1976

Sensitivity of Vertebrate Embryos to Heavy Metals as a Criterion of Water Quality: Phase III: Use of Fish and Amphibian Eggs as Bioindicator Organisms for Evaluating Water Quality

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Research Report No. 91

SENSITIVITY OF VERTEBRATE EMBRYOS TO HEAVY METALS AS A CRITERION OF WATER QUALITY

PHASE III

Use of Fish and Amphibian Eggs and Embryos As Bioindicator Organisms for Evaluating Water Quality

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Project Number: B-044-KY (Completion Report)

Agreement Number: 14-31-0001-5077 (FY-1975)

Period of Project: July 1, 1974 - June 30, 1975

University of Kentucky Water Resources Research Institute
Lexington, Kentucky

The work on which this report is based was supported in part by the Office of Water Research and Technology, U.S. Department of the Interior, under the provisions of Public Law 88-379, as Project Number B-044-KY.

January, 1976

ABSTRACT

Fish and amphibian eggs, embryos and early posthatched (larval) stages were evaluated as bioindicator organisms with which to monitor the quality of natural water resources. Eggs of 9 species were cultured in water collected from each of 11 Inner Bluegrass rivers and streams. The latter were chosen to represent water sources varying in quality from extremely poor to good. Selection was based on the sources and magnitude of pollution, and the diversity and density of piscine populations. Cultures were maintained in vitro, using 12-hr changes of water.

Averaging data for all 9 animal species, egg hatchability (embryonic survival) ranged from 0% for the most contaminated water to 94% for uncontaminated water found to support a healthy aquatic biota. Eggs of the squirrel treefrog and gray treefrog were the most sensitive of 5 amphibian species tested, and of 4 fish species, rainbow trout eggs proved most susceptible to water contaminants.

Egg development provided an index to water quality which compared favorably with independent ecological indicators. In particular, the frequencies of embryonic mortality recorded for the 11 monitoring sites correlated with reductions in density and species diversity of piscine populations. The results indicated that eggs and embryos from a wide selection of fish and amphibian species may be used as sensitive indicators of water quality. Amphibian larvae and early fish fry were considerably less sensitive to variations in water quality.

More contaminated water sources which gave high rates of embryonic mortality also produced high frequencies of teratogenic development, affecting up to 46% of the embryos which survived to complete the hatching process. Defective survivors were rare to absent in cultures where egg hatchability exceeded 80%.

The selected water resources were analyzed for mercury and other trace metals. Appreciable levels of metallic contaminants were found in those water sources which produced high levels of egg mortality. Egg hatchability was precluded or markedly reduced in all test waters where total mercury reached 0.3 ppb or more.

Descriptors: Bioassay, Bioindicator, Heavy Metals, Water Quality

ACKNOWLEDGEMENTS

We are most appreciative for the assistance of Dr. Jarvis Hudson in the analyses of the water samples used in this study. We also are grateful to Doris Westerman, Charles Andre, and John MacGregor for the use of fish collecting data on Inner Bluegrass streams.

We particularly are indebted to Dr. Robert Kuehne for his helpful counsel in selecting monitoring sites and for fish collecting records on the Hickman Creek drainage system.

The research facilities used to conduct these experiments were provided in part by research funds from the National Science Foundation (grant no. G.I. 43623).

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INTRODUCTION

With the advent of the mercury problem, considerable attention was directed to the need for bioindicator and bioassay systems with which to detect and quantify the biological impact of environmental contamination (1, 2, 3). The President's Council on Environmental Quality indicated that the lack of early warning monitoring systems delayed detection of mercury pollution until it had reached critical proportions in certain areas (2).

A number of Federal actions were initiated during the 1960's which were directed to averting similar environmental crises. Under the Water Resources Research Act of 1964, the Department of the Interior (Office of Water Research and Technology) sponsored a system of State Water Resources Research Institutes, to study local problems in water quality. Within the province of the Water Quality Act of 1965, the National Technical Advisory Committee established initial water quality criteria for use in regulating interstate and coastal waters (3), and these criteria later were revised by the Joint Committee on Water Quality Criteria formed under the auspices of the National Academy of Sciences and National Academy of Engineering (4). Finally, the Environmental Protection Agency was established some five years ago to promulgate and enforce protective environmental standards. Despite these developments, certain pollution problems currently have reached major proportions with little forewarning to the American public. Perhaps the foremost example concerns the contamination of major waterways with polychlorinated biphenyls (4, 20). These compounds threaten reproduction in fish and other aquatic organisms, and they pose a health hazard to man due to high levels of PCB accumulation (100-200 ppm) in the tissues of economically important fish species.

Bioassay procedures have been improved and used more extensively in recent years to study such environmental toxicants as PCB (4). However, bioassay test organisms still are used largely in the laboratory to provide acute and chronic toxicity data. Extrapolations from laboratory bioassay data to conditions existing in natural ecosystems are complicated by the physical, chemical and biological interactions which may alter the

availability and/or toxic action of aquatic contaminants. Also, deterioration of aquatic ecosystems frequently involves complex mixtures of toxicants, the combined actions of which are difficult to assess in simulated laboratory tests with bioassay organisms.

Unfortunately, little use has been made of test organisms as bioindicators with which to more directly assess the quality of natural water resources. This has been due in part to restricted availability of reliable, economically feasible test systems. However, properly used, *in situ* tests with bioindicator organisms may aid in the early detection and characterization of aquatic pollution (16). It has been shown in previous investigations that fish and amphibian eggs are highly suitable for bioassay purposes (4, 5, 6). The principal objective of the present study was to evaluate the use of fish and amphibian eggs as bioindicator organisms with which to monitor the quality of natural water resources.

RESEARCH PROCEDURES

Eggs from 9 fish and amphibian test species were maintained in *in vitro* cultures, using water collected from monitoring sites selected on 11 Inner Bluegrass rivers and streams. Frequencies of hatchability were determined for each of the selected water resources, and results were evaluated to ascertain the reliability of fish and amphibian eggs as bioindicators of water quality. As a control baseline, eggs of all test species were cultured in a contamination-free synthetic water shown to give optimum embryonic survival.

