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DETERMINING FISH AVOIDANCE OF POLLUTED WATER*

Stephan R. LARRICK, K. L. DICKSON, D. S. CHERRY & John CAIRNS, JR.

Biology Department and Center for Environmental Studies, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061 USA

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Abstract

A critical evaluation is given of material and methods to be used for the study of behavioral responses of fish towards polluted water. Experiments with Fathead minnows in a steep gradient of hypochlorite are described and discussed. Methods leading towards standarization of fish avoidance reactions are advocated.

Introduction

It is evident that a toxicant may stress fish populations in ways other than acute mortality, but until recently this concept has not been extensively applied. Now substantial attention is being given to sublethal effects which can affect the fertility, growth, susceptibility to disease or predation of organisms (Sprague, 1971). Field studies of fish have shown that toxic solutions may render large portions of an environment undesirable or uninhabitable (Katz & Gaufin, 1952; Sprague *et al.*, 1965; Tsai, 1968, 1970; Cherry *et al.*, 1977a). The behavior of fish that enter these areas is often modified, and any behavior patterns which would allow fish to escape or avoid deleterious conditions and move to more favorable locations within the environment would contribute to their survival and well being. To study such behavior patterns, investigators have employed several laboratory techniques, each designed for a specific application (Scherer, 1977). Presently, prospective investigators must sort through a large amount of information before selecting the technique most appropriate for their research. To facilitate this selection process, this paper will evaluate the merits and shortcomings of behavioral studies and propose certain methods with distinct advantages, leading towards standardization of fish avoidance studies.

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Review of previous avoidance designs

The various experimental troughs used in avoidance studies may be divided into two categories - troughs in which shallow gradients are produced and others with steep gradients(Table 1). The original proponent of fish behavioral studies as related to water quality (Shelford & Allee, 1913) felt that it was impossible to study the reaction of fishes to dissolved gases without the use of shallow concentration gradients. Their apparatus consisted of two parallel boxes, each 120 cm long, enclosed by curtains to minimize external disturbances. Untreated water entered both ends of the reference box. In the experimental box, untreated and treated water entered at opposite ends creating three relatively distinct regions: a region at each end filled with the water introduced on that side, and a central mixing zone. An experimental trial consisted of placing one fish in each trough and recording the relative position of the fish in the trough. Unequal residence time or number of entrances into the treated water indicated avoidance of the toxicant. This 'gradient method' was subsequently modified by Wells (1915). Additional outlets at the center drain prevented the formation of vertical gradients and a series of stop-cocks was placed along the bottom of the trough to facilitate the removal of water samples.

TABLE 1 - Review of the experimental designs of avoidance studies with various treatments and fish species.

Chemical	Species	Reference
	SHALLOW GRADIENTS	
CO_2, O_2, N_2, Acetic acid, NH_4.	*Abramis crysoleucas*, *Ambloplites rupestris*, *Ameiurus melas*, *Catostomus commersoni*, *Etheostoma coeruleum*, *E. zonale*, *Hybopsis kentuckiensis*, *Lepomis cyanellus*, *Micropterus dolomieui*, *Notropis atherinoides*, *N. cornutus*, *Umbra limi*.	Shelford & Allee, 1913; 1914.
H_2CO_3, H_2SO_4, NH_4OH Na_2CO_3.	*Ameiurus melas*, *Lepomis pallidus*, *Pomoxis annularis*.	Wells, 1915.
H^+, H_2CO_3, $CuCl_2$, NH_4OH, OH^-.	*Acheilognathus limbata*, *Carassius auratus*, *Cyprinus carpio*, *Gnathopogon gracilis*, *Lebistes reticulatus*, *Lepomis macrochirus*, *Moroco steindachneri*, *Pimephales promelas*, *Pungtungia herzi*, *Sarcocheilichthys variegatus*, *Tribolodon hakonensis*, *Zacco platypus*.	Ishio, 1964.
ACP, Chlordane, 1-4-Dichloro-2-Nitrobenzene, Isobornyl thiocyanoacetate.	*Lepomis cyanellus*	Summerfelt & Lewis, 1967.
	STEEP GRADIENTS	
Alcohol, chloroform, formalin, mercuric-chloride, copper-sulphate, zinc-sulphate.	*Pygosteus pungitius*	Jones, 1947.

