AN ABSTRACT OF THE THESIS OF

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Rainbow trout (Salmo gairdneri) and bluegill (Lepomis macrochirus) were exposed to flows of water containing low DO concentrations produced by bubbling nitrogen through the water. Abrupt exposure of fish to critical DO concentrations (The standard dose response) and by a more gradual changing of DO levels (The "step-down" approach) were employed to investigate fish reactions. The step-down test was explored as an alternative or supplement to the standard dose response.

Rainbow trout held at 15°C and bluegill at 22°C during acclimation and test periods exhibited lower LC-50s and longer median resistance times than unacclimated fish.

Tests were conducted in which some fish had access to an air/water interface, some had access to a nitrogen/water interface, and some were blocked away from the surface by screens. Both species were shown to benefit from access to an air/water interface.

Hematocrit values for rainbow trout showed that acclimated and unacclimated test fish produced larger numbers of red blood cells when DO concentrations were reduced. This was not the case for bluegills.

THE EFFECTS OF ACCLIMATION AND SURFACE ACCESS ON THE RESISTANCE OF FISH TO HYPOXIC STRESS

by

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DEDICATION

This work is dedicated to my late father, Mr. Goenawan Wirosoebroto, my mother Mrs. Poedjiwati Wirosoebroto, my husband Hartadi, my daughter Harum, and my sons Setiawan and Corvandy.

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THE EFFECTS OF ACCLIMATION AND SURFACE ACCESS ON THE RESISTANCE OF FISH TO HYPOXIC STRESS

INTRODUCTION

A large body of information exists concerning the ability of fish to survive at reduced levels of dissolved oxygen (DO). Unfortunately, findings have tended to be highly variable (Doudoroff and Shumway 1970). Much of this uncertainty probably can be ascribed to flaws or variations in experimental design. The prevalence of oxygen depletion tests in which exposure level continuously changed is one example (e.g. Burdick et al. 1954, Cooper 1960). Another is the lack of consideration given to the influence of acclimation. Shepard (1955) showed, however, that brook trout (Salvelinus fontinalis) acclimated to low DO could tolerate lower DO levels than those not so acclimated. He found the rate of acclimation to be roughly 20-33 hours per mg/L change in DO. Prosser (1957) demonstrated that goldfish (Carassius auratus) likewise benefited from acclimation. Moss and Scott (1961) reported increased tolerance to low DO for acclimated bluegill (Lepomis macrochirus), largemouth bass (Micropterus salmoides) and channel catfish (Ictalurus punctatus). Although the overall degree of advantage conferred by acclimation is not yet clear, it at least should help fish cope with gradual reductions in DO (Doudoroff and Warren, 1965, Davis 1975). Research is warranted to quantify the phenomenon.

It has long been recognized that fish not adapted for atmospheric respiration can benefit from access to the surface during periods of

hypoxia (Baker 1941, Lewis 1970). Burggren (1982) found that goldfish benefited when allowed access to the surface. Of 26 species of North American Great Plains fish studied by Gee et al. (1978), 22 benefited from surface access. Kramer and McClure (1982) found that 29 of 31 tropical freshwater fishes responded to hypoxic stress by engaging in aquatic surface respiration (ASR), and that it enhanced their tolerance. Weber and Kramer (1983) demonstrated the positive influence of surface access on the growth and survival of guppies (Poecilia reticulata) under hypoxic conditions.

Unfortunately, ASR has seldom been systematically accounted for in research on which DO criteria are based. Criteria for species subject to periodic confinement under ice should be based on testing both with and without access to the surface. Oxygen criteria for open water species should not depend significantly upon closed-system tests unless it is shown that the benefits of surface access are so insignificant as to be of little regulatory relevance.

The objectives of the present research were to evaluate the effects of acclimation and surface access on the ability of rainbow trout (Salmo gairdneri) and bluegill to withstand hypoxic stress. Resistance was tested by abrupt exposure of fish to critical DO conditions - the traditional dose response method, and by a more gradual means - the proposed "step-down" method. The latter approach, in which fish were sequentially exposed to increasingly hypoxic conditions, is evaluated as an alternative to the dose-response method. In the surface access test, the tolerance of fish with access to the surface was compared to that of fish to which such access was denied.

MATERIALS AND METHODS

Facilities

Laboratory facilities utilized in this work were located at Western Fish Toxicology Station of the U.S. Environmental Protection Agency's Corvallis Environmental Research Laboratory in Corvallis, Oregon. All tests were of the flow-through variety, using water from a well at the Station. Well water was brought to the desired temperature in a 757 L thermostatically controlled temperature regulating tank and then pumped to headbox #1 (Figure 1). Water in this headbox usually contained less than 4.0 mg/L DO and could be simultaneously directed to the stripping column, the acclimation headbox, and the control headbox.

The stripping apparatus consisted of one glass and three PVC 6 cm ID by 71 cm long vertical columns connected in tandem by PVC fittings. Water from headbox #1 flowed in one end of the assembly, down through the first column, up through a connecting pipe segment, down through the second column, and so on until it exited the fourth column and was routed to the exposure gear. At the base of each column, nitrogen gas was injected at a pressure of 0.84 kg/sq cm through a compressed diatomite airstone, resulting in a counter-current exposure of nitrogen bubbles to the water. The nitrogen escaped through a short standpipe at the top of each column.

