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Behavioral Avoidance of Fluoranthene by Fathead Minnows (*Pimephales Promelas*)

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FARR, A. J., C. C. CHABOT AND D. H. TAYLOR. *Behavioral avoidance of fluoranthene by fathead minnows (*Pimephales promelas*)*. NEUROTOXICOL TERATOL 17(3) 265–271, 1995.—A monitoring system was used to examine the behavioral response of fathead minnows (*Pimephales promelas*) to plumes of the polycyclic aromatic hydrocarbon fluoranthene. Previously unexposed fish and fish surviving acute exposure to fluoranthene were presented with three different concentrations of fluoranthene. Both groups of fish avoided fluoranthene. Pre-exposure did not enhance or diminish avoidance of fluoranthene. The lowest concentration of fluoranthene which produced an avoidance response was 14.7 μ/l, and the concentration of fluoranthene which did not produce an avoidance response was 8.6 μg/l. These results were comparable to the LOEC for survival in 7-day fathead embryo-larval growth and survival tests for fluoranthene. Thus, a fathead minnow could escape from areas highly contaminated with fluoranthene and have a better opportunity to survive, whereas fish would fail to avoid areas where fluoranthene concentrations are below 8.6 μg/l and suffer further toxicosis.

Behavior Avoidance Fluoranthene PAH Fish *Pimephales promelas*

POLYCYCLIC aromatic hydrocarbons (PAHs) are nearly ubiquitous in marine and freshwater ecosystems (22). They are composed of two or more benzene rings fused through the sharing of a pair of carbon atoms. PAHs can be naturally formed by high temperature pyrolysis of organic material, low to moderate temperature diagenesis of sedimentary organic material to form fossil fuels, and direct biosynthesis by microbes and plants (5,22,28). In addition, humans add to this environmental load through the inefficient combustion of carbonaceous material from such industrial activities as oil refinery operations, incineration of industrial and domestic waste and power generation from fossil fuels (1,5,22). PAHs can then reach the aquatic environment through industrial and domestic sewage effluent, surface runoff from land, deposition of airborne particulates, and spillage of petroleum and petroleum products into bodies of water (22). Indeed, the presence of PAHs in the environment is worldwide and total PAH concentrations can range from 0.001 μg/l to 0.01 μg/l in ground water (2) and from 0.025 μg/l to 13.0 μg/l in polluted areas such as the Great Lakes (6,21).

Upon entering the water, PAHs quickly adhere to organic and inorganic particulate matter. Much of the particulate PAH is then deposited in bottom sediments. Leaching or biological activity of these sediments may return a small fraction of the sediment PAH to the water column (22). Extensive research on the uptake, metabolism, and bioconcentration of some PAHs in fish has shown that PAHs are readily accumulated by aquatic biota to levels higher than those in the water. Aquatic organisms are able to accumulate PAHs at low concentrations in the water column, food, or sediment because PAHs are highly hydrophobic and lipophilic (16,22,25,29,44). This favors the rapid transfer of PAHs, via the gills and/or the gut, from the aqueous phase to lipophilic compartments such as the brain, the liver, the gallbladder, the gonads, and the flesh (13,25,46).

Some evidence indicates that PAHs have deleterious effects on fish. For example, Hose et al. (13) documented depressed mitotic rates in the retina and brain, and skeletal malformations in the skull and vertebral column of alevins of rainbow trout (*Oncorhyncus mykiss*) reared in 0.21 to 1.48 μg benzo(a)pyrene/l. In another study, insufficient yolk sacs, lack of body pigment, and abnormalities or absence of the eyes were found in rainbow trout alevins reared in 0.21 to 2.99 μg benzo(a)pyrene/l (9). Decreased reproductive output was observed in fathead minnows (*Pimephales promelas*) exposed to 6 and 12 μg anthracene/l (8). Fry maternally exposed to these con-

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centrations of anthracene, with subsequent exposure to ultraviolet radiation, exhibited developmental effects such as internal hemorrhaging, edema, and eye and yolk deformities (8).