Selection of test species. Piscine species included the largemouth bass (*Micropterus salmoides*), goldfish (*Carassius auratus*), channel catfish (*Ictalurus punctatus*), and the rainbow trout (*Salmo gairdneri*). Amphibians used were all anurans, and included the narrow-mouthed toad (*Gastrophyrne carolinensis*), red-spotted toad (*Bufo punctatus*), pig frog (*Rana grylio*), southern gray treefrog (*Hyla chrysocephala*) and the squirrel treefrog (*Hyla squirella*). Choices were based primarily on seasonal availability, suitable egg production, good handling charac-

teristics for *in vitro* propagation, and representative geographical distribution. Sources of supply, egg production, hatching times and related considerations are summarized in Table 8.

Selection of water monitoring sites. Locations on 11 Kentucky streams and rivers were chosen to reflect variation in water quality ranging from exceptionally poor to good, as follows:

1. Cane Run. The monitoring site was near the junction of Ironworks Pike and Cane Run, approximately 0.5-1.0 mile downstream from an industrial complex (metal plating and alloying) and a small, primary sewage treatment plant (Highlands subdivision).

2. Town Branch. The monitoring site was at the junction of Viley Road and the Southern Railway, approximately 1.2 miles downstream from the Town Branch sewage plant (secondary treatment).

3. Wolf Run. The monitoring site was at the junction of Old Frankfort Pike and Wolf Run, approximately 0.5 mile upstream from Town Branch and 1.0 mile downstream from a small sewage plant (primary treatment).

4. East Hickman Creek. The monitoring site was near Mackey Road, approximately 0.1 mile upstream from the mouth of West Hickman Creek. Pollution sources include storm sewer and septic tank effluents and agricultural waste products.

5. West Hickman Creek. The monitoring site was near Ash Grove Pike, approximately 0.2 mile downstream from the West Hickman sewage plant (secondary treatment).

6. Gainesway Branch. The monitoring site was near the junction of Tates Creek Road and Kirklevington Road, approximately 0.5 mile upstream from West Hickman Creek. This area receives runoff from residential and business districts.

7. Hickman Creek. The monitoring site was near Iron Bridge at Union Mills, off State Highway 169, and approximately 6 miles downstream from the junction of East and West Hickman Creeks. Major pollution is from upstream effluents into East and West Hickman branches. However, over the course of the stream for 4-6 miles above the monitoring site, there is the likelihood of some self-purification due to uptake of contaminants

by bottom sediments, natural aeration, and the influx of spring water.

8. Shelby Branch. The monitoring site was at the junction of Tates Creek Road and East Hickman Road, approximately 0.5 mile upstream from East Hickman Creek. This stream receives runoff from agricultural areas (crops and livestock).

9. Kentucky River. The monitoring site was at Tates Creek Landing, approximately 5 miles upstream from the mouth of Hickman Creek. Pollution sources are diverse, though moderate for a 7th order river.

10. Elkhorn Creek. The monitoring site was near the Frankfort National Hatchery, off Indian Gap Road, and approximately 1.5 miles upstream from the Kentucky River. This stream serves as the water source for the Frankfort hatchery. At least some industrial, domestic and agricultural effluents enter the Elkhorn from small upstream tributaries (e.g., Town Branch).

11. Steele's Run. The monitoring site was near the junction of Old Frankfort Pike and Redd Road, approximately 0.7 mile upstream from the mouth of South Elkhorn Creek. This stream supports a healthy biota, and shows no evidence of significant pollution.

Classification to stream order (12, 13) and general characteristics of the selected monitoring sites are given in Table 7. The 2nd to 4th order streams are within the Hickman and Elkhorn drainage systems, both of which enter the Kentucky River. We have chosen changes in species diversity of fish populations as our principal criterion for assessing water quality, as baseline data for other ecological parameters are largely lacking. Sites 1-3 were selected to represent water resources of extremely poor quality, with animal life markedly reduced to absent. Only two fish species persist at the Wolf Run site, including the bluntnose minnow (Pimephales notatus) and the blacknose dace (Rhinichthys atratulus), and repeated collections over the past two years have failed to produce fish at the Cane Run and Town Branch monitoring locations (8, 9). Records of fish populations for the pre-pollution era are lacking. However, judging from data on streams of similar order in the same hydrological system (10, 11, 13), at least 15 species likely existed before the onset of heavy pollution (Table 6).

Monitoring sites 4-8 were chosen to represent aquatic habitats varying in quality from poor to fair, based largely on reduced diversity of macroinvertebrate and fish populations (7, 8, 9). For example, from 1960 to present time, the number of fish species for the East Hickman, West Hickman and Hickman sites have decreased from 17 to 8, 16 to 11, and 22 to 15, respectively (7; Table 6). Also, since 1972 the number of fish species at sites on Gainesway and Shelby Branches have decreased from 10 to 7 and 17 to 14, respectively (7). Though data for previous years are incomplete, 17 species seem reasonable as a pre-pollution baseline for Shelby Branch. However, prior to the demise in water quality, the number of species for Gainesway may have exceeded 10. These reductions in diversity of fish fauna are summarized for the various monitoring sites in Table 6.

Sites 9 (Kentucky River)and 10 (Elkhorn Creek) were selected to represent generally viable waters which have suffered at least moderate reduction in quality. While collecting records are incomplete, these rivers appear to support generally diverse fish fauna. Though there are no clear indications of substantial losses of piscine species from the Kentucky River during recent years, fish population studies by Williams indicate a decline in the density of game fishes and the probable loss of darter minnows (17). The latter are sensitive (indicator) fish species which generally are absent in polluted waters. Also, interviews with commercial fisherman suggest that channel catfish populations in the Kentucky River have suffered significant reduction (9). Collecting data from the Elkhorn indicates some reduction in the density of fish populations, and at least one species, the rosyfaced shiner (Notropis rubellus) has become rare or absent in recent years (9).