Four short columns were used instead of one long column to minimize the possibility of creating excessive nitrogen super-saturation, which might have led to gas bubble disease in the

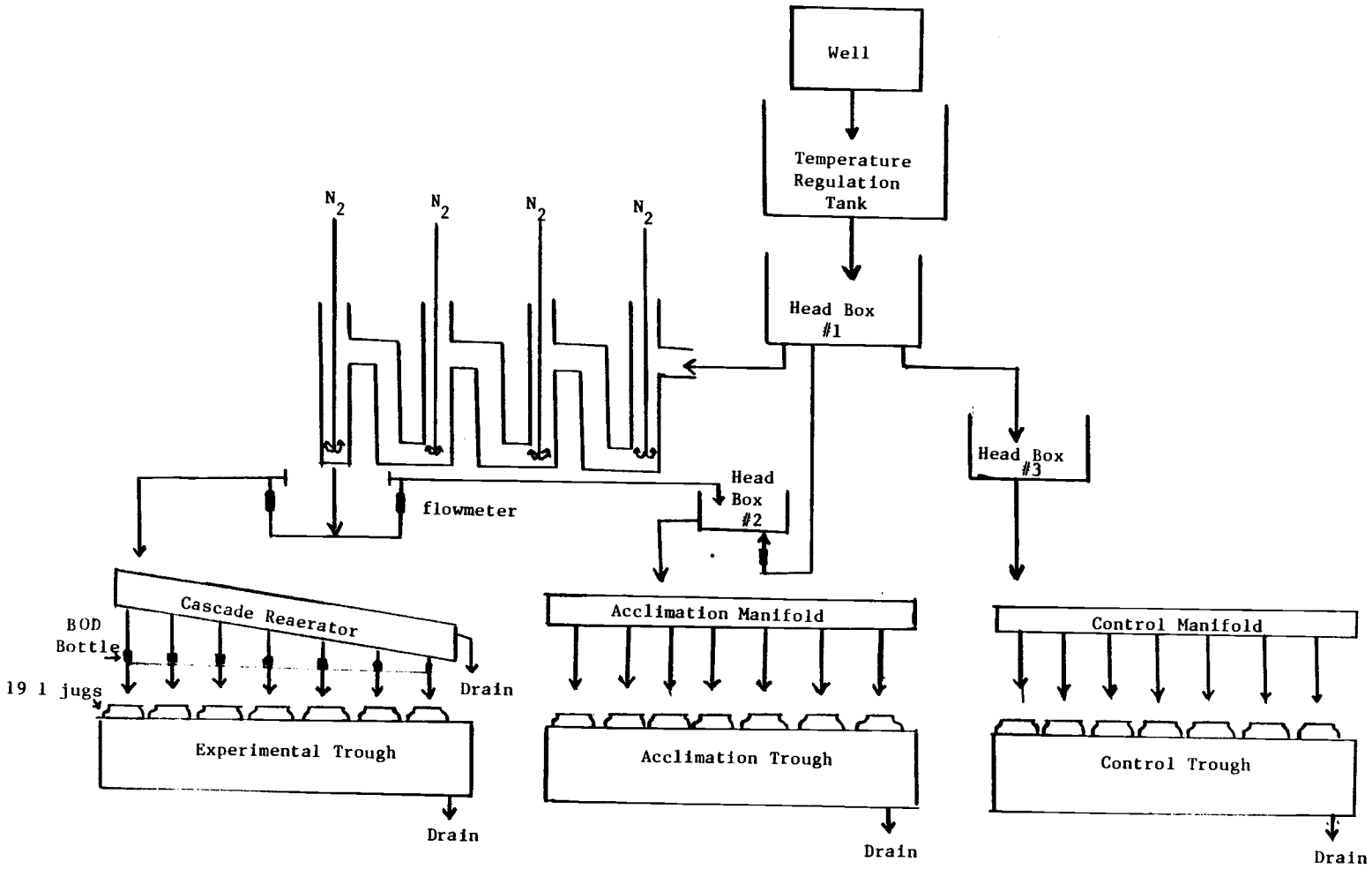

Figure 1. The schematic drawing of the experimental apparatus.

fish. Hereafter, the device will be referred to simply as "the column". The column proved capable of stripping DO down to as little as 0.10 mg/L, depending on nitrogen flow rate. A gas flow meter and valve in the main line was used to make fine adjustment of the nitrogen flow rate to the airstones. Coarse adjustments were made by manipulating clamps on the feed lines to individual airstones. The nitrogen gas originated as evaporate from a 160 L flask of liquid nitrogen.

All DO acclimation and testing of fish were carried out in an array of 19 L wide-mouth jugs confined in linear groups of seven each within three 214 cm long by 33 cm wide by 34 cm deep stainless steel troughs. Water was introduced at the bottom of each jug through 3.2 mm ID flexible PVC tubing, and allowed to overflow and spill into the trough. Each trough was equipped with a drain. A 12 cm long segment of 11.4 cm OD PVC pipe, which will be referred to as a collar, was fit into the neck of each jug. Slots cut into the ends of the collars allowed water to exit the jugs at lip level, but blocked the escape of fish.

Water to be used for DO acclimation was routed by gravity from headbox #1 to headbox #2. There, DO could be raised by sparging with compressed air or lowered by adding water from the stripping column. Valves and flow meters facilitated control and balancing of the two waters. Thorough mixing was promoted by the small size, 5.0 L, of headbox #2. From this headbox, water flowed into a 5.6 cm ID PVC pipe manifold running the length of the first trough. An open standpipe at the distal end of each manifold prevented airlocks. The manifold was

perforated to accomodate a stopper opposite each jug in the trough. Water was tapped off through glass tubes inserted through the stoppers, to which the flexible delivery tubes were attached. Each glass tube was bent into a form that permitted adjustment of the hydraulic head existing at the discharge point in its associated jug. In this way, and by manipulating the lengths of the delivery lines, rates of flow to the jugs were controlled and standardized. Flows to jugs in the other troughs were adjusted in the same manner.

The rate of water flow into a jug was measured by immersing the delivery tube to the bottom of a brim-full 2000 ml graduated cylinder sitting in a shallow pan. The water level in the cylinder was at the same height as that in the jug, so the flow rate was unchanged. After permitting 60 seconds of flow within the cylinder, the volume of over-flow collected in the pass was measured. A flow rate of 220 ml/minute was maintained for all jugs. This flow rate was well in excess of the minimum recommended (EPA 1975) for the fish loading densities used in the tests.

Water for dose-response and stepdown tests passed from the stripping column through a valve and flowmeter to the top chamber of a cascade aerator or "ladder". The ladder consisted of a 16.5 cm wide by 15.2 cm deep by 121.9 cm long topless plexiglass box divided by eleven 10.2 cm high plexiglass baffles into 12 equal chambers. It was operated in an inclined position, with the inflow end (chamber #1) being elevated. The angle of inclination used varied from two to four degrees. Each chamber down the length of the ladder provided water of slightly higher oxygen content. Higher angles of elevation produced

greater differentials between chambers by increasing turbulence within the structure.

