*Fish models in behavioral toxicology: Automated techniques, updates and perspectives**

Andrew S. Kane[1], James D. Salierno[1] and Sandra K. Brewer[2]

[1]*Aquatic Pathobiology Center, University of Maryland, Department of Veterinary Medicine, 8075 Greenmead Drive, College Park, MD 20742*

[2]*U.S. Army Corps of Engineers, Rock Island District Clock Tower Building, Rodman Avenue, Rock Island IL 61201*

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Introduction

Since the science of toxicology began thousands of years ago, behavioral endpoints have been used to study the effects of chemicals and drugs on humans and other mammals. In aquatic toxicology however, the nexus of behavioral sciences with the study of toxicants has only become prominent within the last 5 decades. Behavioral endpoints have been slow to be integrated in aquatic toxicology because, until recently, there was a poor understanding of how alterations in behavior may be related to ecologically-relevant issues such as predation avoidance, prey capture, growth, stress resistance, reproduction and longevity. Further, the ability to achieve repeatable, quantifiable data from a large number of animals or exposures has been challenging. Recent improvements in computer and video automation have made possible significant progress in the ease, utility, and affordability of obtaining, interpreting, and applying behavioral endpoints in a variety of applications from water quality monitoring to use in toxicity identification evaluation (TIE) (Gruber et al. 1994; Balk et al. 1996; Baldwin et al. 1994a,b; Diamond et at. 1990; ATSM, 1995; Drummond et al. 1986, 1990; Rice et al. 1997). Consequently, behavioral endpoints in aquatic toxicology are shifting from being met with skepticism by investigators to being received with greater enthusiasm.

One of the first comprehensive reviews on aquatic behavioral toxicology was published by Rand (1985). Over the past 20 years, the field of behavioral toxicology has grown, in part, because of increased interest in the number of species used, endpoints measured, and methods to collect and interpret data. Numerous reviews have traced these advancements in the field of Behavioral Toxicology (Døving, 1991; Weber and Spieler, 1994; Gray, 1990; Little and Finger, 1990; Little and Brewer 2001). However, the recognition of behavioral toxicology as an important tool in aquatic toxicology is most clearly seen in the acceptance of behavioral endpoints in federal regulations. In 1986, the U.S. government accepted avoidance behavior as

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legal evidence of injury for Natural Resource Damage Assessments under proceedings of the Comprehensive Environmental Response, Compensation, and Liability Act of 1980 (NRDA, 1986).

This chapter provides updated information with an added perspective on automated systems that evaluate quantitative behavioral endpoints. The chapter also provides a compilation of reference material relating to specific and non-specific observations of behavioral alterations in fish that will be of use to both experienced and new practitioners of behavioral toxicology. The reader is reminded that much work has also been done with aquatic invertebrates Boyd et al. 2001) including crabs (Kumari et al, 1987; Bookhout et al., 1984), daphnids (Dodson et al., 1995), clams (Ham and Peterson, 1992; McCloskey and Newman, 1992) and other animals; however, the volume of such work precludes its inclusion in this chapter.

Why study behavior?

Behavior provides a unique perspective linking the physiology and ecology of an organism and its environment: (Little and Brewer 2001). Behavior is both a sequence of quantifiable actions, operating through the central and peripheral nervous systems (Keenleyside 1979), and the cumulative manifestation of genetic, biochemical, and physiologic processes essential to life, such as feeding, reproduction and predator avoidance. Behavior allows an organism to adjust to external and internal stimuli in order to best meet the challenge of surviving in a changing environment. Conversely, behavior is also the result of adaptations to environmental variables. Thus, behavior is a selective response that is constantly adapting through direct interaction with physical, chemical, social, and physiological aspects of the environment. Selective evolutionary processes have conserved stable behavioral patterns in concert with morphologic and physiologic adaptations. This stability provides the best opportunity for survival and reproductive success by enabling organisms to efficiently exploit resources and define suitable habitats (Little and Brewer 2001).

Since behavior is not a random process, but rather a highly structured and predictable sequence of activities designed to ensure maximal fitness and survival (i.e., success) of the individual (and species), behavioral endpoints serve as valuable tools to discern and evaluate effects of exposure to environmental stressors. Behavioral endpoints that integrate endogenous and exogenous factors can link biochemical and physiological processes, thus providing insights into individual- and community-level effects of environmental contamination (Brewer et al., 2001; Vogl et al, 1999). Most importantly, alterations in behavior represent an integrated, whole-organism response. These altered responses, in turn, may be associated with reduced fitness and survival, resulting in adverse consequences at the population level (Bridges 1997).

Rand (1985) stated that behavioral responses most useful in toxicology should be: (1) well-defined endpoints that are practical to measure; (2) well understood relative to environmental factors that cause variation in the response; (3) sensitive to a range of contaminants and adaptable to different species; and, (4) ecologically relevant. To this list, we add endpoints ideally should (5) elucidate different modes of action or chemical classes; (6) be able to "stand alone" and be easily incorporated into a suite of assessments; (7) be simple to automate in order to maximize their utility for a broad range of applications; (8) have representation across species (e.g. reproduction, food acquisition) in order to facilitate investigations into the phylogeny and ontogeny of behavior; (9) include a suite of endpoints that focus on innate behavior of sentinel organisms that can be altered in association with stress exposure; and (10) help delineate ecosystem status i.e., health. Although each of these

considerations has merit, often the application of a specific endpoint, or suite of endpoints, is based on the ability to functionally discern exposure-related alterations, using available techniques, with the most appropriate sentinel species.

The application of behavioral endpoints in any toxicity study must also be based on the stressor(s) to be evaluated. Basic knowledge of the compound/toxicant/stressor of interest is necessary. Stress agents of interest should (a) be "behaviorally toxic," (b) have a route of uptake for the aquatic species in question, and (c) structurally resemble a behavioral or neurotoxicant or one of its active metabolites. Of course, toxicants that may be a behavioral or CNS toxicant to mammals may not have similar affects on aquatic animals, and *vice versa* (Rand 1985).

The mechanism of action, route of uptake, and behavior of the compound of interest in the aquatic environment must all be understood. In adult fish, gill and gut epithelia are major routes of uptake, however physiological differences of different life stages need to be taken in consideration. Larval fish utilize skin as a respiratory interface and may uptake more compounds than adults of the same species that utilize gills for respiration. In contrast, for compounds that directly target the gill lamellae, adult fish will have increased sensitivity compared with larvae due to the increased surface area of the gill surface.

Fish models in behavioral testing.

To date, there are no standardized species or groups of species used for aquatic behavioral toxicology testing. Different species often have different behavioral and physiological responses to stress and toxicant exposure. Therefore, preliminary observations and assays are required in order to determine the feasibility of a particular species, and if aberrant behavioral patterns can be associated with specific exposure scenarios. It is not unreasonable for preliminary testing with a novel species to take months, and in some cases, years, in order to develop biologically-relevant endpoints of exposure.

Fish are ideal sentinels for behavioral assays of various stressors and toxic chemical exposure due to their: (1) constant, direct contact with the aquatic environment where chemical exposure occurs over the entire body surface; (2) ecological relevance in many natural systems (Little et al., 1993a); (3) ease of culture; (4) ability to come into reproductive readiness (Henry & Atchison, 1986), and (5) long history of use in behavioral toxicology. Alterations in fish behavior, particularly in non-migratory species, can also provide important indices for ecosystem assessment.

Ideally, test organisms should have the following characteristics: (1) high ecological relevance; (2) susceptibility to the stressor(s) in question, both in the field and in the laboratory; (3) have wide geographical distributions; (4) be easy to culture and maintain under laboratory conditions; (5) have relatively high reproductive rates and, should have relatively early maturation and easy fertilization in order to produce sufficient numbers of organisms of the proper age and size for testing; (6) have environmental relevance to the potential exposure (have been exposed to the test contaminant in the wild); and (7) have the ability to yield reproducible data under controlled laboratory conditions.

Once the model test species is determined, exposure-related behavioral alterations can be distinguished as fixed action patterns (FAPs) or through specific behaviors discerned from a species' ethogram. FAPs are specific, innate behavioral sequences initiated by specific stimuli that are not a result of gene-environment interactions (Alcock, 1997). These "hard-wired" behaviors are typically under genetic control, may be species specific, go to completion upon initiation, are not regulated through feedback loops, and are not reflexes but complex,

coordinated behaviors (Weber and Spieler, 1994). As such, alteration(s) of FAPs are good endpoints to include in a suite of behavioral tests.