Ishio (1964) developed a method that eliminated vertical gradients and allowed more than one fish to be tested simultaneously. The tank was divided into an upper and lower path by a horizontal layer of sand. Tap water was pumped into the lower path and toxicant was introduced into the upper. Passage of the tap water upward through the sand diluted the toxicant and created a shallow horizontal gradient. Vertical gradients were eliminated by aerating the entire length of the trough with a perforated tube buried in the sand. The movement of twelve fish was monitored in the upper path to obtain a control, followed by introduction of the toxicant. The average position of the fish was plotted against the toxicant to determine the exact concentration that caused an avoidance response.

The second major avoidance method more sharply separates water and toxic solutions, forming steep toxic gradients, and was first proposed by J. R. E. Jones (1947). His apparatus consisted of a 58-cm plexiglass tube with inlets at both ends and outflow from central drains. Untreated water was initially released into the tube at both ends and exited through a central drain. A fish was introduced at the open end of the apparatus, allowed 10-15 min to adjust to the experimental environment, and then monitored in the trough every 30 sec for 10 min. After the control run was completed, a test solution was released at one end of the trough and the movements of the fish were recorded on graph paper for 7-120 min.

The above technique was simple, suitable for many applications, and has been used by many researchers (Jones, 1948, 1951, 1952; Bishai, 1962a, 1962b). The apparatus was further modified by Sprague (1964) for studying the effect of metals on salmon. The 114-cm plexiglass tube was drained by four central hoses that improved the separation of the two bodies of water. This basic design was used with few modifications by Sprague *et al.* (1965), Sprague (1968), Hill (1968), Sprague & Drury (1969) and Rehwoldt & Bida (1970). Scherer & Novak (1973) advanced this design with an automated recorder.

Jones *et al.* (1956) investigated the response of salmonids to industrial wastes with a rectangular wooden avoidance trough that was partitioned into four parallel channels at one end. Water entered each channel at the

TABLE 1 - cont.

Chemical	Species	Reference
	STEEP GRADIENTS (cont.)	
Calcium nitrate, Sodium-sulphide, H^+, Lead-nitrate, Zinc sulphate	*Gasterosteus aculeatus*, *Phoxinus phoxinus*.	Jones, 1948.
Phenol, Para-cresol, Ortho-cresol.	*Phoxinus phoxinus*	Jones, 1951.
O_2	*Gasterosteus aculeatus*, *Phoxinus phoxinus*, *Salmo trutta*.	Jones, 1952.
Sulfate waste, Sulfite waste.	*Oncorhynchus kisutch*, *O. tshawytscha*, *Salmo gairdneri*.	Jones et al., 1956.
O_2, H^+.	*Salmo salar*, *S. trutta*.	Bishai, 1962a,b.
Copper sulfate, Zinc sulfate.	*Salmo salar*	Sprague, 1964.
Cu, Zn.	*Salmo salar*	Sprague et al., 1965.
O_2	*Chologaster agassizi*	Hill, 1968.
ABS, BKME, Phenol, Chlorine.	*Salmo gairdneri*, *S. salar*.	Sprague & Drury, 1969.
DDT, Endrin, Dursban, Malathion, Sevin, 2-4-D.	*Cyprinodon variegatus*	Hansen, 1969.

closed end and exited from a drain at the lower, open end. For each trial, two channels served as controls and the others were treated with a toxic solution. Fish had the option to pass from the lower half of the trough into any of the four upper channels. After exploratory behavior commenced, the number of entries into each channel was recorded. Fewer entries into treated channels signified toxicant avoidance. The apparatus of Bogardus (personal communication, Wapora, Inc., Wash., D.C., USA) was similar except only two separate parallel-flowing bodies of water (one toxicant, another untreated) were produced.