Behavioral studies are a particularly promising means of detecting sublethal effects of contaminants (18). Behavioral changes may occur at quite different concentrations than physiological damage and serve as an important reason why behavioral testing should always be done. A method commonly used to assess the behavioral effects of contaminants is to expose fish to plumes of the contaminant and record their preference or avoidance of the plume. Such experiments have direct relevance to the health of wild populations of fish. We might find a population prevented from using a habitat even though contaminant concentrations were insufficient to cause physiological damage. Conversely, fish might fail to avoid a region of water that would cause severe physiological damage. The ability of fish to behaviorally avoid various pollutants has been demonstrated by several investigators. Investigators have found that several species of fishes can avoid plumes of a variety of heavy metals (3,11,12,30,32,34,35). In laboratory tests, green sunfish (*Lepomis cyanellus*) avoided the pesticide chlordane but not lindane (42). Also, fathead minnows avoided a water soluble fraction of coal liquid containing at least 1.7 and 3.5 mg total phenols/l (4). The preference or avoidance of PAHs has not been documented.

The purpose of this series of experiments was to examine the behavioral responses (preference or avoidance) of fathead minnows to plumes of fluoranthene in a multiple-choice test arena. Second, we also attempted to determine if experimental pre-exposure to levels of fluoranthene sufficient to kill 85% of a population could have an effect on the behavioral responses of the survivors to fluoranthene plumes.

METHOD

Experimental Animals

Immature fathead minnows (Kurtz Hatchery, Elverson, PA) were group housed in flow-through aquaria in charcoal-filtered, dechlorinated water at 24.3 ± 1.2°C (Table 1) under a 16L : 8D cycle. The fish were fed Biodiet starter moist chow morning and night and frozen brine shrimp at mid-day.

These fish were divided into two experimental groups: Prior to behavioral testing, one group of 1000 fish was exposed for 5 days to an average concentration of 87.5 μg fluoranthene/1. Because the toxicity of some PAHs has been shown to be enhanced by exposure to environmental levels of ultraviolet light (type A), these fish were also continuously exposed to 70 $\mu W/cm^2$ UVA throughout the course of the 5-day exposure (8,23,24). The survivors (n = 150), hereafter referred to as "dosed," were then placed in clean water. Two to 4 weeks later, these survivors were behaviorally tested as described next. The second group of fish, hereafter referred to as "naive," was exposed to similar environmental conditions except they were not exposed to fluoranthene.

TABLE 1

WATER QUALITY PARAMETERS MEASURED IN THE AQUARIA HOLDING ALL EXPERIMENTAL ANIMALS AND IN THE 200 L RESERVOIR FOR BEHAVIORAL EXPERIMENTS

Parameter	Mean ± SD
pH	7.42 ± 0.12
Dissolved oxygen (mg O_2/1)	8.45 ± 1.02
Alkalinity (mg $CaCO_3$/1)	293.0 ± 11.7
Ammonia (mg NH_3/1)	0.22 ± 0.04

Behavioral Monitoring System

The behavioral monitoring system consisted of a multiple-choice test arena (octagonal fluviarium), a water delivery system, and an associated video-based data acquisition system (Fig. 1). This system allowed the creations of a discrete plume of toxicant and the assessment of preference and avoidance responses of fathead minnows to water-born, chemical stimuli.

The octagonal fluviarium was an octagonal Plexiglas tank incompletely partitioned into eight equal radial octants by central and peripheral walls, leaving a central octagonal swim chamber in which an animal could move without restriction through all eight octants (27,35,38,45). A laminar flow of water at 1 1/min was established through the radial octants by partitioning of the inaccessible central and peripheral portions by stainless steel mesh (250 μm mesh size). The integrity of toxicant plumes was verified by food dye plumes and by collecting water samples at various depths within the designated octant and adjacent octants and mapping toxicant concentrations. This mapping showed that at a 1 1/min flow through the fluviarium, a toxicant plume could be created in a single octant. Dispersion to adjacent octants was below detectable levels.