Water from the ladder passed through intubated stoppers in the walls of the chambers, through 3.2 mm ID flexible PVC tubing to 300 ml BOD bottles fitted with doubly intubated rubber stoppers, through the bottles, and finally through more flexible tubing to the seven jugs of trough #2. The BOD bottles were placed in line to provide a way to collect water samples from each chamber without influencing turbulence, and hence aeration rates, in downstream chambers.

A third headbox received water directly from headbox #1 and distributed it to jugs in a third trough via a manifold identical to the acclimation manifold. These jugs were sparged with compressed air and served as normoxic controls for the dose-response and stepdown tests.

The surface access tests required six jugs with identical DO levels. To accomplish this water was routed from the stripping column to headbox #3 and distributed through its associated manifold to jugs in the third trough. Normoxic controls for these tests were situated in jugs of trough #2, and received water from headbox #1 via the ladder. The sparging within these jugs compensated for ladder-induced differences in the oxygen levels of their inflows.

For 12 hours each day, the exposure apparatus was illuminated by two pairs of 1.25 m long 40 watt cool white fluorescent lamps augemented by two 100 watt tungsten lamps. Daylight and dark periods were bracketed by half hour "dawn" and "dusk" periods in which the fluorescent lamps were off and the tungsten lamps were automatically

adjusted in brightness by a timer-controlled rheostat. The remaining 11 hours of the cycle were dark. The entire daily cycle was controlled by timers.

Experimental Animals

Tests were performed with rainbow trout and bluegill. These species were selected as being typical of physostomic salmonids preferring cold well-oxygenated water, and physoclistic centrarchids tolerant of warmer, more hypoxic conditions, respectively (Basu 1959, Bond 1979, Carlander 1977).

The trout came from stocks reared at the Western Fish Toxicology Station (WFTS) of the U.S. Environmental Protection Agency's Corvallis, Environmental Research Laboratory in Corvallis, Oregon. They were originally obtained as green eggs from the ODFW Willamette Hatchery in March 1984. Prior to being transfered to acclimation tanks for the experiments, the stocks were kept in indoor 5,000 L tanks at a temperature of 11°C and a DO of approximately 9.5 mg/L. Test fish were fed daily with Oregon Moist Pellet (OMP) at a rate of 2% of live weight per day. Trout used in the experiments averaged 7.6 g (SD 2.18) in weight, and 8.3 cm (SD 0.82) in length.

The bluegill were netted from Oregon Department of Fish and Wildlife ponds near St. Paul, Oregon in March, 1985. Upon arrival at WFTS, they were placed in outdoor 757 L covered tanks at 10°C. Less than 2% mortality was induced by the handling. Fish were maintained in tanks for three weeks prior to use. OMP was offered daily on an ad libitum basis, but it was consumed only by fish being held at higher

temperatures during acclimation. Bluegill used in the experiments averaged 3.4 g (SD 0.99) in weight and 5.1 cm (SD 0.99) in length. Neither species was fed during the exposure phases of tests, or for 24 hours in advance of being handled by net.

Temperature Acclimation

For temperature acclimation, fish were netted from the ambient temperature stock tanks and transferred in buckets of well-oxygenated water to one of two 757 L covered fiberglass tanks equipped with both ambient and thermostatically controlled warm water supplies. Fish were brought from ambient to the target temperature, 15°C for trout and 22°C for bluegill, at a rate of 1°C per day.

The trout were acclimated in groups large enough for a single test, and went on to oxygen acclimation as soon as temperatures reached 15°C. It took 11 days to acclimate the bluegill to 22°C, and time constraints necessitated the acclimation of bluegill in one large group. Test fish were then held at 22°C, for varying lengths of time, until they could be accommodated in the oxygen acclimation apparatus.

Oxygen Acclimation

Fish held at DO concentrations of 4.0 to 4.5 mg/L previous to testing were termed acclimated. Test fish held at DO concentrations greater than 5.86 mg/L were termed unacclimated fish. After completion of temperature acclimation, fish were netted into a bucket of water for transfer to jugs of the oxygen acclimation trough. Ten

fish were put in each jug by stratified random distribution in groups of two. Fish serving as normoxic controls were also transferred at this time, to jugs in trough #3, and aeration was started.

The initial DO in the acclimation jugs was no more than 1.0 mg/L lower than that of the water from which the fish had just been transferred. It was further lowered, when necessary to reach the target DO, by 1.0 mg/L/day (which approximates acclimation rates observed in brook trout by Shepard 1955). These adjustments were made each morning by first adjusting the DO in headbox #2 to the desired level, and then siphoning 75% of the water from each of the jugs. As the jugs refilled, none of the new lower DO water was lost to overflow, and the desired DO level was reached more quickly. In practice, it took about an hour for the jugs to refill. Except as noted, this siphon method was employed whenever the DO in jugs was adjusted.

The target DO was always reached within 5 days and was usually maintained until a total of 7 days elapsed after introduction of the fish to the jugs. On the morning of day 8, the jugs (excepting any normoxic controls) were siphoned as before and transferred to the test trough.

The sole exception to this format involved the fish used for the unacclimated bluegill dose response test. Because of time constraints, it was necessary to move these fish directly from the temperature acclimation tank to the test trough. The normal residence time in the oxygen acclimation trough was eliminated.

Standard Dose Response Tests

Twenty-four hours before starting a test, the stripping column and ladder were activated, adjusted to roughly the desired DO output, and left to stabilize overnight. The next morning, the system was adjusted to produce the desired array of concentrations in the ladder. Then the jugs were siphoned, transferred to the test trough, and supplied with water from various chambers of the ladder. Six concentrations were chosen to produce effects ranging from 0 to 100% mortality. For tests involving acclimated fish, one jug was maintained at 4.00 mg/L to verify that the acclimation process itself and extended confinement in the jugs were not unduly stressful. In addition, for each test a normoxic control was run in the control trough to verify the overall health of the fish used in the test. Test were terminated after 72 hours.