Kleerekoper (1967) described a tank with a unidirectional flow of water and a large (5 x 5 x 0.5 m), open area for monitoring the response of fish to toxic gradients. Water entered through one entire wall, passed through baffles which produced a laminar flow and created a stable gradient in the open test area. When fish passed over any of 1,936 photo-cells embedded in the floor, light from an interfaced collimeter was intercepted and the relative position of the fish was recorded. Each experiment consisted of three consecutive eight-hr recordings of the locomotion of a single fish with 'clean' water initially in the entire tank, with toxicant introduced into one half, and then with toxicant introduced into the opposite half of the tank. Therefore, the time accumulated in either area of the tank, the average radius of each turn, and fish orientation could be studied.

Westlake & Lubinski (1976) described a similar system that was more compact and inexpensive. Water was introduced and mixed in a deep end, allowed to flow through a baffle that laminated the flow, and then entered a shallow area that housed the experimental fish. The proximity of fish movement produced a drop in voltage recorded by an overhead television camera which was processed by a small computer that recorded fish location. In this way sophisticated parameters of locomotion could be analyzed in addition to a simple avoidance response.

Höglund (1951) attempted to integrate both the shallow and steep gradient methods in a 'Fluviarium' that was subdivided into an apportionment box, a central section of nine vertical glass plates creating ten longitudinal

TABLE 1 - cont.

Chemical	Species	Reference
	STEEP GRADIENTS (cont.)	
$CuCl_2$	*Carassius auratus*	Kleerekoper et al., 1970.
Cr, Zn, Cd.	*Roccus lineatus*	Rehwoldt & Bida, 1970.
O_2	*Lepomis macrochirus*, *Micropterus salmoides*, *Oncorhynchus kisutch*, *O. tshawytscha*.	Whitmore et al., 1970.
DDT, Malathion.	*Gambusia affinis*	Hansen, 1972.
$CuCl_2$	*Carassius auratus*	Kleerekoper et al., 1973.
$HgCl_2$	*Carassius auratus*	Scherer & Nowak, 1973.
DDT, Toxaphene, Endrin, Parathion.	*Gambusia affinis*	Kynard, 1974.
Fenitrothion	*Carassius auratus*	Scherer, 1975.
TRC, FRC, CRC.	*Rhinichthys atratulus*	Fava & Tsai, 1976.
BKME	*Lagodon rhomboides*, *Fundulus grandis*	Lewis & Livingston, 1977.

TABLE 1 - cont.

Chemical	Species	Reference
	STEEP GRADIENTS (cont.)	
CRC	*Notropis blennius*, *N. volucellus*, *Pimephales vigilax*	Bogardus et al., (pers. comm.).
TRC, CRC, FRC, HOCl.	*Ambloplites rupestris*, *Micropterus dolomieui*, *M. punctulatus*, *Notropis cornutus*, *N. rubellus*, *Oncorhynchus kisutch*.	Cherry et al,1977a,b in press.
TRC, CRC, FRC, HOCl.	*Notemigonus crysoleucas*	Larrick et al, in press.
	FLUVIARIUM METHODS	
O_2, Nickel nitrate, Ferric nitrate.	*Leuciscus rutilus*	Hoglund, 1951.
2,4,6-Trinitrophenol	*Leuciscus rutilus*	Lindahl & Marcstrom, 1958.
Sulphite Waste Liquor, HCl, O_2, NaOH, NaCl.	*Coregonus nasus*, *Esox lucius*, *Gasterosteus aculeatus*, *Leuciscus idvarus*, *L. rutilus*. *Perca fluviatilis*, *Salmo alpinus*, *S. salar*, *S. trutta*, *Salvelinus fontinalis*, *Tinca tinca*.	Hoglund, 1961.

sections, and a test chamber to house the fish. Toxic substances were released into the apportionment box which created a reproducable series of concentrations. The total number of appearances in each concentration was presented in histograms to show which sections were least frequented or avoided.