The fluviarium was entirely surrounded in black hardware cloth and illuminated with four vita-lights (Durotest Inc., NJ, 15 watts) and four black-lights (General Electric, USA, 15 watts) mounted in a square configuration above the fluviarium. A Macam Photometrics (Livingston, Scotland) Model UV-103 radiometer was used to quantify UVA (320–400 nm). This light configuration minimized shadows in the fluviarium and produced 220–320 $\mu W/cm^2$ UVA.

Behavioral Bioassay Protocol

Both naive (n = 24) and dosed (n = 24) fish were used. Three experimental subgroups (n = 8 for each) were drawn from each of these two groups. One experimental subgroup of naive fish was then presented with one of three measured concentrations of fluoranthene (mean ± SE): 14.7 ± 2.7 μg/1, 22.5 ± 3.1 μg/1, and 43.0 ± 3.9 μg/1. Similarly, one experimental subgroup of dosed fish was presented with one of three concentrations of fluoranthene (mean ± SE): 8.6 ± 2.2 μ/1, 24.4 ± 5.8 μ/1, and 43.8 ± 5.3 μ/1. Originally, both naive and dosed fish were to be presented with the same concentrations of fluoranthene, but due to fluctuations in fluoranthene concentrations within the fluviarium, there were slight differences in the concentrations presented to the two experimental groups.

All fish were deprived of food 72 h prior to behavioral monitoring, because previous studies have reported that partially satiated animals are not as responsive to chemical cues as unsatiated individuals (7,40). A single fish was tested during each experimental period. Once the fish was put into the fluviarium it was given a 10-min adjustment period to insure active swimming and to reduce the likelihood of "freezing" or "escape" responses due to handling (40). After 10 min, the fish's behavioral activity was recorded and quantified for 15 min (the experimental period) using the Videomex-V system described next. This experimental period was divided into three treatment periods, each 5 min in duration. In the first 5 min of the experimental period, charcoal-filtered, dechlorinated