Quantifiable behavioral changes in chemically-exposed fish provide novel information that cannot be gained from traditional toxicological methods, including short-term and sublethal exposure effects, mechanism of effect, interaction with environmental variables, and the potential for mortality (Kleerekoper et al. 1973; Giattina et al. 1981; Birtwell and Kruzynski 1989; Henry & Atchison, 1986; Saglio & Trajasse, 1998; Little & Finger, 1990). Ecologically relevant behaviors affected by sublethal concentrations include: altered vigilance, startle response, schooling, feeding, prey conspicuousness, migration, and diurnal rhythmic behaviors (Little & Finger, 1990; Zhou & Weiss, 1998). Changes in behavior may also alter juvenile recruitment, thereby disrupting population demography and community dynamics over time (Bridges, 1997).

Researchers wishing to develop a new fish model for behavioral toxicology, must consider the life history and ecology of the species. For example, herring form tight schools in nature, yet if kept solitary in the laboratory, will die after a few days (Radakov, 1973). Other clupeids, such as menhaden (*Brevoortia tyrannus*), can be laboratory-maintained over long periods of time (months to years) in large holding tanks, but when transferred to behavior/exposure arenas, become highly stressed and succumb within 72 hours due to sepsis prior to any toxicant/stressor exposure (Kane and Salierno, unpublished observations). These examples demonstrate the importance of having insight into the sentinel species' niche, i.e., habitat, diet, foraging strategies, reproductive strategies, home range, and social structures (school vs. shoal vs. solitary swimmers). These factors will help discern (1) which types of behavior are important for study, (2) appropriate experimental design and exposure parameters and (3) the ecological importance of the behavior on the life history of the fish. Channel catfish (*Ictalurus punctatus*), brown bullhead (*Ictalurus nebulosis*) and striped bass (*Morone saxatilus*), for example, may at times share similar geographical habitats, but differ greatly in life history characteristics (diet, position in the water column, migration, and social structures). These differences directly translate into differential exposure to chemicals in the environment. Of course there may also be vast physiological and biochemical differences between species in the metabolism, tissue distribution and elimination profiles, all of which can alter exposure concentrations at target tissues.

Different species of fish will have different suites of behaviors and adaptive behavior patterns, i.e., responses to stimuli. These patterns may also vary widely under different holding and exposure conditions within individuals of the same species. Therefore, it is critical to carefully document observations of normal baseline behavior under controlled conditions prior to behavioral testing with a chemical or other stress agent. Further, it is important to recognize changes in behavior that are associated not with controlled, laboratory stress exposure, but sub-optimal health. Table 1 provides a consolidation of qualitative comments from the literature that indicate behavioral changes associated with a broad variety of different scenarios. This table illustrates the often non-specific nature of many different behavioral alterations associated with disease agents, biologicals, sub-optimal water quality and contaminants. Further, these collective references suggest the need to provide quantitative data when reporting behavioral alterations to facilitate comparison with other studies or observations.

The requirement to carefully document baseline "normal" behavior should be viewed as a strength of behavioral testing. Traditional (LC_{50}) tests do not require stringent documentation of baseline behavior, other than visual observations of whether the test subjects were "healthy," and

verification that a minimal amount of mortality (i.e., ≤10%) occurs in the control treatment group(s). Behavioral toxicology testing, however, allows the control group to subsequently be exposed, if careful documentation on baseline behavior is made. The statistical power of behavioral tests can be greatly improved by using repeated measures analyses, using each animal as its own control. This type of analysis greatly reduces the inherent variability between individuals.

Descriptive behavioral alterations

Behavioral assays provide biologically relevant endpoints to evaluate sublethal exposure effects and may compliment traditional toxicity testing. In order to evaluate behavioral endpoints, specific descriptive observations regarding behavioral alterations in response to low-level stress (deviation from baseline) need to be demonstrated. The degree of alteration that can be experimentally meaningful is typically based on the ability to statistically discriminate differences between treatment and control groups. However, it should be noted that a common misuse of statistics is to find differences between treatment groups solely due to low *p*-values without empirically-observable, exposure-related changes. Factors that can influence *p*-values include, in addition to sound experimental design, use of proper controls (negative, solvent, positive), time frame of observation(s), sample size, response precision within treatment groups and reproducibility between experiments (refer also to section below on *Preliminary studies*).

Ultimately, the question addressed in behavioral toxicology is: How do alterations in a species' behavior, resulting from sublethal stress exposure, alter individual fitness and have a biologically-relevant effect? Even when biologically- and statistically-significant data are derived from an exposure study, it is typically difficult to extrapolate alterations observed under controlled laboratory conditions to ecologically-relevant field scenarios. The answer to bridging this gap lies in the appropriate selection of behavioral endpoints and adding a similarly-controlled comparison with the same species under more naturally complex or field exposure conditions (e.g., using predator-prey interactions and avoidance-attractance responses) (Bridges 1997; Woodward et al. 1997; Hartwell et al. 1987).

Individual movement and swimming patterns

Avoidance and attraction. When a contaminant triggers a stimulus response, the resulting behavioral reaction (avoidance or attraction) significantly regulates exposure duration of the organism. If the contaminant is perceived by the fish as noxious, the fish responds by avoiding the area containing the chemical. In contrast, if the contaminant triggers an attractance response, the fish will stay in the area, thus increasing exposure duration. Avoidance-attractance responses depend on (1) the substance activating the receptor; (2) sufficient exposure history of the species to evolve adaptive responses or sufficient experience by the organism to acquire a response to the stimulation; and, (3) sufficient directional information from the chemical concentration gradient to orient in the proper direction from the chemical plume (Little and Brewer 2001).

Avoidance and attractance behavior in fish has proven to be an easy and realistic behavioral endpoint of exposure because many contaminants induce avoidance or attractance behavior. The utility of avoidance behavior as an indicator of sublethal toxic exposure has been demonstrated over the past 50 years, and chemically-induced avoidance or attractance may significantly alter the distribution and migration patterns of individuals and groups of fish (Sprague, 1968).

Gray (1990) demonstrated the avoidance of oil-contaminated water and gas-supersaturated water by free-ranging fish in the field. Avoidance of heavy metals (e.g., cadmium, copper, cobolt, aluminum) by a variety of freshwater fish has been documented at low, environmentally relevant concentrations (Grillitch et al., 1999; Hansen et al., 1999; Exley, 2000; Svecevicius, 2001). In addition, fish can actively avoid fluctuations in water quality conditions, such as hypoxia, temperature, acidification, and ammonia (Jones, 1952; Peddler and Maly, 1986; Weinstein and Kimmel, 1998; Wannaker and Rice, 2000). Fish also maintain the ability to avoid anthropogenic compounds released into the environment, including certain pesticides and rotenone (Kynard 1974; Hogue, 1999).

Through the use of elaborate experimental designs, coupled with qualitative and quantitative measures of behavior, avoidance behavior can provide an endpoint that directly correlates to the field. Similar avoidance responses were observed in laboratory and field tests with fathead minnows (*Pimephales promelas*) when exposed to metals characteristic of the Coeur d'Alene River (Idaho, U.S.A) downstream from a large mining extraction operation. Telemetry studies conducted at the confluence of the river with an uncontaminated tributary of the river revealed a similar avoidance of the contaminated water within the concentration range that induced avoidance responses in laboratory studies (Woodward et al. 1997). Hartwell et al. (1987) conducted integrated laboratory-field studies of avoidance and demonstrated that fathead minnows avoided a blend of heavy metals (copper, chromium, arsenic, and selenium) that are typical of effluent from fly ash settling basins of coal-burning electrical plants. Fish avoided a 73.5 $\mu g\ L^{-1}$ mixture of these metals in a natural stream and 34.3 $\mu g\ L^{-1}$ in an artificial stream.

It may appear at first glance that most studies demonstrate avoidance behavior when fish are exposed to contaminants. However, it is difficult to generalize about the avoidance of aquatic contaminants by fish because of the variety of species and experimental designs used to test behavioral responses, as well as variations in the modes and sites of action of the chemicals studied (Giattina and Garton 1982). Beitinger (1990) reviewed the published literature on avoidance for over seventy-five different chemicals. Roughly one-third of the chemicals were avoided, whereas the others either failed to elicit a response or induced inconsistent responses. Many contaminants may cause avoidance reactions but some may attract aquatic organisms: these include detergents (Hara and Thompson 1978), some metals (Timms et al. 1972; Kleerekoper et al. 1973; Black and Birge 1980), and petroleum hydrocarbons (Lawrence and Scherer 1974; Atema 1976). Also, different species may have different avoidance responses. Largemouth bass (*Micropterus salmoides*) have been shown to be insensitive to 50 $\mu g\ L^{-1}$ copper sulfate, whereas goldfish (*Carassius auratus*) and channel catfish were attracted to this concentration (Timms et al. 1972). Part of the explanation for these apparent conflicting results may be the contaminant-induced alterations of chemosensory systems. Hansen et al. (1999a,b) found simultaneous alterations in chemosensory-mediated behavior, in the physiologic responsiveness of the olfactory system of chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*O.* mykiss) to L-serine, and evidence of damage to the olfactory tissue responsible for mucosa production and olfactory receptor cells. McNicol and Scherer (1991) determined that whitefish (*Coregonus clupeaformis*) avoided cadmium concentrations of 1 $\mu g\ L^{-1}$ and less, and also avoided cadmium at 8 $\mu g\ L^{-1}$ and greater, but showed little response to concentrations between this range.