Materials and methods

Cherry *et al.* (1974) described an avoidance apparatus patterned after Meldrim & Gift (1971) that incorporated several desirable features and has been extensively applied (Cherry *et al.*, 1975, 1977a, b). The avoidance unit consisted of two troughts, each 190 by 20 cm, which were filled at opposite ends by two circulating baths. Adjustment of medial drains maintained water one to three cm deep, depending upon fish size. A closed circuit television monitor was used to observed fish movement in troughs which were isolated within a plywood enclosure.

Each trial consisted of a reference phase and a series of experimental runs. Each water bath contained unmodified water pumped directly from the New River at Glen Lyn, Virginia. The water was adjusted to the desired temperature and then fed into both troughs. Fathead minnows *(Pimephales promelas)* were obtained from a commercial warm-water hatchery and were acclimated and tested at 18° C. One fish was placed in each trough and after normal exploratory movements were obtained, the cumulative time spent on the pretreated side of the medial drain was recorded for a 10-min period.

Following this reference phase, a 100 mg/l calcium hypochlorite stock solution was added to one water bath to establish a steep chlorine gradient in each trough. The effect of TRC (total residual chlorine) on fish behavior was investigated by Sprague & Drury (1969) and Dandy (1972) but insufficient information was provided to evaluate fish response to constituents of the FRC (free residual chlorine) – which include the hypochlorous acid (HOCl) and hypochlorite ion (OCl^-) fractions – and the combined residual chlorine (CRC). Fish in both troughs were monitored simultaneously for another 10-min period, and the time accumulated in the chlorinated water was recorded. Chlorine concentrations were doubled after each run so that eight fish were tested individually at doubling exposures from 0.025, 0.050, … 0.800 mg/l TRC.

Water from the troughs was withdrawn through several lateral drains at the beginning and termination of each run. Samples were analyzed for TRC and FRC with a Wallace and Tiernan amperometric titrator. Hydrogen ion concentrations were determined with a Corning Model 610-A portable pH meter and hypochlorous acid concentrations were calculated (Cherry *et al.*, 1977b). The Statistical Analyses System (SAS) by Barr *et al.* (1976) was utilized for all computations. The first TRC at which the residence time differed significantly ($p \leq .05$) from the reference phase (avoidance threshold) was determined by the Wilcoxon Sign Rank Test (Wilcoxon & Wilcox, 1964).

Results and discussion

At 18° C acclimation temperature, the percent residence time remained relatively constant for the first three recordings, then dropped sharply at 0.100 target TRC and remained low at the reamining concentrations. The TRC concentration at the avoidance threshold was 0.115 mg/l, which consisted of 0.079 mg/l CRC, 0.036 mg/l FRC and 0.022 mg/l HOCl.

The execution of any avoidance response presupposes the presence of a directive factor. Directive factors, as defined by Fry (1947), include all cues that enable an organism to determine its own location and the location of other organisms in the environment. In addition, avoidance behavior depends upon the organism's ability to discriminate between two bodies of water, for without this perception, avoidance of lethal doses would not occur. Hasler & Wisbey (1949) showed that a fish's ability to discriminate between different waters is very acute, but little research has been carried out to determine the locus for for toxicant reception, in spite of its great importance to orientation and locomotion in fish. Although quantitative data are scarce, Sprague & Drury (1969) and Dandy (1972) have shown that most fish perceive chlorine at low concentrations. The HOCl avoidance threshold for fathead minnows (0.022 mg/l) is similar to that reported for the common shiner (0.010-0.28 mg/l) (Cherry *et al.*, in press). This precision between fish species is significant because fathead minnows were exposed to a nearly continuous range of values from 0 to 0.168 mg/l HOCl and had ample opportunities to select intermediate or high concentrations.

