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Behavioral Indicators of Sublethal Toxicity in Rainbow Trout

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Abstract. Four measures of behavior—spontaneous swimming activity, swimming capacity, feeding behavior, and vulnerability to predation—were assessed as indicators of sublethal toxicity in rainbow trout (*Oncorhynchus mykiss*) in 96-hr exposures to sublethal concentrations of six agricultural chemicals: carbaryl, chlordane, dimethylamine salt of 2,4-dichlorophenoxyacetic acid (2,4-DMA), tributyl phosphorotrithioate (DEF[1]), methyl parathion, and pentachlorophenol. After exposures, behavioral changes consistently demonstrated sublethal toxicity, but effects on specific behaviors varied with contaminants and their concentrations were altered by the water quality criterion concentration for chlordane (2 μg/L), and at a concentration of DEF (5 μg/L) that had previously been shown to inhibit growth and survival after a 90-day exposure. Feeding behavior was inhibited most by exposure to DEF, 2,4-DMA, and methyl parathion. Vulnerability to predation was heightened most by exposure to carbaryl and pentachlorophenol. Although all chemicals inhibited spontaneous swimming activity, only carbaryl, DEF, and 2,4-DMA influenced swimming capacity.

Behavioral studies of test organisms conducted in conjunction with standard 96-hr toxicity tests provide data beyond those for establishing lethal concentrations of contaminants; they also demonstrate toxic effects caused by sublethal chemical concentrations. However, attempts to assess the sensitivity and usefulness of various behavioral responses as indices of contaminant toxicity have been hampered by insufficient data. Studies published in the literature are usually not directly comparable because they are based on different species, different chemical substances, and different methods (Little *et al.* 1985).

The purpose of this study was to determine the relative effects of sublethal exposure to six chemicals on four types of behavior in rainbow trout (*Oncorhynchus mykiss*). The data were obtained from several different studies. The rainbow trout was selected as the test organism, because it is commonly used in toxicity tests. The four responses evaluated—swimming activity, swimming capacity, feeding, and vulnerability as prey—were chosen because of their implications for survival of fish in their natural environment. The six agricultural chemicals tested represent four distinct chemical groups: carbamates, organochlorines, organophosphates, and phenolics. These compounds vary in environmental persistence, physiological mode of action, and bioaccumulation potential and should be typical of the range of contaminants to which fish are exposed in the wild.

Methods

Test Organisms

Laboratory exposures and behavioral measurements were conducted at the National Fisheries Contaminant Research Center (Columbia, MO) with small rainbow trout weighing 0.5 to 1.0 g. The fish were cultured and exposed in well water (pH 7.8, 237 mg/L alkalinity as $CaCO_3$, and 272 mg/L hardness as $CaCO_3$) at 16°C under a 14:10 light-dark photoperiod and were fed commercial fish food *ad libitum*, supplemented with daphnids or larval chironomids several times weekly.

Toxicant Exposure

Six technical grade compounds were applied during 96-hr static exposures: carbaryl (1 naphthyl *N*-methylcarbamate) from Union Carbide Corp.; chlordane (1,2,3,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7 methanoidene) from Velsico Chemical Corp.; 2,4-DMA (dimethylamine salt of 2,4-dichlorophenoxyacetic acid) from Union Carbide Corp.; DEF (*S,S,S*,tributyl phosphorotrithioate) from Mobay Chemical Corp.; methyl parathion (*O,O*,dimethyl *O*-*p*-nitrophenyl phosphorothioate) from Monsanto Corp.; and pentachlorophenol from Dow Chemical Co. Stock solutions of these compounds were prepared by dissolving appropriate amounts of each (adjusted for percent purity) in reagent grade acetone. Control treatments were exposed to equal amounts (1 ml) of acetone. Exposures shown in Table 1 ranged from subacute to chronically toxic concentrations that may occur in the environment. Static tests with nominal exposure concentrations were used throughout to parallel

[1] Reference to trade names or manufacturers does not imply government endorsement of commercial products.