In recent physiological and behavioral studies (McNichol and Hara, personal communication), electro-olfactorygram (EOG) responses to the lower concentrations were shown to be mediated by the olfactory system. The olfactory system apparently became injured

at cadmium concentrations greater than 1 μg $L^{-1,}$ when avoidance responses ceased to occur and EOG responsiveness to L-serine were abolished. The renewed avoidance response to 8 μg L^{-1} was likely induced by generalized irritation.

Likewise, Hansen and co-workers (1999a,b) found that even brief exposure of chinook salmon and rainbow trout to copper (25 μg L^{-1}) was associated with a significant reduction in EOG responses that recovered over several days; however, exposure to higher concentrations (44 μg L^{-1} chinook salmon; 180 μg L^{-1} rainbow trout) abolished behavioral responses. Futhermore, physiologic recording revealed these higher concentrations diminished both the EOG responses recorded on the mucosa as well as electro-encephalogram (EEG) responses to L-serine recorded from the olfactory tract. Necrosis and reduced density of olfactory receptors were evident injuries to the olfactory epithelium. These studies highlight the versatility and importance of integrating behavioral endpoints into a suite of toxicological studies that include relevant physiological and pathological endpoints to help elucidate mechanism of action. We refer the reader to the many thorough reviews of the well-studied endpoint of avoidance-attractance behavior (e.g., Cherry and Cairns 1982; Hara et al. 1983; Beitinger 1990; Little and Brewer 2001).

Swimming patterns. Avoidance behavior is an amalgam of many behaviors that may culminate in a single endpoint, whereas movement analysis is a finer scale technique investigating the components of movement. Neurotoxicity is frequently observed in changes in form, frequency, or posture of swimming movements, with changes often occurring much earlier than mortality (Little and Finger 1990; Little and Brewer 2001). Sublethal metal and pesticide exposures have demonstrated alterations in swimming behaviors and serve as models for additional stressors (Kleerekoper et al., 1972; Brewer et al. 1999; Allin and Wilson, 2000; Kwak et al., 2002). When bluegill received pulsed doses of the pyrethroid insecticide ES-fenvalerate (0.025 μg L^{-1}), the first indication of toxicity was caudal fin tremors as fish initiated movement (Little et al. 1993b). Exposure of rainbow trout to sublethal concentrations of 40 μg L^{-1} malathion resulted in convulsive movements (Brewer et al. 1999). A review by Little and Finger (1990) revealed that the lowest behaviorally effective toxicant concentration that induced changes in swimming behavior of fish ranged from 0.1 to 5.0 percent of the LC_{50}. When observations were made over time, behavioral changes commonly occurred 75 percent earlier than the onset of mortality. Development of locomotory responses, frequency of swimming movements and duration of activity were significantly inhibited before effects on survival or growth were observed in brook trout (*Salvelinus fontinalis*) alevins exposed to aluminum concentrations (300 mg L^{-1}) under acidic conditions ($pH \leq 6.1$) (Cleveland et al. 1991).

Movement analysis of individuals and groups of fish continues to be refined as computer technology advances. Swimming responses have been used in automated biomonitoring systems because of their consistent sensitivity to numerous contaminants (Miller et al. 1982; Smith and Bailey 1988). Studies of fish movement typically involve videography and quantification of movement parameters. Movement endpoints are designed to discern alterations in general swimming patterns in response to stressor exposure. Behavioral endpoints quantified through movement analysis typically include total distance traveled, velocity, acceleration, turning angles and frequency, time spent swimming, as well as horizontal and vertical distribution of individuals.

The measurements of swimming behavior are usually limited to the laboratory. Assessment of fish at contaminated field sites is currently not possible as species-typical responses have not been defined to permit the evaluation of behavioral function except for the

most extreme aberrations (Little and Brewer 2001). In the laboratory, subtle changes which arise from exposure can be confirmed through comparisons with controls or with responses observed during a pre-exposure period.

Intra- and interspecific interactions. For hazard assessment and environmental regulation it is important to show a causal linkage with the population in order to provide a predictive index of population-level effects. Recently, behavioral toxicology has focused more on complex behaviors such as prey capture, predator avoidance, and courtship and mating. These behaviors maintain high environmental relevance as well as direct fitness consequences to the individual. Hypoactivity and hyperactivity, as well as deviations in adaptive diurnal rhythmicity, may disrupt feeding and increase vulnerability to predation (Steele 1983; Laurence 1972). However, studies on these more complex behaviors are multifaceted and difficult to conduct depending on the amount of ecological realism the researcher wishes to achieve, but the experimental design can also be readily adapted to standard toxicity testing procedures (Matthers et al. 1985). The advantages, however, can be great and ecosystem effects of toxicant and stressor exposure may be more readily implicated. For example, fish exposed to heavy metals (cobolt, lead, cadmium) displayed alterations in dominance, feeding behavior, growth, and predator avoidance (Weis and Weiss, 1998; Sloman et al., 2002; 2003). Faulk et al. (1999) demonstrated that the F_1 generation of fish exposed to DDT had deficits in their response to vibratory and visual stimuli, as well as altered swimming behavior. Feeding and prey vulnerability have been used to examine sublethal contamination because predator and prey may be differentially affected by toxicants (Sandheinrich and Atchison 1990). Exposure to environmental mercury resulted in alterations in foraging (prey strikes and captures) as well as predator avoidance (Smith and Weis, 1997). Female reproductive behavior and nest digging were found to be disturbed upon exposure to increasing levels of acidification (Kitamura and Ikuta, 2001). Alterations in these behaviors can have serious effects to the individual and population of fish exposed, and may induce changes in gene flow and demography (Weiss et al., 2001; Nacci et al., 2002). To date, there are no well-characterized examples of automated systems to detect and evaluate predator-prey interactions. Certainly, this is not to say that the technology is not currently attainable.

Respiratory patterns. Respiration is a rhythmic neuromuscular sequence regulated by an endogenous biofeedback loop as well as by external environmental stimuli. Acute contaminant exposure can induce reflexive cough and gill purge responses to clear the opercular chamber of the irritant, and can also increase rate and amplitude of the respiratory cycle as the fish adjusts the volume of water in the respiratory stream. As exposure continues, the respiration cycle can become irregular, largely through decreased input as well as alterations in the endogenous pacemaker. Diamond et al. (1990) found that the frequency and amplitude of bluegill opercular rhythms and cough responses were altered following exposure to different contaminants. For example, dieldrin, an organochlorine insecticide, increased ventilatory frequency at concentrations above 24 $\mu g\ L^{-1}$ and caused cough responses and erratic movements. In contrast, zinc at 300 $\mu g\ L^{-1}$ reduced the amplitude of the respiratory response.

A variety of biomonitoring systems have been developed to assess changes in respiratory rate relative to stress exposure. These systems have the great advantage of sensitivity since many waterborne stressors, even at low environmental concentrations, affect gill tissue and respiratory function. Respiratory frequency, depth (volume) and cough frequency can be measured, non-invasively, using physiological signals from restrained sentinel fish. One such system to accomplish this with good repeatability has been described by Shedd et al. (2001).

Briefly, small flow-though exposure vessels house individual small fish in their respective chambers. Electrical signals generated by the respiratory and body movements of individual fish are detected by electrodes suspended above and below each fish. The signals are amplified, filtered, and analyzed using various algorithms on a personal computer. The muscular electrical output (0.05-1 mV) from each fish is independently amplified by a high-gain, true differential-input, instrumentation amplifier by a factor of 1000. Signal interference by frequencies above 10 Hz is attenuated by low-pass filters. The ventilatory parameters monitored by the computer include ventilatory rate, ventilatory depth (mean signal height) and gill purge (cough) rate.

Since fish are poikilotherms, temperature may also play an important role in determining exposure effects on a given fish species. Efforts at the UM Aquatic Pathobiology Laboratory to validate a respiratory response system as described above, have recently demonstrated temperature-dependant differences in bluegill (*Lepomis macrochirus*) exposed to brevetoxin at 19˚ versus 25˚C. Interestingly, respiratory responses (increased ventilatory, cough and "other movement") were altered at 25˚, but not 19˚C (Figure 1).