Once the toxicant has been perceived, fish may respond in several ways. The simplest response is a *kinesis* in which the animal's body is not oriented with respect to the source of stimulation. The stimulus may change the swimming speed (orthokinesis) or the rate of turning (klinokinesis) (Hinde, 1970). *Taxes* are more advanced movements directing an animal toward (positive taxis) or away from (negative taxis) a stimulant (Maier & Maier, 1970). A movement that is directed by alternate comparisons (klinotaxis) is often found in animals that have only one receptor or several receptors that are widely dispersed. In order to elicit this behavior, the receptor will be exposed to a potentially toxic solution and fish will compare these sensations with those received in alternate solutions. After a comparison is completed, the fish may orient itself either toward or away from either stimulus. These movements, which are successive in time and space, were clearly demonstrated by Collins (1952). The second taxis – *tropotaxis* – is initiated by a simultaneous comparison of different solutions, usually involving symmetrical or bilateral sense organs. These movements are most successful in very steep gradients and can be identified if one of a pair of receptors is destroyed (Bardach *et al.*, 1967).

It is difficult to separate the different orienting components in vertebrates, which has led to much debate regarding the mechanism that predominates in an avoidance response. Proponents supporting kinetic movements emphasize the erratic but energetic movements of fish that move into poorly oxygenated water (Jones, 1952). However, this idea was refuted by Stott and Cross (1973) who concluded that directed actions dominated the response of roach to low oxygen conditions. They noted that low oxygen tensions increased fish's activity, but did not significantly alter position in a shallow (gradual) gradient, because activity remained high even after the fish returned to the untreated water. In all likelihood, each component is probably present and contributes to a greater or lesser degree depending upon the fish species and the nature of the toxicant (Höglund, 1961).

Fathead minnows demonstrated most of these behavior patterns in our steep gradient. Upon entering the chlorinated water, the fish frequently swam in wide circles. Because of the limited space available, the fish would soon re-enter untreated water, suggesting that ortho- and klinokinetic behaviors were operative. During subsequent entries fish appeared to detect chlorine more quickly. After swimming in decreasing circles at the toxicant-water interface, most individuals chose to move into the untreated water. Because the fish generally moved its entire body into and out of the toxicant, it appeared to be making successive comparisons (klinotasis). There was no evidence for telotaxic behavior. It is not known if a fish has distinct toxicant receptors, but chlorine may simply act as an irritant over large areas of the body surface (A. G. Heath, Biology Dept., VPI & SU, Blacksburg, Va., USA, personal communication), thereby providing the stimulation for toxic behavior.

Prospective investigators must consider the advantages and disadvantages of each method. The most obvious decision to be made is whether to employ an apparatus that creates a shallow or a steep gradient. Shallow gradients have the distinct advantage of simulating the majority of lotic environments. Turbulence normally dilutes effluents downstream creating very gradual horizontal gradients. As fish swim toward the gradient from downstream locations, they must be subjected to ever increasing toxic concentrations.

The use of shallow gradients also presents several problems: (1) they are difficult to reproduce with accuracy, (2) the threshold avoidance concentration is difficult to ascertain in a continuous gradient unless it lies directly at a sampling point, (3) fish placed in a gradual concentration gradient may select a favorable location as opposed to avoiding a certain toxic concentration, and (4), most importantly, shallow gradients cannot provide distinct directional cues for fish orientation. For these reasons, we decided to employ an apparatus which created a steep toxic gradient.

A single toxic concentration, which is the greatest advantage of steep gradients, provides the best opportunity for the sense organs of fish to discriminate between two opposing bodies of water. These gradients provide the necessary cues for successful directed movements such as klinotaxes and tropotaxes. In addition, kineses are apt to be more successfully executed in very steep gradients, due to the proximity of favorable environmental conditions. The chief objection to the use of these gradients concerns their applicability to field situations. Steep gradients are not particularly common in nature, but they are present at the confluence of two bodies of water or in the immediate vicinity of an industry's outfall. The discharge at APCO's Glen Lyn plant in Virginia forms a very steep gradient. Chlorinated discharges remain relatively unmixed with rapidly flowing river water for meters below the outfall area, perhaps because of the differing water temperatures and densities.