Table 1. Chemical compounds and concentrations used in the exposures

Chemical	Type of compound	Compound purity (%)	96-hr LC50[a] (mg/L)	Concentrations tested (mg/L)		
				Low	Medium	High
Carbaryl	Carbamate insecticide	99.5	1.95	0.01	0.1	1.0
Chlordane	Organochlorine insecticide	100.0	0.042	0.0002	0.002	0.02
DEF[b]	Organophosphate defoliant	95.4	0.660	0.005	0.05	0.1
2,4-DMA[c]	Phenoxy herbicide	41.0	100.0	0.5	5.0	50.0
Methyl parathion	Organophosphate pesticide	76.8	3.7	0.01	0.1	—
Pentachlorophenol	Organochlorine insecticide	92.0	0.052	0.0002	0.002	0.02

[a] Values from Johnson and Finley (1980)
[b] *S,S,S,* tributyl phosphorotrithioate
[c] Dimethylamine salt of 2,4-dichlorophenoxyacetic acid

the exposure procedures that are used in toxicant screening tests. Fish were acclimated in 32-L glass exposure aquaria in 28 L of water for 48 hr and exposed in groups of 24 to each treatment under static conditions for 96 hr before initiation of behavioral tests. Pretest mortality was less than 5%. Several groups were exposed at each concentration to ensure that sufficient numbers of test organisms were available for testing. Exposures to different compounds were conducted at different times. Fish were not fed during the acclimation and exposure periods.

Behavioral Measurements

Swimming capacity, spontaneous activity, and feeding behavior were observed from 0800 hr to 1600 hr with fish from each treatment group tested each hour to compensate for diurnal fluctuations in behavioral activity.

Swimming capacity was measured in a plexiglass stamina chamber (12 cm diameter, 152 cm long) modified from Brett (1971). Individual fish were held in the chamber for 5 min at a water velocity of 9.2 cm/sec to ensure they would swim; thereafter, velocity was increased by 6.2 cm/sec every 3 min until the fish was no longer able to swim. Swimming capacity was calculated by the equation, Swimming Capacity (cm/sec) = A + 6.2 (B/180), which integrates water velocity and swimming time: A is the water velocity (cm/sec) during the last successfully completed interval, and B is time (sec) that fish spent at the highest water velocity reached.

For spontaneous swimming activity tests, fish were individually transferred to an opaque plexiglass cylinder (24 cm in diameter × 25 cm tall) containing 3 L of water. Each fish was held in the cylinder for 5 min and observed by an overhead television camera for 2 min to determine the amount of time the fish was in motion. The observation period was timed with a count-down timer and cumulative time in motion was timed with a stopwatch.

After the swimming activity tests, feeding behavior was observed; one *Daphnia magna* was added to the activity chamber during tests of fish exposed to carbaryl and methyl parathion, and 5 daphnids were added for other tests. The number of times fish struck at the daphnids and the number of daphnids eaten during a 5-min period were recorded.

To test vulnerability to predation, fish were marked before exposing them to a toxicant by anesthetizing them with tricaine methanosulfonate (2% solution) and injecting them with red, blue, or green latex dye to differentiate treatments. The subdermal dye marks (0.5 mm dots) were randomized among treatment groups during each replicate test to insure that the marks given to fish of a particular group did not increase their vulnerability to predation. After a 36-hr holding period to allow for handling-induced mortality, fish were exposed to the agricultural chemicals for 96 hr. After exposure, fish from each treatment group (5 fish for carbaryl, chlordane, and pentachlorophenol tests, 10 for the remaining tests because larger predators were used) were added to each of four cylindrical tanks (54 cm in diameter, 73 cm deep) that contained a gravel substrate and plantings of *Elodea* sp. and received a flow of 1 L/min of uncontaminated well water. One predator (a 40 to 60-g largemouth bass, *Micropterus salmoides*) was added to each after a 4 hr acclimation. The unexposed predators had not been fed for 4 days before the test to insure consistent appetite. All fish were removed when about half of the rainbow trout had been eaten (6 to 8 hr after the start of the test), and survivors of each treatment group were counted.

Statistical Analysis

Square root transformations were made on measurements of spontaneous activity, feeding strike frequency, and prey consumption to achieve normality. Predation data were arc sine square root transformed. Analysis of variance and Duncan's multiple range tests were performed on all data by using SAS General Linear Models procedures (Statistical Analysis Systems, Triangle Park, NC) to determine if swimming capacity, spontaneous swimming activity or feeding were significantly affected by the test exposures. Chi-square analysis was used to determine differences in the vulnerability of fish to predation.