As with other behavioral systems, it is essential to properly integrate responses over time in order to achieve a good signal to noise ratio. The accuracy of any computer ventilatory parameter can be established by comparing the computer-generated values with concurrent strip chart recorder tracings (Shedd et al., 2001). Biomonitoring systems that measure fish ventilatory patterns have further application as early warning signals of water quality changes and toxicity (ASTM 1995; http://www.biomon.com/biosensor.html).

Social behavior and group dynamics

Toxicology studies typically focus on the exposure of single fish in the laboratory, when in reality, many fishes tend to congregate in groups and interact with many components of their environment. Group living is a basic life history characteristic of many fishes, with twenty five percent of all species forming schools or shoals during their life, and fifty percent during larval and juvenile stages (Radakov, 1973; Pavlov and Kasumyan, 2000). Pavlov and Kasumyan (2000) define a fish school as having all individuals oriented in the same direction, situated at a certain distance from each other, and unitary in all movements (polarized). Shoaling, in contrast, is a simple, spatial aggregation of fish attracted by a stimulus occurring independently of each other with no mutual attraction between individuals (non-polarized). Schooling and shoaling behaviors are complex social behaviors utilized by a wide diversity of fish species to increase individual fitness and propagate their genes in the population (Partridge, 1982) by providing defense from predation while increasing reproductive, foraging and migration efficiency. These behaviors have predictable structures, shapes, and responses to threats and environmental fluctuations. In addition, these behaviors are intimately tied to, and regulated by, the visual and lateral line systems, and are developed as soon as fish are able to swim and feed (Pavlov and Kasumyan, 2000). Alterations in school structure and density can be caused by individual differences in motivation, physiology, and abiotic and biotic factors of the environment. It has been demonstrated, through the use of shoal choice experiments and frame capture, that pesticide exposures can alter shoaling and schooling behaviors. Atlantic silversides (*Menidia medidia*) exposed to an acetyl cholinesterase inhibiting insecticide, carbaryl, displayed alterations in parallel orientation and increased distances between fish when compared to controls (Weis and Weis, 1975). In addition, swimming orientation in schools of threespine sticklebacks (*Gasterosteus aculeatus*) was disturbed following exposure to the organotin bis(tributyltin)oxide

(Wibe et al., 2001). Schooling declined following exposure of yearling common carp to 0.05 mg L^{-1} DDT and of fathead minnows to 7.43 mg L^{-1} of the herbicide 2,4-dinitrophenol at a pH of 7.57 (Holcombe et al., 1980).

Behavioral analysis systems

Rand (1985) extensively reviewed different exposure and tank designs that have historically been used to evaluate fish responses to stress exposures. These systems include both static and flow-through designs, as well as tube, Y-shape, rectangular, square, round and maze configurations (Bishai, 1962a,b; Davy et al., 1972, 1973; Folmar, 1976; Hansen, 1969; Hansen et al., 1972; Hill, 1968; Hoglund, 1953; Jones, 1947; Jones et al., 1956; Kleerekoper, 1969; Kleerekoper et al., 1972; Rehnoldt and Bida, 1970; Scherer & Nowak, 1973; Sprague, 1964, 1968; Sprague & Drury, 1969; Sprague et al., 1965; Westlake & Lubinski, 1976). The different exposure designs permit gathering data relevant to avoidance and attraction, ability to detect gradients, orientation to changes in light, sound and temperature, altered performance/stamina and learning. Changes associated with toxicant or stress exposure may be gathered visually, or with electrodes (Spoor et al. 1971; Spoor and Drummond 1972; Drummond and Carlson 1977), photocells or photoresistors (Waller and Cairns 1972), or videography. Data has been recorded using event recorders, strip chart recorders, polygraphs, and video processors and computers that can integrate signals and generate x-y coordinate data.

Recent hardware and software updates have been used to develop integrated exposure systems to test suites of behavioral endpoints. These exposure systems are used to investigate the effects of sublethal stressors on fish movement and responses to stimuli. For example, simultaneous video capture from multiple exposure arenas can be used to digitally track movement. Video data can then be used to address questions regarding differences between treatment groups or differences between pre- and post-exposure behavior using repeated measures analyses.

Figure 2 schematically depicts such a system using video cameras with multiple, dedicated analog video decks. In this system, twelve 10-liter exposure arenas were constructed from 14-inch (35.6cm) diameter polyvinyl chloride (PVC) pipes and end caps. Each arena had two 0.25-inch (0.64cm) threaded nipples that served as an input and drain. The input was bifurcated to accept both toxicant and dilution water flow lines. Toxicant and dilution water flow were electronically controlled with digital, multi-channel peristaltic pumps (Masterflex L/S, Cole-Parmer, Vernon Hills, IL) that were supplied by multiple, aerated 600-liter carboys. The exposure arenas were illuminated with 14:10 hour (Light: Dark) shadowless fluorescent lighting combined with a computer-controlled dusk and dawn cycle provided by incandescent lights. Lighting was controlled using X-10 computer hardware with an X-Tension (Sand Hill Engineering, Geneva, FL) software interface on a Macintosh operating system. Dusk-dawn lighting systems are very useful in reducing stress in aquatic holding and testing facilities, and can be developed, relatively inexpensively, using other techniques and hardware systems (e.g., Byers and Unkrich 1983). Twelve color CCD (charged-coupled device) cameras with manual iris and focus control were mounted above the respective arenas and were connected to dedicated VCR decks with time-lapse and dry contact closure capability for recording and computer control. All twelve VCRs were connected to a multiplexer that supported real-time observation (Figure 2). VCR recording and stop functions were synchronously activated remotely using X-10 technology.

Analog video data is then digitized in real time at 3 frames per second on a Macintosh platform (various hardware and software systems, both Macintosh- and Windows-based, are available for this task). Movement data is subsequently imported into a commercial tracking program (Videoscript Professional, version. 2.0©) and converted into x,y coordinate data. This program uses a custom algorithm designed for fish movement, which identifies and tracks the head of each fish target. The x,y coordinate data is then analyzed, using proprietary software designed at the Aquatic Pathobiology Laboratory, to obtain the desired behavioral endpoints. There are a variety of "off the shelf," commercially-available motion tracking and analysis systems (e.g., *Ethovision*, Noldus Information Technology; and *Expert Vision*, Motion Analysis Corporation) that can then be customized for particular research requirements.

A major benefit of this behavioral hardware and analysis system (Figure 2) is that investigators can take conventional behavioral analyses, which have previously been limited to ranks and counts, and quantify it using computer technology. The behavior and hardware analysis system has potential for greater flexibility in behavioral measurements than commercial behavioral quantification systems, but requires that a programmer be on staff. Current system capabilities and development areas include quantification of a wide range of behaviors, including daily swimming patterns, startle-type responses, avoidance behaviors and social interactions (Table 2). In addition, the system can remotely dose and record up to 12 individual fish or 12 groups of up to ten individuals simultaneously, for up to 1 hour, without the need to mark or tag the animals, thus reducing variance in behavior due to observer or handling disturbances. Finally, the system can be adapted for static and flow through exposures of environmentally relevant contaminants, and has the ability to be mobile for real-time field assessment.

Recent efforts have refined a set of behavioral endpoints in order to investigate the effects of sublethal stressors on fish movement and responses to stimuli. A suite of behavioral endpoints has been developed to evaluate the effect(s) of specific compounds at low levels on fish (Table 2). In addition, individual and group models can be utilized with the ability to test different species of small fish. To illustrate the importance of using a suite of exposure endpoints, data from killifish (*Fundulus heteroclitus*) exposed at 25˚C to an environmentally-relevant concentration (40 µg L^{-1}) of dissolved brevetoxin failed to produce exposure-associated alterations in any non-directed movement parameter. (Brevetoxin is an important biotoxin produced by harmful algae (dinoflagellates) associated with "red tides.") However, this acute, sublethal, low level exposure significantly altered startle responses in killifish. Conversely, a low anesthetic dose (60 mg L^{-1}) of the common anesthetic and model toxicant, methane tricanesulfonate (Alpharma 2001), was associated with significantly altered movement patterns (Figure 3) as well as startle responses.

In addition to exposure-related alterations in movement patterns, changes in startle response parameters can lend valuable insight into changes in the CNS that may have significant environmental consequences. Startle response parameters include, but are not limited to: response frequency, response latency, and average velocity. Startle responses can be elicited using a vibratory stimulus, and quantified from small to medium-sized fish using the exposure system described previously in this chapter (Figure 2). Alternatively, more precise acoustical tone pips, generated through underwater speakers, can be used as a stimulus with small fish (Figure 4). Startle response testing can also yield data sets that can be used for screening large groups of animals.