The parameter chosen as an index of avoidance behavior must be compatible with the physiological and behavioral response of each species to each toxicant. Shelford & Allee (1914) recorded the number of entries into the modified water was a measure of avoidance behavior. Whitmore *et al.* (1970) placed markers at varying distances within the experimental solution to determine the depth of penetration for each visit. In both cases, it was assumed that higher concentrations of toxicant would elicit a greater turning frequency and consequently more shallow visits into the treated water. These entry indices account for klinokinetic movements which result in more frequent turns in the treated water and for klinotaxes in which the fish must repeatedly enter the treated water to compare these sensations with those in untreated water. Unfortunately, these methods do not account for orthokinetic responses in which fish move deeply into the toxicant but leave so quickly that less time is actually spent on the modified side. This may account for contradictory opinions in the reporting of entry indices as adequate (Kleerekoper *et al.*, 1973) or inadequate (Fava & Tsai, 1976) measures of avoidance behavior. Elapsed time may be the most accurate measure of both kinetic and taxic behaviors and has been shown to be a sensitive index of avoidnace behavior (Fave & Tsai, 1976; Larrick *et al.*, in press). Time indices account for increases in swimming speed as fish move rapidly through a toxicant and for increased turnings as fish avoid unfavorable conditions. Data from our study show that this parameter is very sensitive to fish movement. The avoidance threshold for fathead minnows dropped sharply from 47 to 26 percent residence time. The measurement of other parameters does not provide such complete information.

The choice of test organisms should also be carefully considered. Perhaps the most widespread solution is to utilize the most important fish in the region being studied. Using this guide the response of fish that are considered 'valuable', either economically or recreationally, are used for regulations concerning the remainder of the population. Unfortunately, fish of uniform age and size are not always available. A second premise is to experiment with as many different species as possible. This choice has been important in creating guidelines for specific discharges, but the results from different studies cannot be adequately compared. Much interest has recently been given to the idea of establishing a standard fish for bioassays similar to the white rat in medicine (Sprague, 1970). If such an organism is tested singly or in conjunction with other species, the ability to either reproduce or compare results among laboratories would be greatly improved (Lennon, 1967). The selection of a specific fish depends upon certain criteria: 1) a uniform response to most toxicants; 2) adequate availability of a certain size, age and condition; 3) ease in handling and transporting; and, 4) preferably one that can successfully complete a life cycle in one year or less (Adelman & Smith, 1976). Suggested species have included the fathead minnow and goldfish *(Carassius auratus)* (Adelman & Smith, 1976) and the bluegill sunfish *(Lepomis macrochirus)* (Cairns, 1969). Of these species, the fathead minnow is readily available, easily manipulated and bred, and most suitable for general bioassay work. The data show that this species responds quite precisely in our avoidance apparatus. Bluegill and goldfish are often too large to be utilized in many apparatuses and are difficult to rear over several generations in small laboratories.

Selection of a specific standard bioassay fish is based upon the unlikely prospect that the species accurately represents the response of every other species. For this reason, a single choice should be broadened to accomodate variations in water quality which often result in different fish species. Because of its widespread distribution, the wealth of supplementary data in the literature, and the refined culturing techniques, rainbow trout *(Salmo gairdneri)* may be substituted as a cold water test organism. In this way, the accumulating data will not be unnecessarily biased by the smaller, warm water fathead minnow.

From the preceding discussion it is evident that few present or past methodologies incorporate every desirable feature. The apparatus described herein contains many useful modifications. Premixing experimental solutions in the treated water bath reservoir enables an investigator to test a wide variety of toxicants under constant temperature conditions. Steep toxic gradients are produced in avoidance troughs which provide the necessary sensory cues for fish to make a quick, sharp decision concerning its desirability. The lateral drains allow for regulation of water depth so that different age classes and species may be accomodated.

Important decisions must soon be made to limit the further degradation of all water resources. Unfortunately, zero discharge is an unrealistic goal (Cairns, 1977). Criteria must be formulated to help establish standards that can adequately protect our water resources. Avoidance thresholds provide valuable insight regarding the survival of fish in toxic solutions that cannot be obtained from static or chronic bioassays. Fish may successfully survive brief exposures to toxic concentrations greater than their 96-hour lethal concentrations by avoidance behavior and retreat to more favorable locations in their environment. Therefore these studied can provide extremely useful information from which such criteria can be established.

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