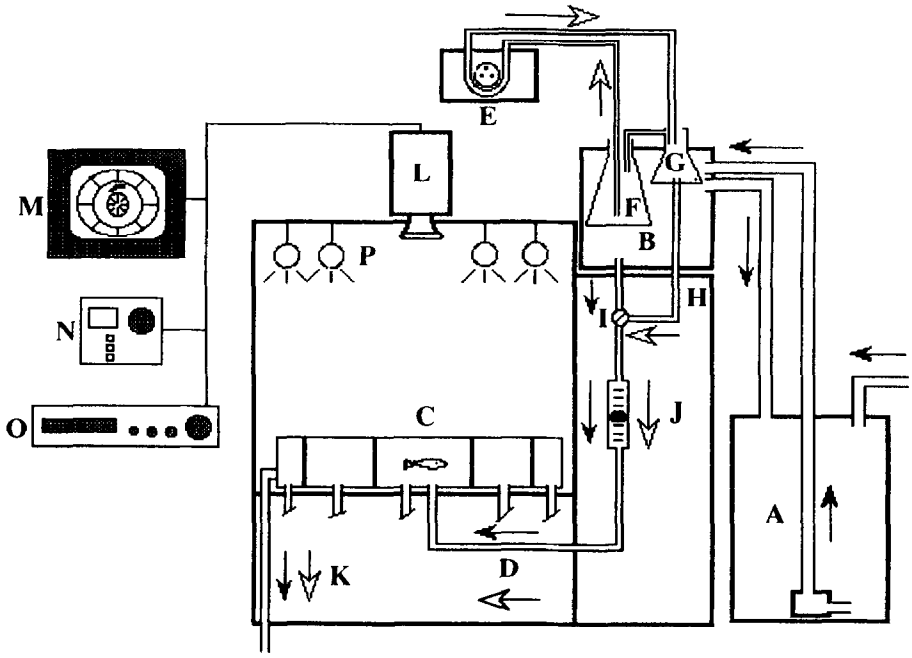

FIG. 1. Schematic diagram of the front view of the behavioral monitoring system, with a 200 L reservoir (A), from which charcoal-filtered, dechlorinated water was then pumped into a constant-overflow head box (B) and then flowed into the octagonal fluviarium (C), through one of eight tygon tubes (D) leading to one of eight infusion chambers. The toxicant was delivered to the fluviarium from a 4 L reservoir (F), floating within the constant-overflow head box, from which the toxicant was then pumped (E) into a 1 L flask (G) also floating within the constant-overflow head box and then flowed into the fluviarium through tygon tubing for the toxicant (H). In the 1 L flask, the toxicant overflowed through a top outlet and was returned to the 4 L reservoir. Flow valves (I) and flow meters (J) were used to produce a constant flow rate through the entire fluviarium of approximately 1 L/min. Water left the fluviarium through peripheral water outlets (K). A video camera (L), video monitor (M), Videomex V (N), video-cassette recorder (O), and vita-lights and black lights (P) were also used. Solid arrows represent the flow of charcoal-filtered, dechlorinated water, and open arrows represent the flow of the toxicant.

water at 25 ± 0.5°C (Table 1) flowed into all eight octants. This served as the preexposure period and produced baseline behavioral data. During the second 5 min, fluoranthene-laden, charcoal-filtered, dechlorinated water flowed into one of the eight octants, the treatment octant, and charcoal-filtered, dechlorinated water flowed into the remaining seven octants (the exposure period). In the last 5 min of the experimental period, again charcoal-filtered, dechlorinated water flowed into all eight octants (the post-exposure period). The octant selected to receive the chemically treated plume was determined systematically such that every other octant (a total of four octants) was used once for each experimental group. This procedure was followed to minimize the effects of possible biases of fish for specific octants in the fluviarium due to cues not evident to the investigators (35). The fluviarium was rinsed with 1N sodium hydroxide after each experimental subgroup to reduce chemical absorption.

Samples of the fluoranthene-laden water plume were collected at low (the floor of the swim chamber) and medium (approximately 2 cm from the floor of the swim chamber) depths, and at the periphery of the swim chamber because the fish spent most of their time there. These samples were stored at −70°C, and later analyzed by reverse-phase high-pressure liquid chromatography to determine the actual concentration to which the fish were exposed. To determine fluoranthene concentrations, 10 μl samples or standards were injected onto a Waters 3.9 mm × 15 mBondPak™ C18 column at 30°C. A mobile phase of 80% acetonitrile and 20% HPLC grade water was used at 0.8 μl/min. A Hitachi F1000 fluorescence detector was used at an excitation of 360 nm and an emission of 460 nm. Peaks were recorded and quantified on an IBM microcomputer based waters 820 Chromatography Data Station.

The fluoranthene-laden water was collected immediately prior to experimentation from a once-through elution column (23,24) and then diluted with charcoal-filtered, dechlorinated water to produce the desired concentrations. For the once-through elution column, 10 g of fluoranthene was dissolved in 500 ml of acetone and added to 1000 g of dried silica sand (0.2% wt/wt) under a fume hood and allowed to dry of 24 h. The treated sand was then added to a 7 × 50 cm (diameter × length) glass column and the column was placed in-line with 25°C charcoal-filtered, dechlorinated water.

Data Acquisition and Analysis

Movements of the fish were quantified with an automated data collection system consisting of a video-digitizer and associated software (Videomex-V with multiple zone distance traveled monitor, Columbus Instruments, Columbus, OH). The Videomex-V system can quantify slight changes in locomotor

activity and record at intervals of 30 s or longer the time that an animal spends in a given number of connecting arenas, the number of entries into and the distance traveled in those arenas, and the speed at which the animal was traveling in each arena. The Videomex-V system eliminates the need for manual frame by frame quantification of movements from video tape. In this study, the Videomex-V system identified the octant position occupied by each fish during each 1-min interval of the test. The data set therefore consisted of dependent variables only, since each fish was exposed to all three treatment periods (pre-exposure, exposure, and post-exposure), and the movement of a fish during 1 min was affected by its movement the minute before. With all dependent variables, the data from each experimental group were analyzed using a doubly repeated analysis of variance (ANOVA) (26). This test calculated a separate ANOVA for treatment effects, time effects among treatment periods, and time effects within each treatment period.