The monitoring site on Steele's Run was selected to reflect one of the healthiest aquatic ecosystems in the Hickman and Elkhorn drainages. The fish population has remained stable during the past decade, with the same 15 species reported at or near our monitoring site in 1966 (11), 1972 (10), and again in 1975 (9). No appreciable reductions in density have been noted for these fish populations. Also, this stream supports

a rich invertebrate fauna and a healthy amphibian population (9).

Egg culture methods. Water samples were collected from the monitoring sites at 2-3 day intervals, analyzed for pH, hardness, dissolved oxygen, and conductivity, and stored in polyethylene containers until needed. Fertilized eggs from the 9 animal test species were cultured in vitro, using water from each of the 11 monitoring sites. The cultures were maintained in deep Petri dishes, modified as described by Birge and Just (6). The culture water was changed regularly at 12 hr intervals, and all cultures were given continuous aeration.

Culture water was monitored routinely for temperature, oxygen, ammonia level, water hardness and pH, using a YSI tele-thermometer with thermocouple, YSI oxygen meter (model 51A), Orion ammonia and and water hardness electrodes, and a Corning digital pH meter (model 110, with expanded millivolt scale). All glassware used in the monitoring experiments was rinsed in acetone, soaked in nitric acid and washed in an excess of distilled, deionized water. Culture temperature was maintained at 55-56° F for trout eggs, 66-67° F for the goldfish, 71-72° F for all amphibian species and the largemouth bass, and at 75-76° F for the channel catfish. Culture pH varied somewhat with different water sources, ranging from 7.1 to 8.4. All cultures were maintained in temperature-regulated, walk-in environmental rooms. Eggs and embryos were inspected daily to determine mortality rates and to remove dead specimens.

Particular attention was given to trout embryos, especially during the "green stage." Harmful exposure to artificial light was avoided (18, 19) and cultures were maintained under semisterile conditions to minimize occurrences of soft egg disease and fungus. Prior to each use, culture rooms were disinfected and irradiated with ultraviolet light for 12 hours, and they were maintained under positive pressure during culture experiments. In the event of fungus or soft egg disease, we treated periodically with formaldehyde (1/1000 for 10 minutes) or malachite green (1/2000 for 30 minutes). Control eggs and embryos were cultured simultaneously with experimentals, and under identical conditions

except they were propagated in synthetic Holtfreter's solution (14). Holtfreter's solution was prepared with distilled, double deionized water (conductivity less than 0.4 μmhos). Two modifications of Holtfreter's original salt formula were used, including 1) reduction of sodium bicarbonate to 0.1 g/liter, and 2) reduction of sodium bicarbonate and sodium chloride to 0.1 g/liter each. Control survival was equally good for the two modifications. Also, both control solutions gave a hardness of 87-93 ppm as $CaCO_3$ and a pH of 7.7-7.9. The conductivity given in Table 7 is for the low salt modification (0.1 g NaCl/liter).

Except for the catfish, eggs and sperm were stripped by hand using procedures given by Leitritz (15). Immediately after fertilization, egg samples were transferred to culture dishes. Catfish spawn was collected from hatchery ponds and eggs were 18-24 hrs into development when transferred to culture dishes. Omission of treatment during the early postfertilization period may have accounted for somewhat higher survival of catfish eggs in water samples from polluted monitoring sites (e.g., Wolf Run; Tables 1,4).

Minimum sample size was set at 100 eggs/culture and survival was defined as completion of the hatching process. Subsequent to hatching, amphibian and catfish embryos were screened for developmental anomalies, to determine the percentages of survivors which were affected by gross teratologies. Except for the trout, all cultures were maintained through development and for 4 days posthatching. Trout cultures were terminated at 15 days of development.

Water samples from all monitoring sites were analyzed for chromium, mercury, nickel, and zinc using a Perkin-Elmer atomic absorption spectrophotometer (model 503). Total mercury was determined using the cold vapor procedure of Hatch and Ott (21), and the other metals were analyzed by flame atomic absorption spectrophotometry (22).

OBSERVATIONS

The hatchability frequencies obtained in the monitoring experiments are summarized in Tables 1 and 2 for fish and amphibian species, respectively. Except for the trout, survival frequencies were determined at hatching. Water samples from the three monitoring sites chosen to represent high degrees of pollution all produced striking reductions in survival of fish and amphibian eggs used for test purposes. Complete lethality was observed for eggs and embryos of all species cultured in water samples from Cane Run and Town Branch, and extremely poor survival was recorded for Wolf Run. Concerning the latter, hatchability ranged from 9% to 40% for 6 amphibian test species, and averaged 27% and 54% for trout and catfish. Trout culture data were taken on the 15th day of development, when the majority of cultures were terminated. However, the Wolf Run trout cultures were continued through the 18th day, at which time there was 100% lethality.

As seen in Table 3, amphibian eggs generally failed to survive for more than one day in Cane Run water, and 2 days in Town Branch cultures. Survival for representative species varied from 3% to 36% through 4 days posthatching for amphibian embryos and larvae cultured in Wolf Run water. Embryonic survival decreased sharply with culture time. Cane Run and Town Branch water produced complete lethality in catfish cultures prior to 4 and 6 days, respectively (Table 4), and survival in Wolf Run water decreased progressively from 100% on day 1 to 54% on day 6 (hatching). However, survival of newly hatched catfish alevins was high through 4 days posthatching, dropping only 4% below the hatching frequency (Table 4).

Eggs cultured in East Hickman Creek water showed markedly higher hatchability, amounting to 40-57% for the amphibian and piscine test species (Tables 1 and 2). Compared to hatchability in control water, survival of East Hickman cultures closely approached the TL_{50} range. The responses were surprisingly uniform among the full spectrum of test species considered.