Step-down Tests

Four step-down tests, 2 with acclimated fish and 2 with unacclimated fish, were carried out for each species. Each test included a 4.0 mg/L control and an aerated normoxic control, and the same conformation of exposure apparatus as in the dose response test.

On the first day of a test, 75% of the water was siphoned from a single jug containing 10 fish acclimated to DO 4.0 mg/L, and the jug was moved from the acclimation trough to the test trough. There it was supplied with water from the ladder having a DO of about 3.5 mg/L. Twenty-four hours later, this jug was again siphoned and then supplied

with water having a DO of about 3.0 mg/L. At this time a jug of fish acclimated to about 7.0 mg/L (which had been moved to the acclimation trough for aerated acclimation a day later than the previous jug) was siphoned and moved to the 3.5 mg/L position in the test trough. Beginning 48 hours later, a second, analogous, pair of jugs followed into the test trough in the same manner. Every 24 hours the jugs were siphoned and brought to successively lower DO levels until all fish had died. Increments between the "steps" were approximately 0.5 mg/L down to an exposure level of 2.0 mg/L, and were about 0.2 mg/L thereafter.

The entire procedure was then repeated for the second species.

Surface Access Tests

The surface access tests required the previously described reconfiguration of the exposure gear to deliver water of the desired DO to the manifold of headbox #3. Water normally routed to headbox #3 for normoxic controls was instead delivered via the ladder to jugs in trough #2. Once the system was adjusted and stable, 6 jugs of fish which had been acclimated to a DO of about 4.0 mg/L were siphoned and transferred to trough #3. They were then exposed to a DO level approximately equal to their previously determined 72 hour LC-50.

In two of the jugs, fish were denied access to the surface by means of screens secured just below the necks of the jugs. Each barrier consisted of a 17.1 cm diameter piece of 2 mm by 3 mm mesh plastic screen which had been cemented to a split ring of rigid PVC plastic. The assembly was folded in half, inserted through a jug's

neck, and re-opened screen-side-up within the jug. Three monofilament nylon lines secured to the screen at one end and to a rubber band at the other were drawn back through the jug's neck and PVC collar. When the rubber band was slipped over a pencil resting across the top of the collar, the screen assembly was pulled up against the shoulder of the jug approximately 10 cm below the water surface. The 1.5 cm thickness of the screen's support ring prevented it from folding back upon itself. A small hole in the screen permitted insertion of the sampling siphon and the thermometer. Previous experience with the materials used in this arrangement had shown them to be non-toxic to fish.

In the remaining 4 jugs, fish had access to the surface. However, in two of them the surface was overlain with nitrogen gas delivered continuously through a hole in the collar. In the latter case, the collars were closed at the top by plastic film secured with a rubber band. The nitrogen gas exited through the water overflow slots.

All surface access tests were 48 hours in duration.

Monitoring

Oxygen levels throughout the exposure gear were determined by modified Winkler analysis (EPA 1979) of water samples collected in 300 ml BOD bottles in one of two ways. Bottles inserted "in line" between the ladder and the jugs of trough #2 were, except for a brief period just after being exchanged, continuously full and undergoing turnover of their contents. Analysis of these samples provided information on individual ladder chambers. All other samples were collected by

siphoning water to the bottom of 300 ml BOD bottles and allowing the bottles to overflow approximately one volume after filling. Siphon tubes were then withdrawn while still discharging and the bottles were stoppered. Chemical analysis was usually completed within 20 minutes.

Occasional fluctuations in the DO of the well water were capable of causing corresponding changes throughout the exposure gear in the absence of compensatory adjustments. To detect these changes, a YSI model 57 DO meter equipped with a recorder was used to monitor the column output frequently each day, and to record it continuously day and night. The meter was calibrated each morning against a winkler sample siphoned from the vicinity of its sensor in the top chamber of the ladder.

DO exposure levels reported for fish are based on water samples siphoned from the vessels holding the fish. During oxygen acclimation, DO was generally checked twice per day. In the rainbow trout dose response tests, jugs were sampled four to five times in the first 24 hours, and two to three times per day thereafter. In the other tests, test jugs were sampled every 15 to 60 minutes while fish were showing signs of stress. At other times, jugs were sampled at least four times per day, and usually no less than every hour. DO levels in control jugs were measured daily, since they tended to be more stable and never approached critical values. Frequent monitoring of the headboxes and ladder provided valuable additional information on system performance. Night sampling was generally avoided as being a possible source of extraneous stress.

Water temperature in headbox #2 was continuously recorded by a thermograph. Fluctuations at this point were reflected throughout the system, so the instrument's reading was observed frequently each day. The thermograph was calibrated daily against a laboratory thermometer graduated to 0.2°C, which had in turn been calibrated against a National Bureau of Standards certified reference thermomemeter. The laboratory thermometer was used to make all the temperature readings reported for vessels containing fish. During acclimation, the temperature of each jug was generally checked twice each day. Jugs were usually checked twice per day during the rainbow dose response tests, and hourly during the day in the bluegill tests.

Total hardness, pH, and total alkalinity were measured at least once, and usually twice, during each test. These factors tended to be fairly stable and to fall well within acceptable limits with respect to the fish. In each case, samples were siphoned from one or more jugs containing fish and were promptly analyzed. Total hardness was measured by titration with EDTA (APHA 1980). An Orion model 701A pH meter equipped with a Ross-type combination electrode was used to measure pH, and for potentiometric titration of total alkalinity (APHA 1980).

Fish were considered dead upon cessation of opercular movements. During periods in which fish were showing signs of hypoxic stress, mortality was checked at least every hour, and usually no less than every 30 minutes. To avoid the creation of additional stress, observations were seldom made at night. Dead fish were left in place in an effort to maintain a stable BOD throughout each test.

Equipment capable of measuring light intensity within the jugs was not available. A weston Model 756 illumination meter was used to measure light intensity at the shoulder of each jug, yielding values from 23 to 69 foot-candles. However, the complexity of shadow patterns cast by the gear make it doubtful that these readings are particularly respresentative of conditions within the jugs. Also, because the walls of the laboratory were translucent, the amount of variation between jugs undoubtedly changed somewhat as the intensity of sunlight changed.