Three requirements were established for an acceptable test.

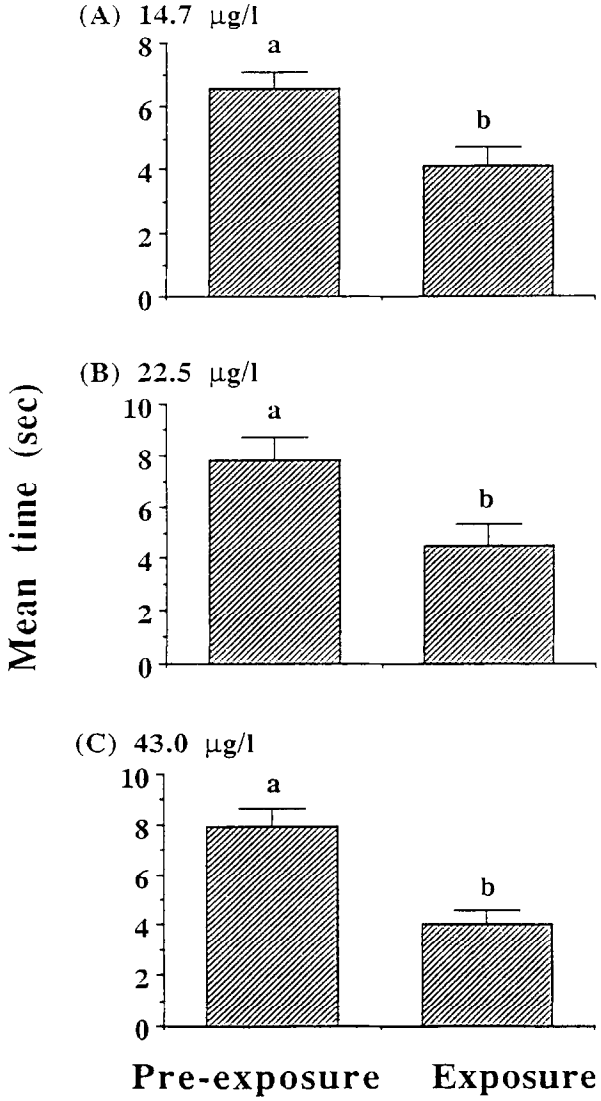

FIG. 2. Mean time spent in the octant (± SEM) into which fluoranthene flowed during the exposure period as compared to the time spent in that octant during the pre-exposure period for individual naive fathead minnows (i.e., never before exposed to fluoranthene). Different letters above bars indicate significance at $p \leq 0.05$. Fluoranthene concentrations were (A) 14.7 μ/l, (B) 22.5 μ/l, and (C) 43.0 μ/l. For (A) and (C), the experimental subgroups were composed of eight fish. For (B) the experimental subgroups was composed of six fish due to the failure of two fish to meet the requirements for an acceptable test.