TABLE 1

EFFECTS OF WATER QUALITY OF SELECTED KENTUCKY STREAMS ON SURVIVAL OF FISH EMBRYOS USED AS BIOINDICATOR ORGANISMS

MONITORING SITE	PERCENT SURVIVAL[1]				
	Trout	Bass	Goldfish	Catfish	All Species
Cane Run	0	-	-	0	0
Town Branch	0	0	0	0	0
Wolf Run	27	-	-	54	40
East Hickman Creek	54	-	-	57	56
West Hickman Creek	61	-	-	65	63
Gainesway Branch	60	-	-	79	70
Hickman Creek	63	-	-	84	74
Shelby Branch	75	-	-	87	81
Kentucky River	78	-	-	88	83
Elkhorn Creek	72	90	90	92	86
Steele's Run	73	98	96	95	91
Control Water	81	98	98	95	93

[1]Values represent hatching frequencies for all species except trout. Survival for trout embryos was determined at 15 days of development.

TABLE 2

EFFECTS OF WATER QUALITY OF SELECTED KENTUCKY STREAMS ON SURVIVAL OF AMPHIBIAN EMBRYOS USED AS BIOINDICATOR ORGANISMS

MONITORING SITE	PERCENT HATCHABILITY[1]					
	Squirrel Treefrog	Gray Tree-frog	Narrow-mouthed Toad	Pig Frog	Red-spotted Toad	All Species
Cane Run	0	0	0	0	0	0
Town Branch	0	0	0	0	0	0
Wolf Run	9(11)	9	10(46)	21(23)	40	18
East Hickman Creek	40(4)	40	41(7)	51	55	45
West Hickman Creek	48	49	59	67	69	58
Gainesway Branch	67(2)	70	74	80	80	74
Hickman Creek	79(3)	81	78(6)	86	87	82
Shelby Branch	90	86(1)	89	87(1)	96	90
Kentucky River	85(1)	86	89(1)	93	94	89
Elkhorn Creek	91	89	91	94	96	92
Steele's Run	96	96	96	97	98	97
Control Water	94	97	94	98	97	96

[1]Percentages include all animals which lived to complete hatching, and percentages of survivors bearing embryonic anomalies are given parenthetically.

With the exception of the trout, hatchability of fish and amphibian eggs increased progressively in waters collected from East Hickman, West Hickman, Gainesway, Hickman and Shelby Branches (Tables 1 and 2). Gainesway water gave intermediate results, with embryonic survival ranging from 60% for the trout to 80% for the pig frog and red-spotted toad. Hatchability of Shelby Branch cultures averaged 81% and 90% for piscine and amphibian species, respectively. Trout eggs gave the lowest survival rate, amounting to 75%.

Embryonic survival in Kentucky River water was essentially the same for amphibian species (89%) as that noted for Shelby Branch. However, hatchability was moderately higher for fish, particularly the trout (78%). Only slightly higher values were obtained for Elkhorn Creek water, with fish and amphibian hatchability averaging 86% and 92%, respectively (Tables 1 and 2).

Steele's Run was selected to represent an uncontaminated water source supporting a healthy aquatic biota. Except for the trout, hatchability in Steele's Run water was uniformly high for all species, ranging from 95-98%. By comparison, hatchability in control water varied from 94% for the squirrel treefrog to 98% for the pig frog, bass, and goldfish. Steele's Run was the only natural water source to give embryonic survival equal to that found for the synthetic culture medium. The survival of trout eggs in Steele's Run water may have been moderately reduced by malachite green treatment used to control fungus.

Embryonic lethality generally was proportional with culture time for all species (Tables 3, 4 and 5). Also, no correlations were found between embryonic survival and pH, conductivity or water hardness (Tables 6 and 7). Postembryonic (larval) survival was less affected by water quality. As seen in Tables 3, 4 and 5, survival values at 4 days posthatching usually dropped below hatching frequencies by only 1-6%. Even in Wolf Run water, catfish alevins survived surprisingly well for 4 days. The only notable exceptions were larvae of the gray treefrog and narrow-mouthed toad which suffered 56% to 70% mortality in Wolf Run water (Table 3).

TABLE 3

SURVIVAL WITH DURATION OF EXPOSURE OF AMPHIBIAN EMBRYOS AND LARVAE CULTURED IN NATURAL WATERS OF VARYING QUALITY

MONITORING SITE	PERCENT SURVIVAL[1,2]																	
	Gray Treefrog				Narrow-mouthed Toad				Pig Frog						Red-spotted Toad			
	1	2	3*	7	1	2	3*	7	1	2	4	5	6*	10	1	2	3*	7
Cane Run	23	0	0	0	24	0	0	0	40	0	0	0	0	0	42	6	0	0
Town Branch	57	12	0	0	64	18	0	0	69	46	13	0	0	0	64	27	0	0
Wolf Run	73	48	9	4	65	51	10	3	93	78	66	51	21	20	89	74	40	36
E. Hickman Cr.	88	64	40	34	77	67	41	38	91	86	74	68	51	50	92	78	55	50
W. Hickman Cr.	81	75	49	47	86	78	59	55	93	85	81	79	67	65	93	87	69	67
Gainesway Br.	94	86	70	63	90	82	74	73	99	97	93	89	80	79	94	88	80	79
Hickman Cr.	96	90	81	80	96	89	78	78	100	99	98	96	86	85	97	95	87	85
Shelby Br.	97	89	86	86	97	95	89	89	99	98	96	94	87	87	99	98	96	94
Kentucky R.	95	92	86	85	97	96	89	88	100	99	99	98	93	91	99	98	94	93
Elkhorn Cr.	96	93	89	87	97	95	91	90	100	99	98	97	94	94	99	98	96	96
Steele's Run	100	99	96	96	100	98	96	95	100	100	100	100	97	96	99	99	98	97
Control Water	100	98	97	96	99	97	94	93	100	99	99	98	98	97	99	99	97	96

[1]Exposure was continuous through 4 days posthatching. Asterisks denote hatching times.

[2]Duration of exposure given in days.