Results

None of the six agricultural chemicals induced mortality during the 96-hr exposures at the nominal concentrations tested, but the behavior of rainbow trout was impaired in different ways by the chemicals (Table 2).

Swimming capacity was not affected by any exposure concentration of chlordane, methyl parathion, or pentachlorophenol, and was significantly reduced only in fish exposed to the highest concentration of carbaryl (Table 2). Exposure to DEF and 2,4-DMA induced significant increases in swimming capacity at low concentrations and significant decreases in swimming capacity at high concentrations (Table 2).

Changes in spontaneous swimming activity were observed after exposure to each chemical (Table 2). Fish exposed to 1.0 mg/L carbaryl—50% of the LC50 reported by Johnson and Finley (1980)—were significantly less active than controls. In contrast, methyl parathion in concentrations as

Table 2. Influence of a 96-hr exposure to six agricultural chemicals on the behavioral responses of rainbow trout

Chemical (mg/L)[a]	Mean behavioral response[b]					
	Swimming capacity (cm/sec)	Swimming activity (secs)	Strike frequency $(5\ min)^{-1}$	Daphnia consumed	% Consuming daphnia	% Survival from predation
Carbaryl						
0	24.7 x	116 x	1.0 x	1.0 x	100 x	85 x
0.01	25.4 x	119 x	1.2 x	0.7 x	70 x	35 yz
0.1	23.6 x	113 x	1.1 x	0.6 x	60 x	50 y
1.0	19.9 y	79 y	0.6 x	0.3 y	30 y	25 z
S.E.[c]	0.8	4.0	0.3	0.1		0.3
N[d]	26	10	10	10	10	4
Chlordane						
0	17.2 x	115 x	6.8 x	4.8 x	100 x	67 x
0.0002	18.1 x	98 xy	4.6 xy	3.5 xy	90 x	73 x
0.002	17.4 x	77 y	3.8 y	2.2 y	50 y	20 y
0.02	16.1 x	69 y	2.5 y	1.7 y	50 y	7 y
S.E.	0.8	12.0	1.0	0.6		0.3
N	17	10	10	10	10	3
DEF[e]						
0	25.5 x	118 x	2.3 x	1.9 x	80 x	62 x
0.005	26.6 y	90 xy	1.6 x	0.8 y	60 x	60 x
0.05	23.0 yz	63 y	0.6 y	0.4 yz	20 y	42 y
0.10	20.8 z	30 z	0.1 y	0.0 z	0 y	32 y
S.E.	1.2	11.0	0.3	0.2		0.5
N	10	10	10	10	10	4
2,4-DMA[f]						
0	18.2 x	109 x	8.1 x	2.3 x	100 x	58 x
0.5	19.4 y	110 x	9.6 x	2.0 x	90 x	48 x
5.0	18.1 x	86 xy	7.0 x	1.6 x	60 y	52 x
50.0	16.7 z	81 y	1.9 y	0.0 y	0 y	0 y
S.E.	0.5	9.0	1.4	0.5		0.8
N	50	10	10	10	10	4
Methyl parathion						
0	19.9 x	115 x	0.9 x	0.9 x	90 x	83 x
0.01	21.5 x	100 y	0.9 x	0.5 y	50 x	57 xy
0.1	21.0 x	70 z	0.2 y	0.2 y	20 y	33 y
S.E.	0.8	5.0	0.2	0.1		0.7
N	12	10	10	10	10	3
Pentachlorophenol						
0	19.1 x	111 x	6.0 xy	4.1 x	100 x	72 x
0.0002	17.9 x	111 x	6.7 xy	2.8 xy	100 x	32 y
0.002	17.7 x	86 y	7.2 x	2.1 y	86 x	52 xy
0.02	17.5 x	85 y	3.8 y	2.2 y	93 x	32 y
S.E.	0.8	5.0	1.1	0.5		0.5
N	15	14	14	14	14	5