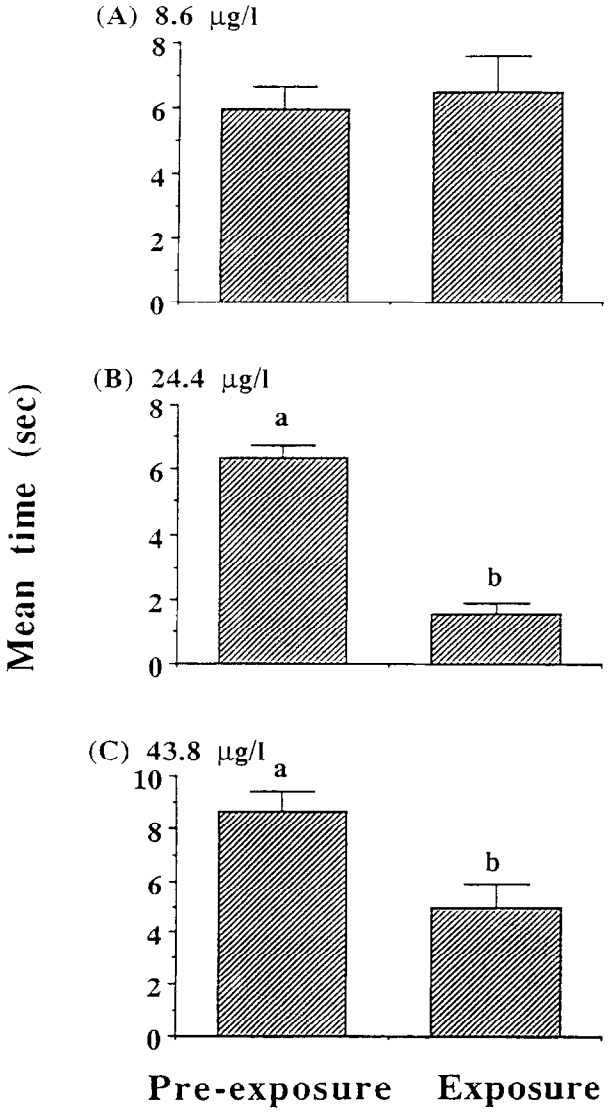

FIG. 3. Mean time spent in the octant (± SEM) into which fluoranthene flowed during the exposure period as compared to the time spent in that octant during the preexposure period for individual dosed fathead minnows (i.e., acutely exposed to fluoranthene prior to behavioral monitoring). Different letters above bars indicate significance at $p \leq 0.05$. Fluoranthene concentrations were (A) 8.6 μ/l, (B) 24.4 μ/l, and (C) 43.8 μ/l. For (A) and (C), the experimental subgroups were composed of eight fish. For (B) the experimental subgroup was composed of five fish due to the failure of three fish to meet the requirements for an acceptable test.

First, a fish had to pass through all eight octants by the end of the second minute during any of the three treatment periods. If the fish failed to meet this requirement then it was eliminated from the data set. Only three fish (all dosed fish from the experimental subgroup presented with 24.4 μ fluoranthene/l) out of the 48 total fish tested failed to meet this first requirement. Second, there could be no confounding time effects. Pre-exposure behavior could not be statistically different from post-exposure behavior. The test subject had to show recovery to preexposure behavior during the postexposure period. To insure this, a doubly repeated ANOVA was used to compare all three treatment periods (pre-exposure, exposure, and post-exposure) on a per-minute basis for time spent in the treatment octant. Nonsignificant results from this three way comparison verified that there were no time effects among treatment periods or time effects within each treatment period, and therefore any behavioral modifications were concluded to be fluoranthene induced. Finally, if a fish's mean time in the treatment octant exceeded 3 SDs from the mean, it was considered an outlier and removed from the data set. Only two fish (both naive fish from the experimental subgroup presented with 22.5 μ fluoranthene/l) were outliers and excluded from data analysis.

Subsequently, to test whether exposure to fluoranthene plumes would cause preference or avoidance, a doubly repeated ANOVA was used to compare the preexposure period to the exposure period, on a per-minute basis. Therefore, all preference and avoidance analyses were two-way comparisons (pre-exposure period to exposure period) only. Significance was declared at $p \leq 0.05$.

RESULTS AND DISCUSSION

Naive fish spent significantly less time in an octant with a fluoranthene plume of 43.0 μ/l, 22.5 μ/l, or 14.7 μ/l than they did in that octant during the preexposure period, $F(1, 7) = 16.21$, $p = 0.0051$; $F(1, 5) = 6.78$, $p = 0.0480$; $F(1, 7) = 8.73$, $p = 0.0212$, respectively; Fig. 2). Similarly, dosed fish spent significantly less time in an octant with a fluoranthene plume of 43.8 μ/l or 24.4 μ/l than they did in that octant during the pre-exposure period, $F(1, 7) = 8.10$, $p = 0.0248$; $F(1, 4) = 58.16$, $p = 0.0016$, respectively). No statistically significant change in time spent in an octant between the pre-exposure and exposure periods was detected in a fluoranthene plume of 8.6 μ/l, $F(1, 7) = 0.14$, $p = 0.7205$; Fig. 3.