While our primary objective was devoted to characterizing the selected water resources according to their effects on embryonic and larval development, attention also was given to the differential sensitivity of animal test species. With respect to amphibian developmental stages, the squirrel treefrog and gray treefrog proved most sensitive (Table 2, Figure 1), giving generally lower hatchability frequencies than other species. Eggs of the red-spotted toad and the pig frog showed the highest tolerances to waterborne contaminants, giving higher survival values than other amphibian species. The catfish gave hatchability frequencies similar to those of the red-spotted toad (Figure 2). The trout proved to be most sensitive of all species. However, cultures for Steele's Run, Elkhorn Creek and several other monitoring sites were treated during late development with malachite green to control fungus. This may have reduced embryonic survival to some extent.

Embryonic anomalies were most common in water resources which produced higher frequencies of lethality (Tables 2 and 4). For example, 11% and 46% of squirrel treefrog and narrow-mouthed toad larvae which survived treatment with Wolf Run water were defective (Table 2). Anomalous survivors were rare to absent when hatchability exceeded 80%. The more common anomalies included defective vertebral columns, skulls and jaws; various deficiencies of the brain and/or absent sense organs (e.g., eyes); absent or defective fins; defects of the gut and yolk sac; and various degrees of partial twinning. These classes of teratological defects have been discussed earlier by Birge and Just (5).

Embryonic survival was averaged for piscine species, amphibian species, and for all test species, to provide convenient hatchability indices for use in evaluating the selected monitoring sites for water quality. The results are given in Table 6, together with data on the diversity of fish fauna for the selected water resources. There is a positive correlation between egg hatchability and the diversity of fish fauna.

TABLE 4

CORRELATION OF PERCENT SURVIVAL WITH EXPOSURE TIME FOR CATFISH EMBRYOS CULTURED IN NATURAL WATERS OF VARYING QUALITY

MONITORING SITE	PERCENT SURVIVAL WITH CULTURE DAYS[1]							
	1	2	3	4	6	7	9	10
Cane Run	100	68	21	0	0	0	0	0
Town Branch	100	85	67	34	0	0	0	0
Wolf Run	100	95	89	78	54(1)	52	51	50
East Hickman Creek	100	93	85	75	57(2)	56	56	56
West Hickman Creek	100	95	91	83	65(4)	63	63	63
Gainesway Branch	100	98	96	90	79(2)	78	77	77
Hickman Creek	100	97	94	91	84	83	79	79
Shelby Branch	100	99	98	96	87	87	87	87
Kentucky River	100	98	96	94	88	87	86	86
Elkhorn Creek	100	98	96	94	92	91	91	91
Steele's Run	100	99	98	98	95	94	94	94
Control Water	100	99	98	98	95	94	94	94

[1]Exposure was continuous through 4 days posthatching. Percentages of survivors bearing embryonic anomalies at hatching (6 days) are given parenthetically.

TABLE 5

CORRELATION OF PERCENT SURVIVAL WITH EXPOSURE TIME FOR BASS AND GOLDFISH EMBRYOS CULTURED IN NATURAL WATERS OF VARYING QUALITY

MONITORING SITE	PERCENT SURVIVAL WITH CULTURE DAYS[1]								
	Bass[2]				Goldfish[2]				
	1	2	3*	7	1	3	5*	7	9
Town Branch	72	45	0	0	81	59	0	0	0
Elkhorn Creek	97	94	90	89	98	95	90	90	87
Steele's Run	99	98	98	98	99	98	96	96	96
Control Water	99	98	98	98	99	98	98	96	95

[1]Cultures were maintained through 4 days posthatching

[2]Asterisk denotes day of hatching.

Water samples from the monitoring sites were analyzed for selected trace metals at 4 intervals during the hatchability study. Unfiltered water was used in all instances. The detection limits were 0.1 ppb for mercury, 2.0 ppb for zinc, 10 ppb for chromium, and 10 ppb for nickel. The results of metal analyses are presented in Table 9. Maximum concentrations of total mercury were 0.3 ppb for Cane Run and Wolf Run and 1.4 ppb for Town Branch, averaging 0.5 ppb for the latter. Mercury levels ranged up to 0.2 ppb in water samples from East Hickman, Gainesway and Hickman, and 0.1 ppb was found in Shelby Branch, Kentucky River and Elkhorn River. Chromium was detected only in water samples from Town Branch and Wolf Run, while nickel was found in all waters except Cane Run and Gainesway Branch (Table 9). Zinc was found to exceed 1 ppm in most waters analyzed during the first sampling period. Thereafter, zinc was most persistent in Town Branch, ranging from 84-104 ppb. Detectable levels of all 4 metals were found only in Town Branch and Wolf Run, and Steele's Run was the least affected by trace metals of all the monitoring sites considered.

DISCUSSION

The principal objective of this investigation was to examine fish and amphibian eggs, embryos and larvae as reliable, economically feasible test organisms for monitoring the quality of water resources. The results indicate that eggs and embryos of aquatic vertebrates may be used in simple *in vitro* cultures to detect deterioration in water quality. The frequency of egg hatchability gives quantitative data which compare favorably with independent, ecological indicators of water quality, such as species diversity and population density.

As shown in Table 6, a strong positive correlation was found between egg hatchability and the retention of species diversity in fish fauna. In water sources where fish species have not been lost due to pollution (*e.g.*, Steele's Run), embryonic development was normal and egg hatchability was fully comparable to that obtained under optimum culture conditions using contamination-free, synthetic

TABLE 6

CORRELATION OF EMBRYONIC SURVIVAL WITH SPECIES DIVERSITY OF PISCINE FAUNA

MONITORING SITE	Percent Embryonic Survival[1]			Diversity of Fish Fauna[2]	
	Fish	Amphibians	Fish & Amphibians	# Species/Expected	% Species Remaining
Cane Run	0	0	0	0/15	0
Town Branch	0	0	0	0/15	0
Wolf Run	40	18	24	2/15	13
East Hickman Creek	56	45	48	8/17	47
West Hickman Creek	63	58	60	11/16	69
Gainesway Branch	70	74	73	7/10	70
Hickman Creek	74	82	80	15/22	68
Shelby Branch	81	90	87	14/17	82
Kentucky River	83	89	87	-	-
Elkhorn Creek	86	92	89	-	-
Steele's Run	91	97	94	15/15	100

[1]Data combined and averaged from Tables 1 and 2.