At the conclusion of each test, 10 fish from a jug in which no mortality had ocurred were sized. Because fish had been assigned to jugs by stratified random distribution, it was assumed that each group of 10 fish would be representative of the other groups in the same test.

After being anaesthetized with MS-222, each fish was blotted, measured to standard length, placed individually in a tared aluminum dish, and weighed with the dish to the nearest mg on a Mettler Model PC180 electronic balance. Total weight minus the tare was taken as the wet weight of the fish.

The fish-containing dishes were then placed in a static-air drying oven set at 80°C. They were re-weighed in 72 hours, and every 24 hours thereafter until all weights were stable within ± 5 mg. Before each weighing, dishes were cooled to ambient temperature in a dessicator. The new total weight minus the tare was taken as the dry weight of the fish.

Hematocrit levels were measured for stressed and unstressed fish at the conclusion of each test in which appropriate survivors were available. After a fish was anaesthetized with MS-222 and thoroughly blotted, a sharp knife was used to slice off the tail at the posterior base of the adipose fin. A 25 mm column of blood was then drawn into a heparinized microcapillary tube touched to the wound, and the tube was sealed with Critoseal compound. After centrifuging the tube for 4 minutes at 11,500 RPM, the percent of contents represented by packed solids was determined using an IEC Model CR microcapillary reader.

RESULTS

Standard Dose Response Tests for Rainbow Trout

The Spearman-Karber method (Hamilton et al. 1977, 1978) was used to calculate the 72 hr LC-50s in the standard dose response tests (Table 1). The standard error of the difference method (Sprague and Fogels 1976) was used to statistically compare LC-50s.

Rainbow trout acclimated to 4.2 mg/L DO had LC-50 values of 1.47 and 1.61 mg/L, respectively. Those acclimated to 5.86 and 6.24 mg/L DO had higher LC-50 values of 1.86 and 1.80 mg/L, respectively. The differences between the LC-50s of fish acclimated to low and high DO levels (0.19-0.39 mg/L) were statistically significant ($P \leq 0.05$). The difference between the LC-50s of the two low-acclimated groups (0.14 mg/L), while smaller than the inter-treatment difference, was also significant. The LC-50s of the high-acclimated fish were not significantly different from each other.

The tests give an indication of the general thresholds of low DOs that will result in a total mortality or total survival for rainbow trout. The thresholds were calculated as the geometric mean of the mean DO concentrations bracketing the effect of interest. For fish acclimated at about 4.2 mg/L DO the DO threshold causing 100% mortality appeared to be between 1.40 and 1.50 mg/L DO. The total mortality threshold for fish maintained at 5.86 or 6.24 mg/L DO before testing was in the range of 1.64 to 1.69 mg/L.

Table 1. Results of the standard dose-response DO test for acclimated and unacclimated rainbow trout.

Test No	DO acclimation (mg/L)	N	Mortality (%)	Median resistance times (minutes)		DO ± SD (mg/L)	LC-50 (mg/L)
				Computed	Observed		
1	4.24	10	100	139	109	1.16 ± 0.05	1.47
		10	100	189	172	1.31 ± 0.08	(1.44-1.51)*
		10	60	1033	1002	1.50 ± 0.10	
		10	0		-	1.54 ± 0.08	
2	5.86	10	100	76	76	1.23 ± 0.14	1.86
		10	100	102	104	1.42 ± 0.13	(1.78-1.94)*
		10	100	154	128	1.60 ± 0.13	
		10	70	223	137	1.79 ± 0.21	
		10	20		-	1.95 ± 0.22	
		10	0		-	2.28 ± 0.19	
3	4.22	10	100	136	118	1.26 ± 0.08	1.61
		10	100	257	213	1.34 ± 0.18	(1.55-1.67)*
		10	60	2248	2825	1.68 ± 0.11	
		10	0		-	1.69 ± 0.09	
4	6.24	10	100	54	54	1.35 ± 0.07	1.80
		10	100	63	65	1.53 ± 0.13	(1.72-1.89)*
		10	100	100	121	1.64 ± 0.13	
		10	60	223	187	1.65 ± 0.06	
		10	40		-	1.88 ± 0.18	
		10	0		-	2.16 ± 0.22	

* 95% confidence limits

Calculated thresholds of total survival in the standard dose response test differ for fish held at low and high DO concentrations prior to testing. At acclimations of about 4.2 mg/L DO the thresholds of total survival were 1.68 and 1.53 mg/L DO, respectively. Higher total survival thresholds of 2.11 and 2.02 mg/L were calculated for fish held at pretest DO concentrations of 5.86 and 6.24 mg/L. Nearly all low-acclimated fish survived at concentrations that were lethal to 100% of the high-acclimated fish.

Times to death were observed and recorded during the tests. The elapsed time of exposure until 50% mortality at each concentration was recorded as the median resistance time (MRT). The MRT values were also estimated from the individual times to death at each DO using the Spearman-Karber method, inputting time instead of concentration.

Rainbow trout in tests 1 and 3 that had been acclimated to about 4.2 mg/L DO showed longer MRTs than the fish held at 5.86 or 6.24 mg/L DO when tested at similar DO concentrations. The MRT values were not easily compared but were always greater for acclimated fish at comparable DO levels. Differences exceeded an order of magnitude when the LC-50s were approached. For example, the MRTs between 1.50 and 1.68 mg/L ranged from 1,002 to 2,825 minutes for acclimated fish and comparable values ranged from 65 to 187 minutes for unacclimated fish.

Step-Down Tests for Rainbow Trout

Two series of step-down tests utilizing acclimated and unacclimated rainbow trout were performed to compare results with the standard dose response tests. Because the mortality within each test

was spread over several exposure levels, it was possible to calculate LC-50s. It was assumed that the sensitive fish died first (ie. at higher DO concentrations) and that the more resistant fish died last (at lower DO concentrations). Thus, as a test progressed, mortality was treated cumulatively. For example, the 3 fish dying at 1.36 mg/L plus the 1 fish dying previously at 1.41 mg/L (Test 1 Table 2) were assumed to be equivalent to 4 deaths at 1.36 mg/L. The rainbow trout acclimated to 4.0 mg/L had LC-50s of 1.29 and 1.33 mg/L while those acclimated 7.0 mg/L yielded slightly higher LC-50s of 1.42 and 1.37 mg/L. The LC-50s for unacclimated fish were only slightly higher than for acclimated fish. The differences between LC-50s were significant only between test 1 and test 2.

