
524	Volatile Organic Compunds (VOCs)	No Detections	69	70	68
531	Carbamates	No Detections	11	11	11
547	Glyphosate	No Detections	1	1	1
548	Endothall	No Detections	1	1	1
549	Diquat & Paraquat	No Detections	2	2	2
550	Polyaromatic Hydrocarbons	No Detections	16	16	16
632	Diuron	Detection(s) (see Table 4b)	1		
632	Diuron	No Detections		1	1

Note: [a] Detection limits are listed in Appendix C

Table 4b Summary of Detections of Organic Compounds

EPA Method	Description of Analytes	Analyte Name	Minimum Concentration	Maximum Concentration	Reportable Detection Limit
507	Nitrogen & Phosphorus containing Pesticides	Simazine	ND	0.10	0.10
524	Volatile Organic Compunds (VOCs)	Chloroform	ND	TR	0.50
524	Volatile Organic Compunds (VOCs)	Total THMs	ND	TR	0.50
632	Diuron	Diuron	ND	2.10	1.00

Note: Units are micrograms per liter

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Table 5
Chemical Analyses Summary of Organic Compounds for Control Water

Table 5a Summary of Analyses

			Number of Parameters Analyzed by Sample Date	
EPA Method	Description of Analytes	Test Results[a]	3/15/2000	10/2/2000
504	EDB & DBCP	No Detections	2	2
506	Phthalates & Adipates	No Detections	7	7
507	Nitrogen & Phosphorus containing Pesticides	No Detections	20	20
508	Chlorinated Pesticides	No Detections	41	41
515	Chlorinated Acides	No Detections	8	8
524	Volatile Organic Compunds (VOCs)	Detection(s) (see Table 5b)		4
524	Volatile Organic Compunds (VOCs)	No Detections	71	67
531	Carbamates	No Detections	11	11
547	Glyphosate	No Detections	1	1
548	Endothall	No Detections	1	1
549	Diquat & Paraquat	No Detections	2	2
550	Polyaromatic Hydrocarbons	No Detections	16	16
632	Diuron	No Detections	1	1

Note: [a] Detection limits are listed in Appendix C

Table 5b Summary of Detections of Organic Compounds

EPA Method	Description of Analytes	Analyte Name	Minimum Concentration	Maximum Concentration
524	Volatile Organic Compunds (VOCs)	Bromodichloromethane	ND	2.00
524	Volatile Organic Compunds (VOCs)	Chloroform	ND	3.40
524	Volatile Organic Compunds (VOCs)	Dibromochloromethane	ND	0.60
524	Volatile Organic Compunds (VOCs)	Total THMs	ND	6.00

Notes: Units are micrograms per liter
The reportable detection limit for the VOCs with detections was 0.50 micrograms per liter

exposure to test water, microscopic tissue evaluation is critical in determining if cellular mechanisms or developmental processes were altered in any way by the nine-month exposure. Exposure to test water was interrupted for a four-month period approximately three months into the test. This non-continuous exposure may limit the ultimate data interpretation, although the exposure was continuous during the early life stages of the medaka. (Medaka are sexually mature after about three months of age, with the majority of their overall growth occurring in the first three months.) Rapid cell division associated with growth is particularly vulnerable to toxic insult, so exposure to the early life stage is the most critical portion of the life cycle to evaluate. Cellular aberrations resulting from early life stage toxin exposure are usually expressed through time in adverse health outcomes. No gross lesions were observed on medaka externally during the nine-month exposure, nor were they observed on internal organs at necropsy. It is recommended that funding for this evaluation be obtained and that histology, pathology, and statistical analyses of the findings be performed. The preserved medaka are being held in storage by GEO-CENTERS, INC., at USACEHR, Fort Detrick, MD. An American College of Veterinary Pathologists (ACVP) certified pathologist should read up to five step sections per fish to look for changes in the following tissues: bone (vertebra), brain, chromaffin tissue, corpuscle of Stannius, esophagus, eye, gallbladder, gill, heart, hematopoietic tissue, interrenal tissue, intestine, kidney, liver, nares, ovary, pancreas, peripheral nerve, pineal organ, pituitary gland, pseudobranch, skeletal muscle, skin, spinal cord, spleen, stato-acoustic organ, swim bladder, testis, thymus, thyroid tissue, urinary bladder, and gross lesions. A summary pathology report should be issued on the findings. Statistical analyses of these findings would be instrumental in designing future studies of this nature for Orange County.

Staff Recommendations. During the Biomonitoring Project, several procedures were modified and more desirable outcomes were obtained. So that future researchers may benefit from these lessons learned, a summary of staff recommendations follows.

- For testing of 100% water, a diluter was not required.
- Water distribution via an eight-port nylon manifold with 1/8-inch orifices and tubing consistently provided the needed supply of water.
- It is desirable that the manifold be centered over the destination aquaria for best flows.
- Needle valves (3/4-inch) worked well to control water flows.
- Two submersible heaters should be located in each water bath.
- The cast iron filter housing should be replaced with bag-type filters for ease of maintenance.
- Chillers or air conditioning should be used to lower the temperature of thermally heated water.
- The aeration vats should be re-plumbed so that each type of water goes through an aeration vat.
- A chemical fume hood vented to the exterior of the mobile lab should be installed for test sacrifice procedures.

CONCLUSIONS

The objectives of the Biomonitoring Demonstration Project were met. Fish biomonitoring is feasible and provided toxicity information that is not elicited from single compound chemical analyses. Test water drawn from underneath the Off-River System did not impair fish survival or growth. Water quality of the test water varied through the nine-month evaluation period but did not appear to adversely affect the test outcome. Engineering challenges, such as providing aeration to the test water and lowering the water temperature, encountered during the test were met successfully. Of the three species of fish tested, the Japanese medaka had the highest survival rate over the test duration. Poor survival of zebrafish in both test and comparison waters demonstrated that the lot tested was not suitable for fish biomonitoring. Bluegill sunfish survival of 100% over a three-month period indicated that short-term testing with a Real-Time Environmental Protection System (REPS) using bluegill is feasible for future water monitoring efforts. All three species of fish had overlapping ranges (mean ± standard deviation) when test water fish growth was compared to comparison water fish growth within a species, strongly suggesting that there were no underlying fish toxicity mechanisms in the test water relative to the comparison water. Although medaka histopathology was not within the scope of this demonstration project, the fish were preserved at test termination so that the tissue would be available if funding for future evaluation became available. Incorporation of medaka histopathology into this and future studies of this nature would provide a more comprehensive evaluation of all potential health outcomes of the study models. The mobile laboratory proved to be a valuable public relations asset for the Biomonitoring Demonstration Project. Many groups of people, from local interest groups, to school children, to newspaper reporters and television crews, toured through the mobile laboratory and were educated about fish biomonitoring. Through furthering understanding of the safety of using the Santa Ana River for groundwater recharge, fish biomonitoring contributed positively to OCWD's approach to ensuring the clean water needs of its constituents.

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