Preliminary studies. When developing or applying automated systems, preliminary studies are essential in order to optimize and validate behavioral hardware and maximize the

sensitivity of individual endpoints. For example, it is important to know (1) how a given species will acclimate to the exposure arenas, (2) effects of flow or toxicant infusion relative to distribution within the exposure arena as well as the sentinel's response to flow (attractance/avoidance to dilution flow), and (3) the optimal duration of observation time intervals. Discerning appropriate observation time intervals (both timing and duration) is critical. Shorter observation time tends to introduce more noise (and a tendency for false positives; type I errors), while longer observation time tends to average out potentially important exposure-related differences (tendency for negatives; type II errors). Figures 5 and 6 provide sample data indicating changes in movement patterns during an acclimation study, and toxicant distribution visualized in a dye experiment, respectively.

The importance of understanding flow and mixing dynamics in test chambers should not be underestimated (Kleerekoper et al. 1973). Most studies use steep gradient test systems in which the chemical concentration increases sharply over a short distance; in most instances the gradient is unknown to the experimenter but not to the fish. Fish must choose between clean water or contaminated water. When the gradient is steep, it may not mimic what fish actually experience in the field, except under unusual conditions, e.g., immediately downstream from an effluent pipe or mine tailing. Kleerekoper et al. (1973) showed that when goldfish (*Carassius auratus*) were attracted to copper concentrations between 11 and 17µg L^{-1} under shallow gradient conditions, but when the gradient was steep, fish avoided concentrations as low as 5 µg L^{-1} (Westlake et al. 1974).

Conclusions

Behavioral toxicology is a useful indicator of sublethal contamination because behavioral endpoints frequently occur below concentrations that are chronically lethal and at lower concentrations than those that affect growth (Little et al. 1993b; Cleveland 1991). As such, careful selection and design of behavioral tests could produce definable, interpretive endpoints for use in regulatory applications of product registration, damage assessments, formulation of water quality criteria and standards, and understanding changes in fish population dynamics based on anthropogenic effects. Behavioral endpoints could also, through the increased use and decreasing costs, be included in regulation of effluent standards. Although behavioral endpoints may often provide excellent sensitivity, utility or biologic significance, behavioral responses have historically not been routinely included in standard aquatic toxicity assessment programs.

In developing behavioral test methods for contaminant assessment, behavioral toxicological endpoints must address one or more of three assessment questions: (1) How well does the response measure effect or injury arising from exposure? (2) Does the response aid in the identification of the toxic agent? and (3) Does the use of the behavioral response increase the capability of contaminant assessments to predict the ecologic consequences of exposure? Eleven years ago, Little et al. (1993a,b) explicitly defined the challenges for behavioral toxicology: "(1) more and better behavioral testing procedures…must be developed and refined into effective tools which can be readily and clearly applied in contaminant assessments; (2) establishment of the links between behavioral effects observed in the laboratory and ecological effects observed in the field to aid in the development of contaminant assessment that are adequately predictive of population and community response; and (3) behavioral toxicologists must continue to work to dispel the erroneous paradigm prevalent in aquatic toxicology that suggests that behavioral responses are not suitable for inclusion in contaminant evaluation and assessment because they

are either unquantifiable, too complex, too variable, extraordinarily difficult to measure, not biologically significant, or lacking in ecologic relevance."

Great strides have been made towards elucidating and developing better testing procedures, in large associated with improvements in videography and the power of personal computers, and with the availability of video equipment to fit almost any budget and work requirement. However, additional work is required to validate the use of many individual and group behaviors as endpoints of sublethal toxicant and stressor exposure.

Today, the relationship between many exposure-related behavioral alterations observed in the laboratory and their relevance to ecological effects observed in the field remains poorly understood. A noteable exception is avoidance responses that have been intensively studied in the laboratory and verified in the field through the use of telemetry. As a result, avoidance is the only behavioral response that has legal standing in the United States as evidence of environmental injury under proceedings of the Comprehensive Environmental Response and Compensation Liability Act. Consequently, there is opportunity for researchers to actively contribute to the development of contaminant assessment paradigms that are predictive of population and community response. To accomplish this goal, sound experimental design, combined with proper statistical analyses, are essential for developing approaches using fish movement for the bioindication of stressors or assessing mechanism of action.

Continued interest and new developments in automated behavioral monitoring and analysis techniques, especially those that can be verified in field trials, are being used to make functional inroads to dispel the erroneous paradigm that "behavioral responses are not suitable for inclusion in contaminant evaluation and assessment" (as stated by Little et al., 1993). Behavioral responses should be more routinely included and integrated in contaminant evaluation as researchers continue to learn about and develop new automated techniques that allow for relatively inexpensive and rapid collection, processing, and verification of behavioral disruptions associated with contaminant exposure.

The behavioral exposure systems and endpoints described in this chapter can be applied to quantify differences in behavior associated with exposure to metals, organics, pesticides, algal bloom toxins, alterations in water quality and agricultural waste. Behavioral endpoints also provide a valuable tool for the quantification of differences in reproductive behaviors, predator-prey interactions, behavioral changes across environmental gradients, and differences in swimming behavior between species. Analysis of behavioral responses to a variety of stimuli can provide quantitative measures of neural and mechanical disruption, reflecting biochemical and physiological alterations (Brewer et al., 2001). These additional tools support toxicological investigation with endpoints other than traditional LC50s, and may aid in determining new no observed effect levels (NOELs) and lowest observed effect levels (LOELs), as well as investigating the environmental relevance of various low-level exposures. Behavioral toxicology may ultimately provide sensitive, non-invasive, and broadly applicable endpoints for the description of integrated, whole animal responses associated with exposure to a wide variety of stressors.

Acknowledgements
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Table 1. Behavioral alterations observed in fish associated with different stress agents.

Host species	Stressor	Behavior/Movement Comments	Reference
	Biologicals		
Salmonids	*Aeromonas salmonicida*	Lethargy, inappetence, loss of orientation, abnormal swimming behavior	Munro & Hastings, 1993; Austin & Austin, 1999
Fish	*Bacillus* spp.	Weakness, lethargy	Austin & Austin, 1999
Cold freshwater fish	Bacterial gill disease	Lethargy, flared opercula, coughing, dyspnea	Noga, 1996
Fish	Bacterial gill disease	Lethargy, anorexia, increased respiration	Bullock & Conroy, 1971; Ferguson et al., 1991
Fish	Blood flukes	Lethargy, flashing	Noga, 1996
Bluegill	40 ppb brevetoxin	Altered ventilatory responses; reversible after 1 hr	Kane et al., 2000
Channel catfish	Channel catfish virus disease	Hanging head up in the water, disorientation, corkscrew swimming	Noga, 1996
Fish	*Chryseobacterium scopthalmum*	Lethargy	Mudarris & Austin, 1989; Mudarris & Austin, 1992
Coho salmon and farmed trout	*Clostridium botulinum*	Sluggishness, erratic swimming, listlessness, may alternately float and sink before showing temporary rejuvination	Cann & Taylor,1982
Warm and cold freshwater fish, cold water marine fish	Columnaris	Dyspnea	Noga, 1996
Striped bass	*Corynebacterium aquaticum*	Inappetance, swimming more slowly	Austin & Austin, 1999
Deep angelfish	Deep angelfish disease (herpes-like)	Loss of equilibrium, headstanding	Mellergaard & Bloch, 1988
Catfish	*Edwardsiella ictaluri*	Fish hang listlessly at surface in a head up-tail down posture, sometimes swimming rapidly in circles. Corkscrew spiral swimming, depression	Plumb, 1993; Noga, 1996.
Lake trout, Lake trout x brook trout	Epizootic epitheliotropic disease (herpes virus-like)	Sporadic flashing, corkscrew swimming	Bradley et al., 1989
Striped mullet	*Eubacterium tarantellus*	Erratic swimming, loss of equilibrium, spiral swimming; floating at surface & sinking to the bottom repeatedly	Udey et al., 1976
Salmonids	*Exophiala salmonis*	Erratic swimming	Richards et al., 1978; Alderman, 1982
Farmed barramundi	*Flavobacterium johnsoniae*	Listlessness, anorexia	Carson et al., 1993
Coho salmon	*Flexibacter psychrophilus*	Nervous spinning	Holt et al., 1993
Fish	Gill *Cryptobia* infestation	Anorexia	Noga, 1996