The DO thresholds of total mortality were calculated at 1.14 and 1.21 mg/L for acclimated fish, and 1.24 and 1.28 mg/L for unacclimated fish. The thresholds for total survival were calculated at 1.57 and 1.58 mg/L for acclimated fish and 1.66 and 1.65 mg/L for unacclimated fish.

Surface Access Tests for Rainbow Trout

These trials with replicated bioassays were performed to compare the percentage of mortalities that resulted from blocking rainbow trout from the surface or atmosphere at low DO concentrations. Of secondary importance were comparisons of MRTs between the following three trials: with access to the surface (WA), screened from the surface (SB), and blocked from the surface by a layer of nitrogen

Table 2. Results of step-down DO tests for rainbow trout.

Test No.	DO acclimation (mg/L)	N	Cumulative mortality	DO (mg/L)	Elapsed time within a concentration (minutes)	LC-50 (mg/L)
1	4.02	10	0	2.25	1440	1.29
		10	0	1.95	1440	(1.23-1.35)*
		10	0	1.74	1440	
		10	1	1.42	87	
		9	2	1.36	192	
		8	3	1.36	210	
		7	4	1.36	305	
		6	5	1.18	195	
		5	6	1.18	200	
		4	7	1.18	205	
		3	8	1.11	90	
		2	9	1.11	240	
		1	10	1.11	360	
2	7.08	10	0	2.31	1440	1.42
		10	0	1.98	1440	(1.34-1.50)*
		10	0	1.81	1440	
		10	1	1.53	120	
		9	2	1.53	150	
		8	3	1.53	270	
		7	5	1.33	1205	
		5	9	1.26	875	
		1	10	1.22	1240	
3	4.08	10	0	2.85	1440	1.33
		10	0	2.73	1440	(1.25-1.40)*
		10	0	2.46	1440	
		10	0	2.07	1440	
		10	0	1.75	1440	
		10	1	1.42	225	
		9	2	1.42	255	
		8	3	1.42	1255	
		7	4	1.30	490	
		6	5	1.13	30	
		5	6	1.13	60	
		4	7	1.13	87	
		3	8	1.13	152	
		2	9	1.13	183	
		1	10	1.13	525	
4	7.02	10	0	3.35	1440	1.37
		10	0	3.07	1440	(1.30-1.44)*
		10	0	2.66	1440	
		10	0	2.14	1440	
		10	0	1.77	1440	
		10	1	1.53	1282	
		9	2	1.53	1282	
		8	3	1.53	1282	
		7	4	1.32	1270	
		6	5	1.22	40	
		5	6	1.22	120	
		4	7	1.22	150	
		3	8	1.22	180	
		2	9	1.22	270	
		1	10	1.22	670	

* 95% confidence limits

(NB). MRTs were calculated for tests with more than 50% mortality by using the Spearman-Karber method (Table 3).

In the first test mean DO concentrations of 1.29 and 1.33 mg/L were maintained in the NB exposures. Total mortality occurred, as might have been expected from the results of the dose response tests. The remaining NB trials had mean DO concentrations of 1.52 to 1.64 mg/L and had mortality ranging from 80 to 90%.

A pattern of decreased mortality was evident for rainbow trout held at similar DO concentrations but with access to the surface (Table 3). Test fish in SB groups had as much as 20% higher mortality and NB groups had up to 50% more mortality than comparably exposed WA groups.

The computed and observed MRTs occupied a relatively narrow range of values. Except for the large MRTs in test $1A_1$ and $1A_2$, the other values tend to overlap and are probably not meaningful (Table 3).

Standard Dose Response Test for Bluegill

Experimental efforts with bluegill were directed toward evaluating the standard dose response test in relation to step-down tests.

One experiment was used to determine an LC-50 of 0.81 mg/L (0.73-0.90 mg/L) for fish maintained at DO of 7.62 mg/L prior to testing (Table 4). Estimates of threshold DO concentrations causing total mortality or total survival of bluegill were 0.61 mg/L and 1.18 mg/L respectively.

The MRTs increased rapidly as DO concentrations increased slightly from the total mortality caused by a DO of 0.53 mg/L. Increasing the

Table 3. Results of surface access DO tests for rainbow trout.

Test No.	Treatment	N	Mortality (%)	DO ± SD (mg/L)	Median resistance times (minutes)	
					Computed	Observed
$1A_1$	WA	10	50	1.53 ± 0.21	-	1780
$1A_2$	WA	10	50	1.53 ± 0.16	-	1765
$1B_1$	SB	10	70	1.53 ± 0.27	155	123
$1B_2$	SB	10	70	1.54 ± 0.24	127	123
$1C_1$	NB	10	100	1.29 ± 0.25	92	98
$1C_2$	NB	10	100	1.33 ± 0.19	68	64
$2A_1$	WA	10	50	1.59 ± 0.12	-	188
$2A_2$	WA	10	60	1.61 ± 0.13	295	215
$2B_1$	SB	10	60	1.59 ± 0.16	368	393
$2B_2$	SB	10	70	1.62 ± 0.15	156	103
$2C_1$	NB	10	90	1.52 ± 0.19	82	83
$2C_2$	NB	10	-	-	-	-
$3A_1$	WA	10	30	1.62 ± 0.09	-	-
$3A_2$	WA	10	30	1.63 ± 0.10	-	-
$3B_1$	SB	10	50	1.67 ± 0.14	-	220
$3B_2$	SB	10	50	1.67 ± 0.12	-	211
$3C_1$	NB	10	80	1.61 ± 0.16	112	105
$3C_2$	NB	10	80	1.64 ± 0.20	213	268

WA = Fish with access to the surface
SB = Fish blocked from the surface by a screen
NB = Fish blocked from the surface by a layer of nitrogen

Table 4. Results of standard dose response DO test for bluegill.