[a] Nominal concentration used during exposure
[b] Means within a given column accompanied by a different letter (x,y,z) are significantly different from each other at $p \leq 0.05$
[c] Pooled standard error of the mean
[d] Number of fish tested per treatment
[e] *S,S,S,* tributyl phosphorotrithioate
[f] Dimethylamine salt of 2,4-dichlorophenoxyacetic acid

low as 0.3% (0.01 mg/L) of the LC50 significantly reduced the swimming activity of rainbow trout. Exposure to intermediate and high concentrations (5 and 50% of LC50) of chlordane (2 and 20 μg/L), DEF (50 and 100 μg/L), and pentachlorophenol (2 and 20 μg/L) significantly reduced swimming activity.

The ability of rainbow trout to capture and consume prey organisms was also diminished by exposure to each chemical (Table 2). Generally, the proportion of fish that failed to capture and eat prey differed significantly from that of the controls at the highest exposure concentration of each chemical. Feeding behavior was not disrupted by concentrations lower than 50% of the LC50 for carbaryl. In contrast, strike frequency and prey capture were significantly inhibited by relatively low concentrations of methyl parathion (1.3% of LC50), DEF (0.7% of LC50), and chlordane (5% of LC50). The frequency of strikes at prey was generally less sensitive to the toxicants than prey capture. For example,

strike frequency was reduced by pentachlorophenol at 20 μg/L, whereas prey consumption was reduced at 2 μg/L. Feeding efficiency—calculated from Table 2 by dividing number of prey captured by strike frequency—declined among pentachlorophenol-exposed fish as more strikes were required to capture prey, compared with controls. Similar trends in strike frequency, prey consumption, and feeding efficiency were observed in fish exposed to methyl parathion, DEF, and carbaryl. Inasmuch as fish that captured prey consumed the prey, chemosensory impairment was not a factor in the feeding inhibitions observed during these studies.

Exposure to the agricultural chemicals increased the vulnerability of rainbow trout to capture by predators. The degree of vulnerability, as measured by survival from predation, varied with contaminant type and concentration (Table 2). Exposure to carbaryl at all concentrations, or 2,4-DMA at the highest concentration, increased the vulnerability of rainbow trout to predation by largemouth bass. Low concentrations of the other five compounds also increased the vulnerability of exposed fish. However, this vulnerability did not always show a clear dose-related response. Pentachlorophenol increased the predation on rainbow trout at concentrations of 0.5 and 50% of the LC50, but exposure to a concentration of 5% had no effect.

Discussion

To be a valid part of toxicity assessment, behavioral measurements should be sensitive to a range of contaminants at sublethal concentrations and these responses should be relevant to survival of fish in the natural environment. Swimming capacity integrates the physiological capacity of fish to generate and coordinate the locomotive energy required for essential functions such as migration or escaping predators. Swimming capacity is particularly important for a species such as rainbow trout that must maintain position against flowing water while feeding. Deviations in spontaneous swimming activity may influence the ability of fish to capture prey by limiting the area of food search and may increase their conspicuousness to predators (Laurence 1972). Inhibited feeding behavior can decrease survival among young fish by causing starvation at the time of yolk sac depletion (Laurence 1972), extending their period of vulnerability to predation by reducing their growth rate (Werner and Hall 1974) and impairing their ability to overwinter (Oliver *et al.* 1979). Tests evaluating the vulnerability of exposed fish to predation assess a predominant cause of mortality in contaminant-stressed populations (Sprague 1971). Predation tests conducted in conjunction with bioconcentration studies can also provide a measure of bioaccumulation from contaminant-laden prey (Goodyear 1972) and allow an estimation of the dietary exposure that would occur as a result of selective predation of organisms that have been exposed to contaminants.

The behavioral measurements of this study had sufficient sensitivity to provide an assessment of sublethal effects of a wide variety of agricultural chemicals. For example, the lowest concentration of DEF to impair any aspect of behavior (5μg/L) in 96 hr was similar to the DEF concentration found to reduce growth in rainbow trout after 90 days of exposure (Cleveland and Hamilton 1983). Behavioral changes were also evident at regulatory threshold limits within 96 hr of exposure. The lowest concentration of chlordane (2.0 μg/L) that caused behavioral change in rainbow trout was lower than the not-to-be-exceeded concentration of 2.4 μg/L established for chlordane by the US Environmental Protection Agency (1980). Chemically less persistent compounds, such as carbaryl and methyl parathion, also impaired fish behavior at concentrations that might be expected to occur in the environment—particularly after repeated applications (Eichelberger and Lichtenberg 1971).