Cherry salmon	*Hafnia alvei*	Slow swimming	Teshima et al., 1992
Salmonids	Infectious hematopoietic necrosis virus	Lethargy, sporadic hyperactivity	Noga, 1996
Salmonids	Infectious pancreatic necrosis virus	Corkscrew spiral swimming, whirling	Noga, 1996
Fish	*Lactococcus gravieae*	Moribund fish swim erratically just below the surface of the water	Austin & Austin, 1999
Fish	Motile Aeromonads	May swim normally or hang in the water, on their sides	Roberts, 1993
Fish	Mycobacteria	Listlessness, anorexia, dyspnea, inappetence	Wolke & Stroud, 1978; Dulin, 1979; Giavenni et al., 1980; van Duijn, 1981; Austin & Austin, 1999
Mainly salmonids	*Myxobolus cerebralis*	Whirling or frenzied, tail-chasing behavior, impaired balance	Hoffman, 1976
Fish	*Nocardia*	Inactivity, anorexia	Frerichs, 1993
Bluegill	*Pfiesteria piscicida* (laboratory exposure to non-axenic cultures)	Decreased aggression and social interactions, followed by solitary time spent on or near the bottom. Subsequent sporadic bursts of activity including tailstanding, bobbing, corkscrewing in place, breaking at the surface, followed by inactivity at a 45 degree angle in water column, or resting on the bottom prior to morbidity. Strong elevations in cough rate and % movement without notable changes in respiratory rate.	Kane et al., unpublished data Kane et al., 2000
Fish	*Pfiesteria piscicida* cultures	Pigmentation changes, lethargy, episodic hyperactivity and decreased respiration.	Burkholder et al., 1992; Burkholder et al., 1995; Marshall Hget al., 2000; Noga et al., 1993
Fish	*Plesiomonas shigelloides*	Inappetence	Klein et al., 1993
Fish	*Pricirickettsia salmonis*	Gathering at the surface of cages, sluggishness, inappetence	Austin & Austin, 1999
Fish	Protozoan ectoparasites	Dyspnea	Noga, 1996
Rio Grande cichlid; zilli cichlid	Rio Grande cichlid rhabdovirus disease	Lethargy	Malsberger & Lautenslager, 1980
Salmonids	Salmonid rickettsial septicaemia	Lethargy, swimming near the surface or at the side of the net	Turnbull, 1993

Rabbitfish	*Shewanella putrefaciens*	Lethargy	Saeed et al., 1987
Carps; sheatfish; guppy; Northern pike	Spring viremia of carp (*Rhabdovirus carpio*)	Decreased swimming ability	Fijan, 1972
Fish	*Staphylococcus aureus*	Lethargy	Austin & Austin, 1999
Fish	*Streptococcus*	Erratic swimming	Kitao, 1993
Tilapia	*Streptococcus difficilis*	Lethargy, erratic swimming, showing signs of dorsal rigidity	Austin & Austin, 1999
Farmed Atlantic salmon	Unidentified gram-negative rod	Lethargy, swimming close to surface, loss of balance	Austin & Austin, 1999
Warm marine fish	Uronemosis	Dyspnea, hyperactivity, then lethargy.	Noga, 1996
Rainbow trout	*Vagococcus salmoninarum*	Listless behavior, impaired swimming	Michel et al., 1997
Fish	*Vibrio alginolyticus*	Sluggishness	Colorni et al., 1981; Austin et al., 1993
Fish	*Vibrio anguillarum*	Anorexia, inactivity	Hjeltnes & Roberts, 1993; Austin & Austin, 1999
Sharks	*Vibrio harveyi*	Lethargy, stopped swimming, appearing disorientated	Austin & Austin, 1999
Fish	*Vibrio salmonicida*	Inappetence, disorganized swimming	Hjeltnes & Roberts, 1993
Japanese horse mackerel	*Vibrio trachuri*	Erratic swimming	Austin & Austin, 1999
Cold freshwater fish (mainly salmonids)	Viral hemorrhagic septicemia	Lethargy, congregating away from the current on the edges of the pond or raceway, looping swimming behavior, darting through the water and spiraling at the bottom of the pond	Noga, 1996
Atlantic salmon	*Yersinia intermedia*	Lazy movements, congregating at the surface of the water	Carson & Schmidtke, 1993
Rainbow trout	*Yersinia ruckeri*	Sluggishness	Stevenson et al., 1993
	Contaminants		
Rainbow trout	Al	Avoidance behavior(s)	Exley, 2000
Rainbow trout	Al	2 minute clips, position holding, slow and burst type swimming	Allen & Wilson, 2000
Atlantic salmon, rainbow trout	Cu & Zn (salmon) Zinc sulfate (trout)	Avoidance behavior(s) 53 ppb (salmon) 5.6 ppb (trout)	Sprague, 1964

3-Spined sticklebacks	BBP (butyl benzyl phthalate)	Shoal choice	Wibe et al., 2001
Rainbow trout	Carbaryl	Velocity, school size NNA @ 1fps for 1 min (60frames)	Weis and Weiss, 1974
Rainbow trout	Carbaryl	Swimming capacity, feeding activity, strikes	Little et al., 1990
Fathead minnow	Cd	Decreased predator avoidance, 25-375 ppb	Sullivan et al., 1978
Rainbow trout	Cd	Altered dominance, feeding & aggression	Sloman et al., 2003
Zebrafish	Cd	Avoidance behavior(s)	Grillitch et al., 1999
Bluegill	Cd, Cr, Zn	Hyperactivity	Ellgaard, 1978
Green sunfish	Chlordane	Avoidance behavior(s) 20, 10, 5 mg/L	Summerfelt & Lewis, 1967
Rainbow trout	Co	Dominance hierarchy, growth, food intake and coloration	Sloman et al., 2001
Rainbow trout	Copper sulfate, dalapon, acrolein, dimethylamine salt of 2,4 D, xylene	Avoidance behavior(s)	Folmar, 1976
Pink salmon	Crude Oil	Avoidance behavior(s) 1.6 mg/L	Rice, 1976
Estuarine Fish	Cu	8 ppb olfactory disruption	Hara et al., 1976
Goldfish	Cu	Velocity, TDT, turning angles	Kleerekoper et al., 1972
Rainbow trout	Cu	Attraction 460-470 ppb Avoidance 70ppb	Black & Birge, 1980
Salmon	Cu	Altered chemoreception and home stream recognition	Sutterlin & Gray, 1973
Rainbow trout	Cu & Ni	Attraction 390 ppb (Cu), 6ppb (Ni) Avoidance 4.4 ppb (Cu), 24ppb (Ni)	Giattina et al., 1982
Rainbow trout	Cu, Co	Avoidance behavior(s)	Hansen et al., 1999
Atlantic salmon	DDT	Alteration in temperature preference, 5-50 ppb	Ogilvie & Anderson, 1965
Bluegill	DDT	Hyperactivity	Ellgaard, 1977
Brook trout	DDT	Biphasic conc.-response relationship for temperature preference and DDT	Miller & Ogilvie, 1975
Croaker	DDT	Effects on the F_1 generation vibratory/visual stimuli, burst speed	Faulk et al., 1999
Goldfish	DDT	Increases in velocity, turns and area occupied	Weis & Weiss, 1974

Brook trout	DDT and methoxychlor analogs	Alteration in temperature preference for methoxychlor analogs	Gardner, 1973
Mosquitofish	Endrin, toxaphene, parathion	Avoidance behavior(s)	Kynard, 1974
Mummichog	Environmental MetHg	Prey capture (strikes and captures), predator avoidance, lab & field validation	Smith & Weis, 1997
Rainbow trout	Heavy Metal Mix	Avoidance behavior(s)	Svecevicius, 2001
Chinook salmon	Kraft Mill extract	Avoidance behavior(s) 2.5 – 10%	Jones et al., 1956
Smelt	Kraft Mill extract	Avoidance behavior(s) 0.5%	Smith & Saalfeld, 1955
3-Spine stickleback	Lead nitrate	Attraction at high concentration Avoidance at low concentration	Jones, 1947
Shiners	Malathion	Concentration-dependent decrease in temperature selection	Domanik & Zar, 1978
Medaka	OPs	Vertical path analysis in 1 minute clips, velocity, meandering, TDT, smooth vs. erratic swimming, 4 fps	Kwak et al., 2002
Mosquitofish	OPs	Avoidance behavior(s)	Kynard, 1974
Goldfish	Parathion	Hypoactivity and alteration in angular change	Rand, 1977a
Mummichog	Pb	Feeding activity and performance, predator avoidance	Weis & Weiss, 1998
Rainbow trout fingerlings	Phenol	Decrease in predator avoidance to adults, 0.5-18 mg/L	Schneider et al., 1980
Minnow	Phenol and p-chlorophenol	Avoidance behavior(s)	Hasler & Wisby, 1950
Herring	Pulp Mill extract	Avoidance behavior(s)	Wildish et al., 1976
Rainbow trout	Rotenone	Avoidance behavior(s)	Hogue, 1999
Roach	2,4,6 trinitrophenol	Attraction	Lindahl & Marcstrom, 1958
3-Spine stickleback	Zinc and Copper Sulfate	Avoidance, “stupefied and motionless”	Jones, 1947
	Water Quality		
Brook trout	Acidification	Avoidance behavior(s)	Peddler and Maly, 1986
Trout	Acidification	Female repro. behavior, nest digging	Kitamura and Ikuta, 2001
Carp	Ammonia	Center of gravity of a group of fish/vertical location	Weinstein and Kimmel, 1998
Largemouth bass and mosquitofish	Ammonia	Decreased prey consumption of bass, less effect on mosquitofish	Woltering et al., 1978
Fish	Ammonia poisoning	Hyperexcitability, fish often stop feeding	Daoust & Ferguson, 1985
Fish	Chlorine poisoning	Dyspnea	Noga, 1996