DO acclimation (mg/L)	N	Mortality (%)	DO ± SD (mg/L)	Median resistance times (minutes)		LC-50 (mg/L)
				Computed*	Observed	
7.62	10	0	1.28 ± 0.18	-	-	0.81 (0.73-0.90)**
	10	30	1.09 ± 0.10	-	-	
	10	60	0.87 ± 0.08	826	820	
	10	90	0.70 ± 0.10	468	358	
	10	100	0.53 ± 0.03	40	42	

* Spearman-Karber method was used to compute MRT
** 95% confidence limits

DO from 0.53 to 0.70 mg/L increased the observed MRT from 42 to 358 minutes. At a DO of 0.87 mg/L the observed MRT increased to 820 minutes.

Step-down Tests for Bluegill

The step-down tests were conducted with bluegill acclimated to about 4.0 mg/L DO and unacclimated bluegill held at DO concentrations of about 7.0 mg/L. There were no apparent differences between any groups of test fish. Both acclimated and unacclimated bluegills survived at concentrations of about 0.5 mg/L and suffered complete mortality at about 0.35 mg/L (Table 5).

Surface Access Tests for Bluegill

The bluegill were tested to determine the effects of not reaching the atmosphere at low DO concentrations. The DO concentrations were held slightly higher than DOs causing a total mortality in the standard dose response tests. Three replicated trials were used in two tests (Table 6). The trials consisted of allowing bluegills access to the surface (WA), blocking fish from the atmosphere by a screen (SB) or preventing normal surface access by placing a layer of nitrogen over the test jug (NB).

Mortality of fish screened from the surface (SB) or in treatments with a nitrogen atmosphere (NB) was 100% in all tests. Fish in treatments with access to an air/water interface suffered from 40 to 90% mortality and had longer MRTs than fish in the other treatments. MRTs for fish in treatments having a nitrogen atmosphere were slightly

Table 5. Results of step-down DO tests for bluegill.

Test No.	DO Acclimation (mg/L)	N	Mortality (%)	DO ± SD (mg/L)
1	4.05	10	0	0.48 ± 0.04
		10	100	0.31 ± 0.02
2	7.05	10	0	0.54 ± 0.02
		10	100	0.38 ± 0.01
3	3.99	10	0	0.55 ± 0.03
		10	100	0.35 ± 0.02
4	7.10	10	0	0.55 ± 0.02
		10	100	0.37 ± 0.01

Table 6. Results of surface access DO tests for bluegill.

Test No.	Treatment	N	Mortality (%)	DO ± SD (mg/L)	Median resistance times (minutes)	
					Computed	Observed
$1A_1$	WA	10	90	0.62 ± 0.04	323	290
$1A_2$	WA	10	80	0.63 ± 0.03	351	290
$1B_1$	SB	10	100	0.62 ± 0.06	122	105
$1B_2$	SB	10	100	0.63 ± 0.06	101	100
$1C_1$	NB	10	100	0.61 ± 0.02	81	75
$1C_2$	NB	10	100	0.63 ± 0.04	83	90
$2A_1$	WA	10	40	0.72 ± 0.03	-	-
$2A_2$	WA	10	40	0.73 ± 0.03	-	-
$2B_1$	SB	10	100	0.67 ± 0.04	90	126
$2B_2$	SB	10	100	0.72 ± 0.02	98	85
$2C_1$	NB	10	100	0.68 ± 0.07	81	76
$2C_2$	NB	10	100	0.68 ± 0.04	74	66

WA = Fish with access to the surface
SB = Fish blocked from the surface by a screen
NB = Fish blocked from the surface by a layer of nitrogen

but consistantly lower than those for fish screened from surface access.

Hematocrit Values in the Test Fish

During the dose response tests blood samples were taken from, and hematocrit values determined for, fish held at different dissolved oxygen concentrations (Table 7). A general increase in hematocrit levels was noted in rainbow trout held at low DO concentrations. However, the bluegill showed a narrow range of hematocrit values when held at different DO concentrations. There were no statistically significant differences between hematocrit levels of test and control bluegill.

Table 7. Mean hematocrit values for rainbow trout and bluegill at different DO concentrations.

Species	Test No.	DO acclimation (mg/L)	DO concentration ± SD (mg/L)	Mean hematocrit ± SD
Rainbow Trout	1	4.24	1.50 ± 0.10	47 ± 3.0
			1.54 ± 0.08	48 ± 3.2
			4.15 ± 0.60	46 ± 3.6
			9.27 ± 0.19	41 ± 2.3
	2	5.86	1.79 ± 0.21	50 ± 1.5
			1.95 ± 0.22	48 ± 2.8
			3.70 ± 0.86	48 ± 3.6
			8.54 ± 0.25	46 ± 4.3
	3	4.22	1.68 ± 0.11	46 ± 1.2
			4.31 ± 0.25	45 ± 4.7
			8.44 ± 1.06	42 ± 2.5
	4	6.24	1.65 ± 0.06	50 ± 2.4
			1.88 ± 0.18	51 ± 2.5
			4.05 ± 0.08	48 ± 3.9
			9.42 ± 0.04	45 ± 2.7
Bluegill		7.62	0.87 ± 0.08	34 ± 3.3
			1.09 ± 0.10	36 ± 2.4
			1.28 ± 0.18	33 ± 5.3
			7.20 ± 0.20	34 ± 2.9

DISCUSSION

Comparisons of the LC-50s for DO in the standard dose response tests showed slight but statistically significant increases in tolerance of acclimated rainbow trout. This finding is in agreement with previous studies (Beamish 1964, McLeod and Smith 1966 and Shepard 1955). The differences in the LC-50s might have been larger if the low and high acclimation concentrations had been more divergent (eg. 3 and 9 mg/L). However, fish acclimated to about 4 mg/L were clearly more tolerant of lower DO than fish acclimated to about 6 mg/L, indicating that even small differences in acclimation concentration can be consequential.

The large differences in the MRTs between acclimated and unacclimated rainbow trout are further evidence of the influence of acclimation. Additionally, the DO concentrations for thresholds of total mortality and total survival were significantly lower in acclimated fish and supported the validity of the LC-50 and MRT differences.