[2]Data on fish fauna for past 15 years taken from Kuehne (7), MacGregor and Andre (8), Westerman and Westerman (9), and Small(10).

culture water (Tables 1 and 2). As noted above, the monitoring sites chosen on Elkhorn Creek and the Kentucky River represented areas which possessed viable fish fauna, but where at least some reduction has occurred in species diversity and population density (9, 17). For example, during recent years, the rosyfaced shiner has become rare to absent at the Elkhorn monitoring site (9) as have darter minnows in the section of the Kentucky River considered in this study (17). Hatchability in these waters was reduced from the control baseline by an average of 7 to 10% for fish and 4 to 7% for amphibian species. This response was quite uniform among both fish and amphibians. Considering all eleven test species used, there was no single case in which embryonic survival in these waters equalled or exceeded that found in control cultures.

To facilitate further comparisons between egg hatchability and changes in species diversity of fish fauna, an index was prepared on the percent of fish species remaining at the various collecting sites (Table 6). Values were determined as the number of fish species now present in the selected water resources/the number expected on the basis of collecting data recorded in earlier years. Using collecting records dating back to 1960, the frequencies at which original species have been retained in East Hickman, West Hickman, Gainesway, Hickman and Shelby Branches varied progressively from 47% to 82%. A close correlation exists between species retention for these water resources and egg hatchability (Table 6). The latter varied progressively from 56-81% for fish species, 45-90% for amphibian species or 48-87% when fish and amphibian data were combined. Also, there is a positive correlation between reduced egg hatchability and reductions in the diversity of macroinvertebrate fauna of these water sources (7).

In those instances where most or all fish species have been lost due to pollution (e.g., Cane Run; Town Branch; Wolf Run), fish and amphibian eggs either failed to develop or exhibited low frequencies of hatchability. Also, a treatment differential was found in which more

contaminated waters gave shorter survival times for eggs and embryos. As seen in Table 3, Cane Run water produced complete lethality of amphibian eggs on the first day of treatment, and there was a general decrease in mortality/time responses with waters giving increased hatchability.

The developmental responses considered in this investigation included frequency of egg hatchability (embryonic survival), frequency of anomalous survivors (teratogenesis), and survival rate of early posthatched (larval) stages. Egg hatchability clearly proved to be a useful criterion for evaluating the contamination of water resources. However, under the culture conditions imposed in these experiments, neither teratogenesis nor larval survival proved to be dependable bioindicator responses for evaluating water quality. Higher frequencies of teratogenesis and larval mortality occurred in water sources which produced embryonic mortality of 20% or more. However, these responses were absent or inconsistent in cultures of Shelby Branch, Kentucky River and Elkhorn waters, all of which significantly repressed egg hatchability.

As previously noted by Birge et al.(23), frequencies of teratogenesis among fish embryos treated with trace metal contaminants are high at concentrations which produce low embryonic survival. However, anomalous development is absent or infrequent at lower concentrations which approach threshold (80-90% survival). This generally is in agreement with the results of the present study.

Several investigators have considered fish fry to be more sensitive than fish eggs and embryos to environmental pollutants, including polychlorinated biphenyl compounds (24) and trace metallic contaminants such as zinc and copper (25-28). However, our findings indicate that early postembryonic stages of fish and amphibians are less sensitive than eggs and embryos as bioindicator organisms.

The presence of metallic contaminants likely contributed to the toxic effects of Town Branch and Wolf Run water (Tables 6, 9). Also,

it is likely that in Cane Run and several other streams, mercury levels of 0.1-0.3 ppb contributed in some measure to the reduced hatchability of fish and amphibian eggs. Using a nonflow culture system, Birge et al. found a concentration of 0.7 ppb inorganic mercury to produce mortality in populations of rainbow trout eggs (23), and experiments just completed show that trout eggs suffer up to 100% mortality when treated with 0.2 ppb inorganic mercury in a continuous-flow bioassay system. All water monitoring sites found to contain up to 0.1 ppb or more of total mercury gave significantly lower egg hatchability frequencies than those obtained for Steele's Run and control water (Tables 6, 9). However, considering the sources of pollution to the Elkhorn and Hickman drainage systems, it is evident that a heterogeneous mixture of inorganic and organic contaminants affect the quality of many of the Inner Bluegrass streams selected for study, and that reductions noted in egg hatchability likely are not due solely to trace metal contaminants.

The laboratory culture conditions employed in this study likely produced higher survival rates than would have been obtained in in situ cultures maintained at the actual monitoring sites. In the latter case, there would have been continuous exposure to waterborne toxicants. The in vitro culture procedure, consisting of 12-hour changes of culture water, undoubtedly minimized exposure. As noted by Nebeker and Puglisi (29), intermittent treatment of bioassay organisms does not provide for the continual replacement of toxicants assimilated by the test organisms or otherwise lost from the culture. However, the in vitro egg culture procedure used appears sufficiently sensitive for monitoring purposes. The economy and simplicity of this technique render it suitable for wide applications in evaluating the quality of water resources.

TABLE 7

CHARACTERISTICS OF WATER RESOURCES SELECTED AS MONITORING SITES[1]

MONITORING SITE	Stream Order	pH Mean & Range	Conductivity (µmhos)	Hardness (ppm $CaCO_3$) Mean & Range	Dissolved Oxygen (% Saturation)
Cane Run	2nd	7.6(7.5-7.8)	280	264(225-375)	95
Town Branch	3rd	7.9(7.5-8.1)	350	247(225-275)	85
Wolf Run	2nd	8.0(7.6-8.2)	230	252(173-275)	98
East Hickman Creek	4th	7.9(7.6-8.1)	180	232(173-275)	90
West Hickman Creek	3rd	7.9(7.6-8.1)	195	225(173-275)	100
Gainesway Branch	3rd	8.1(8.0-8.2)	210	256(185-290)	97
Hickman Creek	4th	8.0(7.8-8.2)	180	238(173-265)	100
Shelby Branch	3rd	8.0(8.0-8.1)	165	234(173-275)	90
Kentucky River	7th	7.6(7.4-7.8)	80	109(90-150)	90
Elkhorn Creek	5th	7.7(7.5-8.0)	150	154(90-225)	98
Steele's Run	2nd	8.3(7.9-9.0)	170	262(173-350)	100
Control Water	-	7.8(7.7-7.9)	340	90(87-93)	100

[1]All values for pH, hardness, conductivity and dissolved oxygen are based on a minimum of six determinations.