Fish	Environmental hypoxia	Fish piping for air, gathering at water inflow, depression	Scott & Rogers, 1980; Francis-Floyd, 1988
Cold freshwater fish	Hypercarbia	Dyspnea	Noga, 1996
Bluegill	Hypoxia	Altered ventilatory & cough responses	Kane et al., 2000
Mullet, menhaden, spot, croaker, pinfish, and mummichog	Hypoxia	Avoidance behavior(s)	Wannaker and Rice, 2000
3-spine sticklebacks, minnows, and brown trout	Hypoxia & temperature change	Avoidance behavior(s)	Jones, 1952
Fish	Nitrite poisoning	Lethargy, congregating near the water surface	Noga, 1996
Fish	Low pH	Hyperactivity, dyspnea, tremors	Schweldler et al., 1985
Fish	Total suspended solids	Coughing to clear gills	Noga, 1996
Fish	Low temperature stress	Inactivity, depression	Noga, 1996

Table 2. Individual and group endpoints for movement analysis.

Individual Endpoints	Definition
Percent Movement	The # of seconds the fish satisfies movement criteria divided by the total # seconds spent swimming, multiplied by 100.
Velocity	Average velocity (cm/sec) while the fish is moving during the experimental period.
Angular Change	The difference (0-180°) between the angular components of two consecutive 1 second movement vectors (degrees/sec) divided by the total # of consecutive 1 second movement events. Angular change was only calculated when two consecutive movement vectors met the movement criteria.
Path Tortuosity (Fractal dimension)	Fractal dimension (D) is calculated using the hybrid divider method (Hayward et al., 1989) and is an indicator of path complexity. A series of path generalizations are created at various step sizes and the data are mathematically drawn on a Richardson plot (Log path length vs. Log step length). D is then calculated as 1 minus the slope of the Richardson plot. D=1 if the path is a straight line and 2 if it completely fills the 2 dimensional plane.
Space Allocation	The # of frames fish spend in predefined regions of the exposure arena divided by the total # frames.
Distance from Center	Sum of individuals distance from the center of the exposure arena (cm) divided by the total # frames. This is a measure of how close the fish swims to the walls of the arena.
Relative Burst Frequency	The # of frames that velocity is > 3 SDs above mean velocity.
Startle Response	Duration of movement, latency to response, percent response, and burst swimming. (Response to vibratory/auditory stimulus)
Anti-predator response	Percent fish halting movement, latency to response, percent exhibiting startle-type response, direction of movement (toward or away visual stimuli), and group endpoints. (Response to overhead "fly-by" of bird silhouette)

Group endpoints	Definition
Interactions	The number of times two fish swim within 0.1 body lengths of each other.
Percent Shoaling	Number of frames satisfying shoaling criteria divided by the total number of frames.
Shoal NNA	Angle of trajectory between 2 fish in a shoal, must be greater than 45°.
Shoal NND	Distance between nearest and second nearest neighbor for each fish in a shoal (minimum 3 fish).
Percent Schooling	Number of frames satisfying schooling criteria divided by the total number of frames.
School NNA	Angle of trajectory between 2 fish in a shoal, must be less than or equal to 45°.
School NND	Distance between nearest and second nearest neighbor for each fish in a shoal (minimum 3 fish).
Percent Solitary	Number of frames not satisfying shoaling or schooling criteria divided by the total number of frames.
Solitary NND	Distance between nearest and second nearest neighbor for each individual fish not in a shoal or school.
Velocity	Speed of fish calculated in centimeters per second.

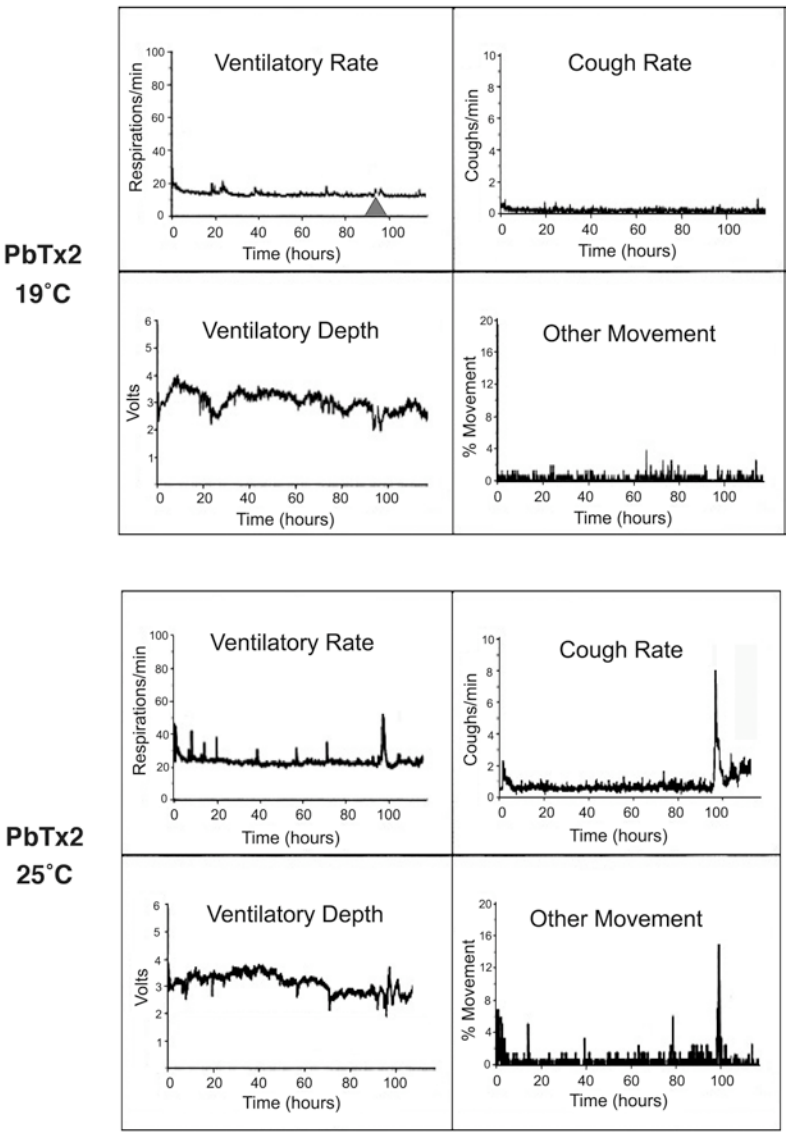

Figure 1. Ventilatory responses of bluegill to brevetoxin (PbTx2, 40 µg L^{-1}) at 19˚ and 25˚C using a flow-through exposure system as described by Shedd et al (2001). These data illustrate the importance of water quality variables (temperature in this case) in determining the behavioral effects of certain toxicants. The upper panel of four graphs shows respiratory responses of bluegill at 19˚C; no significant responses are noted in these data (gray triangle in upper-right graph indicates start of exposure after 96 hours of baseline acclimation). At 25˚C, bluegill respond to exposure with significantly increased ventilatory rate (but not ventilatory depth), cough rate, and "other movement." This latter category represents data that does not fall into one of the three previous categories, based on the analytical algorithms; it often reflects whole body movement in the exposure chambers.