The results indicated that the strict application of the standard dose response test to establish DO criteria is limited. For trout in streams with chronically low DO the standard dose response test sets limits about 0.5 mg/L above the point of immediate lethal levels of DO. The rainbow trout living in streams with DO below a desirable level of 5.0 mg/L may be able to live quite well for short periods at even lower DO levels (Doudoroff and Shumway 1970). However, the successful completion of the life cycle of the rainbow trout may require DO concentrations near saturation values. The rates and

possible scope of acclimation to changing conditions can not be taken into account by simple dose response tests. Data reflective of these factors would seem to be more appropriate as a basis for oxygen criteria, especially at sites where hypoxic conditions are known to develop rather gradually.

Research was directed at understanding several aspects of DO bioassay tests rather than establishing a more complete definition of the value of acclimation in a standard dose response test. The step-down modification of the standard dose response procedure is intended to provide bioassay data incorporating more information, and to be more conservative of fish, equipment, and time. It avoids the unnaturally abrupt DO reductions common to routine dose response tests and provides a reasonable opportunity for acclimation. Also fish are more equally challenged than in dose response tests, in which fish are exposed to ranges of DO decreases.

The difference (0.11 mg/L) between the mean LC-50s for acclimated and unacclimated rainbow trout in the step-down tests was small in comparison to the analogous difference (0.29 mg/L) produced by the standard dose response test. Apparently the time allotted between each reduction of DO in the step-down tests was sufficient to allow significant acclimation to occur. However, the most striking differences between the step-down and dose response test results for both fish species concern the threshold of total mortality. Not only were the step-down thresholds considerably lower, but, in the case of the trout, there was much less difference between acclimated and unacclimated fish than in the dose response tests. Moreover,

acclimated rainbow trout tested by the dose response method had thresholds of total survival similar to those in the step-down tests. Thus, it appears that the step-down approach is capable of allowing for at least as much acclimation as was conferred by pre-test acclimation at 4.0 mg/L. In terms of thresholds of total mortality or LC-50s, acclimation occurring within step-down tests seems even more significant.

The step-down LC-50s are not strictly comparable to dose response LC-50s. They are founded upon reactions of fish to a different pattern of stress, and they may need to be interpreted differently. For example, step-down LC-50s may be misleading if applied to situations in which major changes in DO levels occur abruptly. The facts that the step-down LC-50s were lower than their dose response counterparts and were influenced less by pre-test acclimation indicate that step-down data integrate information about acclimation capacity. Unfortunately for such a promising scalar, it cannot be used when all mortality is compressed into one or two steps. Such was the case for all four bluegill tests.

It is clear that, for the trout, pre-test acclimation was less important in the step-down tests than in the dose response tests. The same cannot be said for bluegill, because no acclimated bluegill dose response test was run. For both species, it is evident that acclimation occurred during the step-down tests to a degree not approached during the dose response tests. While the rate of stress build-up in step-down tests may or may not have been realistic, the capacities of the fish to adjust to changing conditions were shown to

influence the results. It would seem that such information-enriched data could be put to good use in managing water quality.

Surface access tests were conducted to demonstrate the importance of considering aquatic surface respiration (ASR) when assessing the DO needs of fish, regardless of whether they are physoclistic or physostomic. In terms of observed and calculated median resistance times, and total mortality, both trout and bluegill did best when they had access to an air/water interface. When oxygen conditions became sufficiently severe, they would invariably move from immobility near the bottom of the jug (where fresh water was injected) to positions near the water surface. There they would push their mouths into the surface film and engage in vigorous gulping motions. It could not be determined with certainty whether gaseous oxygen was pulled over their gills, but fish were never observed to discharge bubbles from beneath their opercula. Likewise, the presence of an oxygen-enriched microlayer of water at the surface was never confirmed. Nevertheless, their behavior could be interpreted as taking advantage of a source of oxygen.

Fish died sooner and in greater numbers when they were screened from the air/water interface. This could have been due to the withholding of a supplementary source of oxygen. However, it could also be explained as being a consequence of the metabolic demands of panic-stricken swimming that ensued when fish were foiled in their attempts to reach the surface.

Fish with access to a nitrogen/water interface fared worst of all. Their swimming and gulping behavior was indistinguishable from that

of fish with access to an air/water interface. Whether the nitrogen resulted in the absence of an oxygen-enriched microlayer, or created an oxygen-depressed microlayer, or blocked direct access to the atmospheric oxygen was not determined. The fact that, in spite of their vigorous swimming, the screened fish did better than the groups under nitrogen suggests that the nitrogen created some condition more hostile than the mere denial of access to supplemental oxygen.

Differences between the results of the three treatments were not always large, but they fell into a pattern which was quite consistent. The few exceptions to the trend were always contradicted by an accompanying replicate which conformed. The implication of these findings is that data on the reactions of fish to hypoxic conditions in open water should be generated in open test vessels. Data to be applied to systems under ice should be generated in closed test vessels.

The hematocrit results for rainbow trout showed that test fish tend to produce higher number of red blood cells when DO concentrations were reduced. It is indication that the acclimation phenomenon needs to be considered before applying the results of standard dose response tests to streams with chronically low DO concentrations.

Hematocrit levels were measured for bluegill held at dose response concentrations as low as 0.87 mg/L, and were found to be similar to those of the 7.0 mg/L controls. It may be that the mechanisms of bluegill acclimation does not involve a quick elaboration of red blood cells. However, too few measurements were made to reach a firm

conclusion. Examination of fish held for longer periods of time at lower concentrations might yield results more in line with those of the trout.

Overall, the results of this study indicate that the step-down test should be considered as an alternative or a supplement to the traditional dose response approach used in bioassays. The step-down tests apparently allowed fish to acclimate and that capacity was systematically accounted for in the test. Certainly the physical nature of the step-down test, incoporating savings of fish, space, time, and supplies, make it more efficient than the standard dose response bioassay. The experiments indicate that a thorough evaluation of the step-down test as a replacement for the standard dose response bioassay is needed.

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