TABLE 8

SPECIES SELECTED FOR USE AS EMBRYONIC BIOINDICATORS

SPECIES	Developmental Time in Days	Number of Eggs* Per Female	Breeder's Availibility	Source
Channel Catfish (*Ictalurus punctatus*)	6 (72°F)	10,000-15,000	May-August	National Fish Hatcheries Frankfort, Ky. and Senacaville, Ohio
Goldfish (*Carassius auratus*)	5 (66°F)	15,000-25,000	March-July	National Fish Hatchery Frankfort, Kentucky
Largemouth Bass (*Micropterus salmoides*)	4 (65°F)	10,000-15,000	April-June	National Fish Hatchery Frankfort, Kentucky
Rainbow Trout (*Salmo gairdneri*)	24 (55°F)	6,000-8,000	Sept.-April	National Fish Hatchery Wytheville, Virginia
Narrow-mouthed Toad (*Gastrophryne carolinensis*)	3 (72°F)	1,000-3,000	May-August	Charles D. Sullivan Nashville, Tennessee
Pig Frog (*Rana grylio*)	6 (72°F)	6,000-10,000	March-June	Charles D. Sullivan Nashville, Tennessee
Red-spotted Toad (*Bufo punctatus*)	3 (72°F)	2,000-4,000	April-August	Charles D. Sullivan Nashville, Tennessee
Southern Gray Treefrog (*Hyla chrysocephala*)	3 (72°F)	100-500	May-June	Charles D. Sullivan Nashville, Tennessee
Squirrel Treefrog (*Hyla squirella*)	3 (72°F)	80-150	May-August	Charles D. Sullivan Nashville, Tennessee

*Egg production dependent upon size and maturity.

TABLE 9

TRACE METAL ANALYSES OF SELECTED WATER RESOURCES

MONITORING SITE	Mean Metal Concentrations (ppb) for 4 Sampling Periods[1]															
	Chromium				Mercury				Nickel				Zinc			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Cane Run	-	0	-	0	-	0	-	0.3	-	0	-	0	-	14	-	0
Town Branch	20	20	0	30	0.1	1.4	0.3	0.3	170	20	100	130	1530	84	100	104
Wolf Run	20	0	0	0	0	0	0.3	0.3	90	0	0	0	1460	0	13	0
East Hickman Creek	0	-	0	0	0	-	0	0.2	110	-	0	0	1640	-	16	0
West Hickman Creek	0	-	0	0	0	-	0	0	100	-	0	0	1390	-	0	0
Gainesway Branch	0	-	0	0	0	-	0	0.2	0	-	0	0	0	-	0	0
Hickman Creek	0	-	0	0	0.1	-	0	0.2	70	-	0	0	1230	-	0	0
Shelby Branch	0	-	0	0	0.1	-	0	0.1	90	-	0	0	1570	-	0	0
Kentucky River	0	-	0	0	0.1	-	0	0.1	50	-	0	0	1000	-	12	0
Elkhorn Creek	0	-	0	0	0.1	-	0	0	80	-	0	0	1510	-	0	0
Steele's Run	0	0	0	0	0	0	0	0	80	0	0	0	1420	0	0	0

[1]Limits of AAS detection for total mercury, zinc, chromium and nickel were 0.1, 2.0, 10.0 and 10.0 ppb, respectively. Zero denotes no detectability.