Avoidance responses have been described as very sensitive in studies of a variety of heavy metals (3,11,12,30,32,34,35), a water soluble fraction of coal liquid (4), and monocyclic aromatic hydrocarbons (20). Sprague (33) reported a mean avoidance concentration of 5.6 μ/l of zinc for rainbow trout which was only 1.0% of the lethal threshold concentration. A mean avoidance concentration of 1.5 μ/l of a water soluble fraction of coal liquid was reported for fathead minnows, which was 24% of the lethal concentration (4). Others have reported similar threshold avoidance levels far lower than lethal concentrations (14,43). This sensitivity was even observed for relatively insoluble pollutants such as DDT and chlordane (10,42). In our study the avoidance response of fathead minnows to fluoranthene appeared to be only as sensitive as lethality experiments (Diamond et al., personal communication). From the results on the time spent in a fluoranthene plume, for all six concentrations, the No Observed Effect Concentration for avoidance (NOEC), that concentration of fluoranthene that had no statistically significant behavioral effects on the population, was 8.6 μ fluoranthene/l, and the Lowest Observed Effect Concentration for avoidance (LOEC), the lowest concentration of fluoranthene that had a statistically significant effect on the population, was between 8.6 and 14.7 μ fluoranthene/l. The NOEC and LOEC for avoidance are comparable to the concentrations that can be found in the environment, which can range from < 1 μ/l to as much as 13 μ/l in polluted areas (15,31). Furthermore, the LOEC for avoidance was similar to the LOEC for survival in 7-day fathead embryo-larval growth and survival tests for fluoranthene, which was between 1 μ/l and 10 μ/l (Diamond et al., personal communication).

A fathead minnow could escape from areas highly contaminated with fluoranthene and thus have a better opportunity to survive. However, this may only be a short-term benefit to the individual because displacement from preferred habitats may result in increased mortality through predation, decreased growth, or impaired reproduction (18). In areas where fluoranthene concentrations are below the NOEC, a fathead minnow could not avoid these contaminated areas, and these concentrations would likely prove to be lethal over longer exposures.

Our results clearly demonstrate that both naive and dosed fish can avoid fluoranthene. This avoidance by both naive and dosed fish suggested that pre-exposure did not compromise the fish's ability to detect fluoranthene. Dosed fish retained their ability to detect fluoranthene. The documented avoidance reaction also appeared quickly.

Dauble et al. (4) concluded that the avoidance of a toxicant during a 2-day exposure would not have been detectable at all individual times. In our study, however, a 5-min observation of fish presented with fluoranthene was sufficient to document an avoidance response and suggests that these fish can quickly detect and avoid fluoranthene.

From the sensitivity of the avoidance response observed in our study, this behavioral monitoring system seems to be a good toxicological tool, and should be used as a standard protocol in aquatic toxicity assessment, along with the traditional lethal and sublethal tests. The concentration and dispersion of the toxicant can be well controlled. With the automated data acquisition system described here, large amounts of data can be obtained for reliable analysis. A wide variety of organisms can be used, and in fact, this behavioral monitoring system has been used to document preference and avoidance responses for zebra fish (*Brachydanio rerio*), and several species of killifish (27,36,37,39), bullfrog (*Rana catesbeiana*) and green frog (*R. clamitans*) tadpoles (38,45, respectively), and crayfish (*Procambarus clarkii*, *Orconectes rusticus*, *Cambakrus bartoni*; 41). Furthermore, these behavioral experiments are nondestructive. They do not use death as an endpoint. They can be conducted with minimal stress to the fish, and potentially allow for retesting of the same individual at later times (19). Also, behavior integrates many cellular processes and is essential to the viability of the organism, the population, and the community (17), therefore, behavioral effects should be tested for their own sake not as a predictor of physiological damage.

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