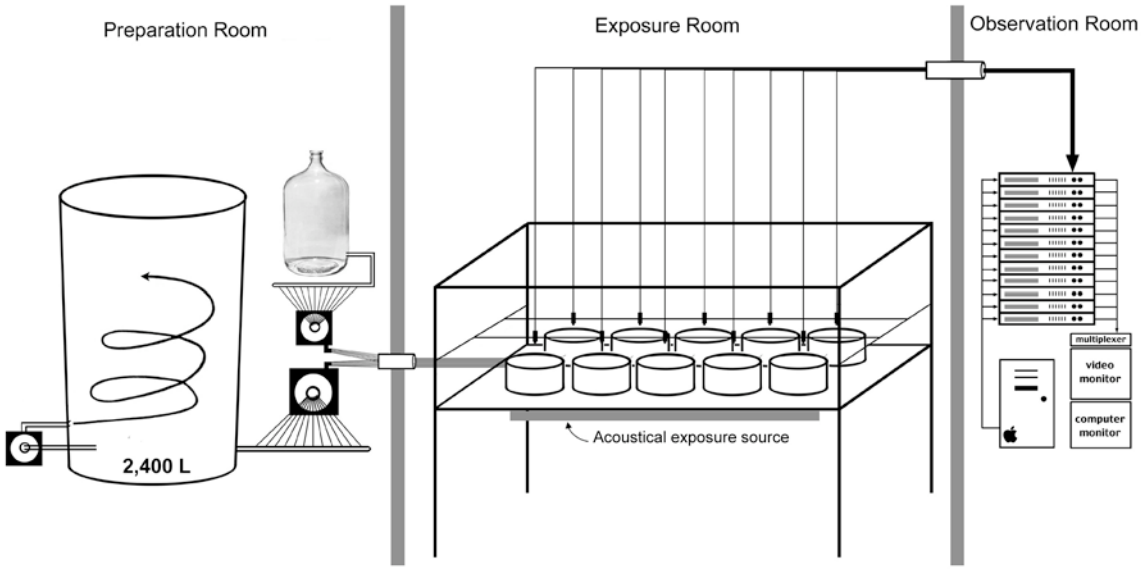

Figure 2. Schematic showing flow-through exposure arenas with dedicated, overhead video cameras (shadowless lighting and drainage not shown) in the behavioral toxicology exposure suite at the University of Maryland Aquatic Pathobiology Laboratory. Water is prepared, temperature adjusted and delivered from an adjacent "preparation room," while video data is amassed by computer-controlled video decks. Toxicants or aqueous stress agents, and flow-through dilution water, are pumped from the preparation room by computer-controlled peristaltic pumps. An instantaneous acoustic/vibratory stimulus can be provided to discern differences in startle response behavior. In this diagram, an acoustical exposure source is indicated under the platform supporting the exposure arenas. The source in one experiment consisted of three carefully-positioned, spring-loaded mouse traps that could be tripped remotely with conjoined pull strings.

Video response data is transformed into x,y coordinate data, and relevant endpoints are discerned using our tracking and analytical software. Each of 12 arenas can house individual or multiple fish (up to 120 fish can be monitored simultaneously), or individual arenas can be replaced with multi-chamber arenas to aid in identifying individual animals that respond (or fail to respond) to different stimuli (see Figure 4 describing chambers used to discern startle response with small fish).

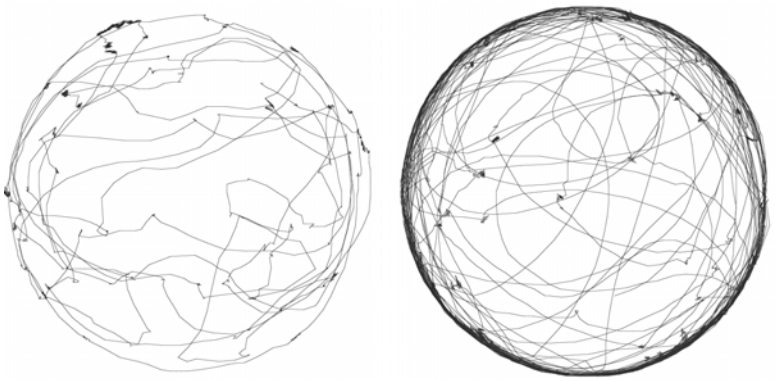

Figure 3. Paths of an individual killifish (*Fundulus heteroclitus*) before (left) and after (right) exposure to a sub-anesthetic dose (60 mg/L) of MS-222. Analysis of these 30-min paths indicate that exposure to low doses of this anesthetic agent causes an increase in percent time in motion (from 12 to 49%) and movement velocity (9.1 to 11.9 cm/sec), a decrease in path complexity (fractal dimension of 1.082 to 1.027), and a tendency to swim close to the arena periphery (change in distance from center). All of these endpoints describe quantifiably significant alterations in movement associated with exposure. Functionally, exposed animals tended to increase their speed and stay in motion to compensate for slight loss of equilibrium. The "intoxicated state" was depicted by "hugging" the vessel walls during movement and failing to maintain vigilance (loss of path complexity). MS-222 exposure also significantly altered the startle response of exposed fish such that there was a decrease in the number of responses, a decline in the response duration, and an increased response latency ($P<0.02$). Startle responses to MS-222 and other chemical stressors were illicited using instantaneous vibratory stimulus as described in the legend for Figure xxx.

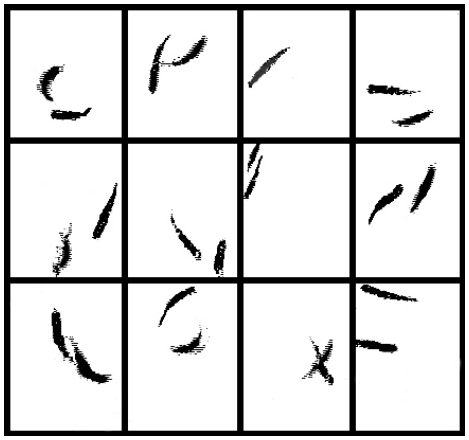

Figure 4. An alternative, more precise mechanism to illicit startle response. Video frame captures showing startle response from zebrafish (*Danio rerio*) exposed to instantaneously-pulsed, tone pip (400 Hz, 150 db). The stimulus was generated using a tone generator, an amplifier, and a calibrated underwater speaker. Fish were acclimated individually in each of twelve, small chambers. Video frames were captured 33 ms prior to the stimulus and 33 ms after the stimulus to visualize the position of the fish prior to and after eliciting startle response. A fish doublet is seen in all chambers where the animal startled in response to the stimulus. All animals startled in this image except for one (top row, 3rd column). In this trial, typical of others at this sound pressure level, 12 out of 12 fish in 8 out of 10 replicate trials startled; 11 out of 12 fish in the other two replicates startled. Note that the acoustic stimulus described here is different from the "acoustical sound delivery board" as illustrated and described in Figure 2.

Figure 5. Acclimation experiment with killifish showing plotted x,y coordinate data for 30 minutes of movement from a single animal 1 hour after entry into control exposure area (left), after 24 hours (center) and after 48 hours (right). These plots indicated that, over time, there was a significant, repeatable decrease in path complexity (fractal dimension), a reduction in angular change and time spent in the center of the vessel (swimming close to vessel wall). These data were useful in determining appropriate timing to expose and observe these fish. We found that after 24 hours of arena acclimation, animals maintained sufficient movement complexity and repertoire to discern changes in non-directed swimming patterns in response to certain stress exposure scenarios.

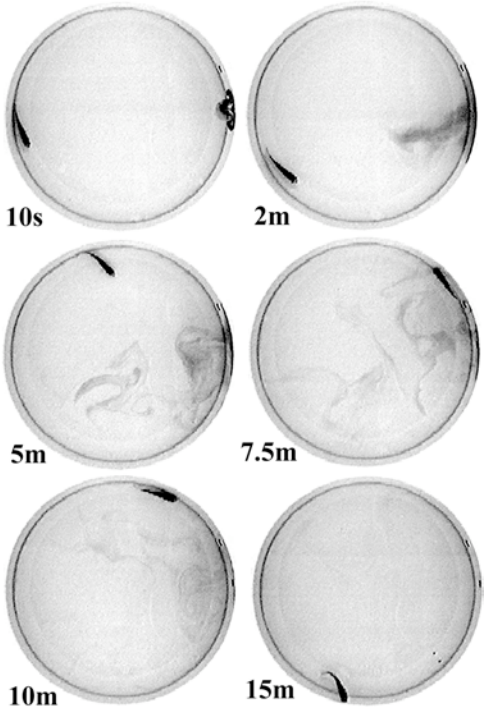

Figure 6. Preliminary experiment using non-toxic food-grade dye to discern patterns and timing of "toxicant" dispersion in exposure arenas. A 5-mL bolus of diluted dye was infused into multiple vessels and video frames were captured at 10 seconds, 2 minutes, 5 minutes, 7.5 minutes, 10 minutes and 15 minutes. These sequential images from a single arena indicate that avoidance/attractance behavior can be observed within the initial 2-5 minutes of exposure, while the "toxicant" is not yet dispersed throughout the arena and there is still a concentration gradient in the "sector" of the arena where the stressor is pumped. After 15 minutes the dye is well-mixed with the dilution water throughout the vessel. A number of factors will influence the rate of dispersion throughout the vessel, including the flow rate of the infused stress agent, the density and temperature of the mixtures, the number and species of test fish in the arena, and the shape and volume of the arena.

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