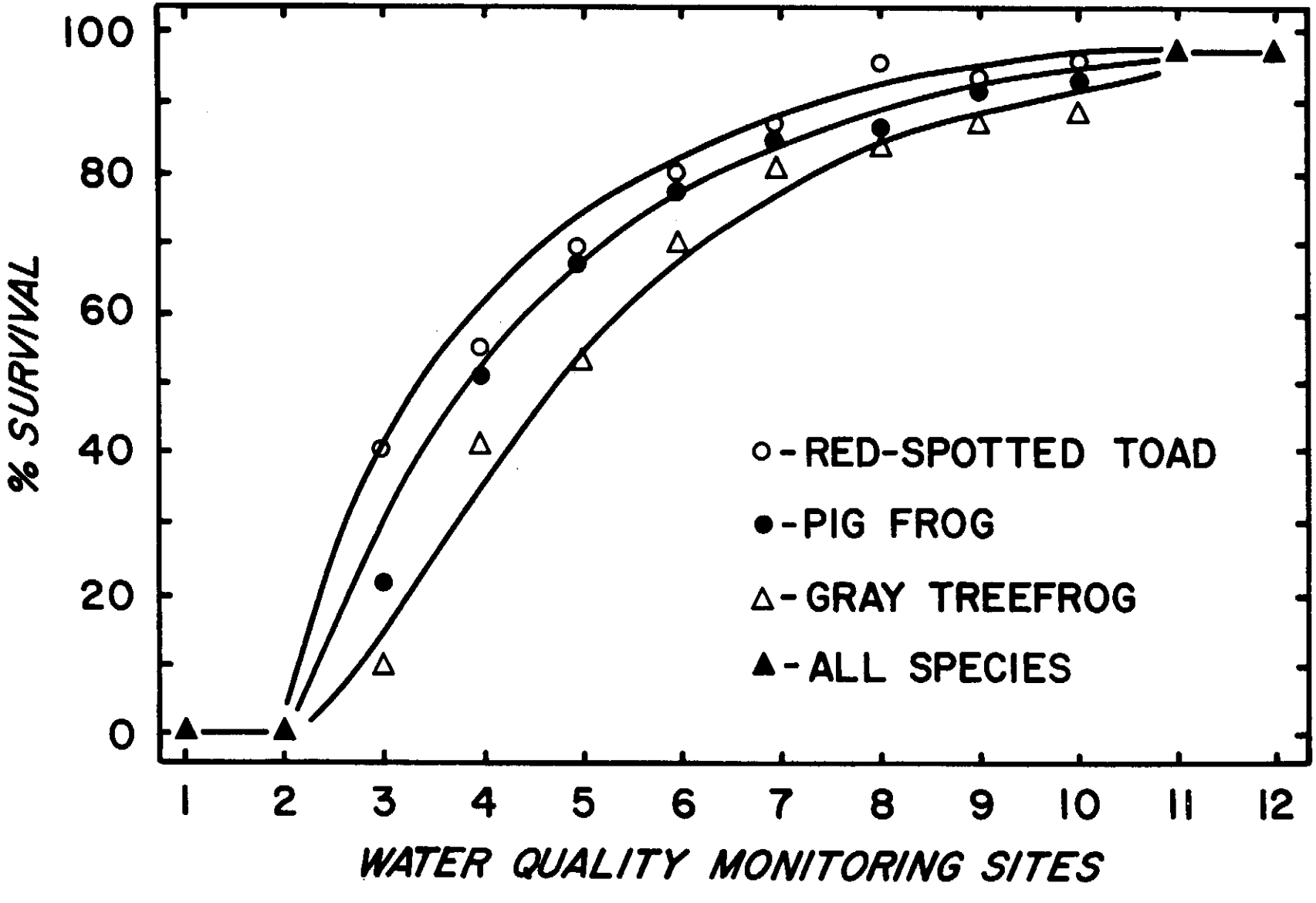

FIGURE 1. EFFECTS OF WATER QUALITY ON SURVIVAL OF AMPHIBIAN EMBRYOS

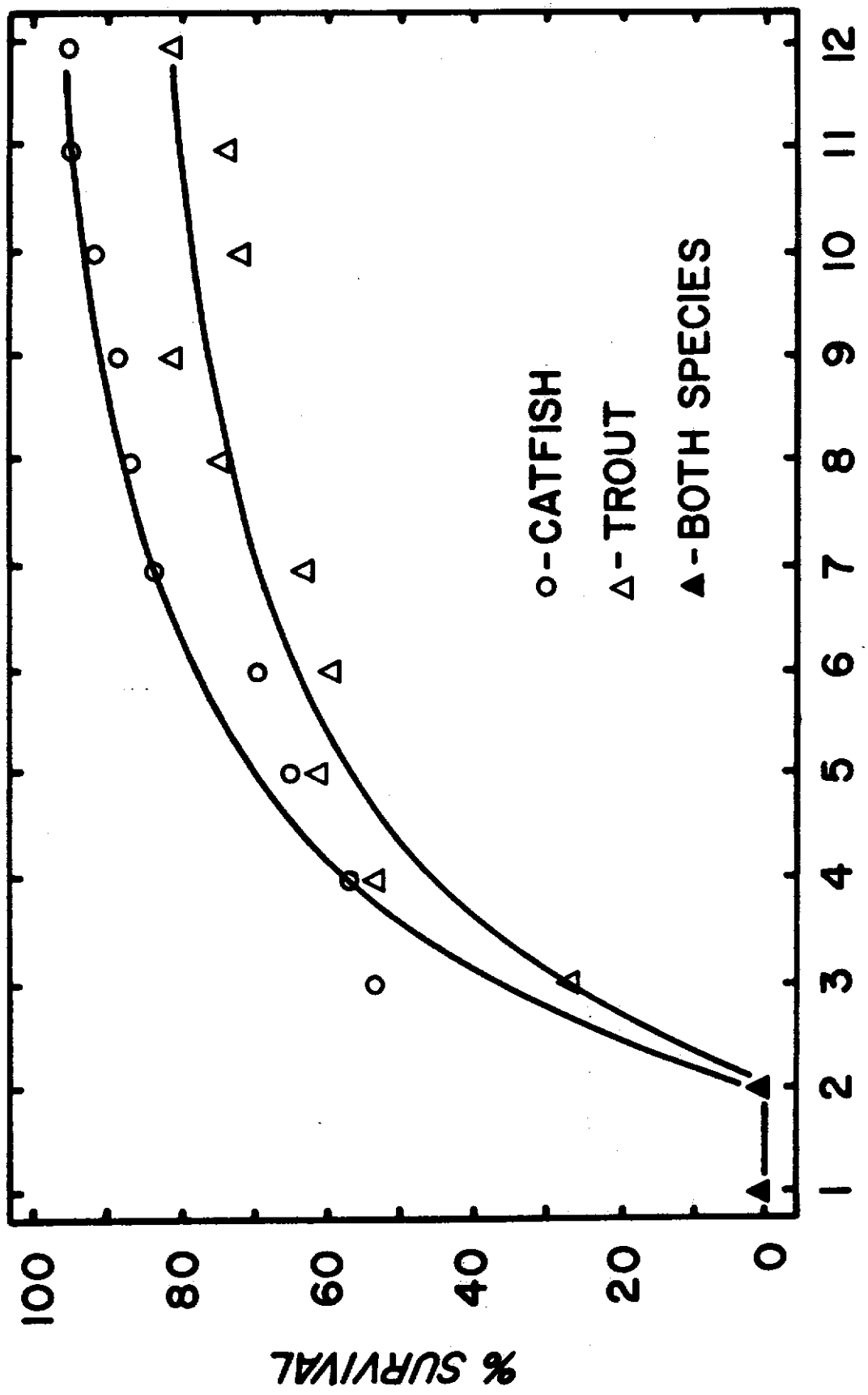

FIGURE 2. EFFECTS OF WATER QUALITY ON SURVIVAL OF FISH EMBRYOS

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