The behavioral responses varied in their sensitivity to different toxicants during this study and may also vary over the period of exposure. Swimming capacity was least sensitive to the toxicants we examined. Neither chlordane, methyl parathion, nor pentachlorophenol impaired swimming capacity at the concentrations applied. Only the highest concentration of carbaryl reduced that capacity. Previous studies have shown that swimming capacity was reduced by other organophosphate compounds, including malathion and fenitrothion, at approximately 33% of the LC50 (Peterson 1974; Post and Leasure 1974). In the present study, swimming capacity was differentially influenced by exposure to DEF and 2,4-DMA; capacity for strenuous swimming was heightened at 0.7% of the LC50 for DEF and 0.5% of the LC50 for 2,4-DMA, and was diminished at higher concentrations. Similarly, swimming capacity of the bluegill (*Lepomis macrochirus*) was significantly reduced by exposure to 1.0 mg/L fluorene, yet significantly increased among fish exposed to 0.10 and 0.25 mg/L (Finger *et al.* 1985).

Swimming activity is one of the most common and most easily measured behavioral responses observed during toxicity studies and can be evaluated by observing orientation and posture and the frequency, duration, and speed of swimming movements. Even though locomotory responses may vary—given the transient changes reported to occur with duration (Drummond *et al.* 1973; Ellgaard *et al.* 1977) and concentration (Steele 1983) of exposure—the single measurement of activity made during the present study after 96 hr of exposure was sufficient to document contaminant effects. Changes in spontaneous swimming activity were consistently induced by low concentrations of each toxicant.

Growth is commonly assessed during toxicity studies—frequently to establish no-observed effect concentrations—and is often used to calculate the maximum acceptable toxicant concentration. Feeding behavior is rarely assessed, in spite of its obvious link with growth. Reductions in feeding have been observed after sublethal exposure to metals (Atchison *et al.* 1987), aluminum in acidic water (Cleveland *et al.* 1986), organophosphates (Bull and McInerney 1974), dioxins (Mehrle *et al.* 1988), and petroleum hydrocarbons (Woodward *et al.* 1987). Feeding behavior has proven to be a consistent, highly sensitive endpoint in these studies. Several aspects of feeding behavior can be evaluated during toxicity studies: motivation to feed; the frequency of patterned activities that occur during the feeding sequence, such as orientation to prey, fixation, strikes, bites, mistakes, or misses (Brown *et al.* 1987); and mechanistic aspects of feeding such as the efficiency of feeding (captures/strikes), and latencies, reaction distances, and food handling times (Sandheinrich and Atchison 1988).

Two aspects of feeding behavior measured during the present study—frequency of strikes at daphnid prey and prey captured—were selected because these measures can be readily incorporated in routine toxicity test designs, can be easily assessed without specialized equipment, and are applicable to a variety of aquatic species including the early life stages of fish. Strike frequency and prey capture were significantly impaired by each of the six contaminants. Inhibitions in the motivation to feed appeared to be the predominant effect of exposure to high toxicant concentrations, whereas reduced feeding efficiency and reduced strike frequencies were predominant impairments resulting from exposure to lower concentrations.

Measurements of the vulnerability of exposed prey to predation was also a sensitive index of toxicity. Predation on exposed prey increased at concentrations ranging from 0.5 to 50% of the LC50, depending on the compound tested. Impurities in the composition of the pentachlorophenol formulation may underlie the lower threshold of sensitivity (0.2, 2.0 μg/L) observed in the present study in contrast to the 500 μg/L threshold reported previously for the guppy, *Poecilia reticulata* (Brown *et al.* 1985). The formulation we used was shown in other studies (Cleveland *et al.* 1982) to contain impurities that included phenoxyphenols. These impurities were nonlethal but inhibited the growth of the fathead minnow (*Pimephales promelas*) at 0.09 μg/L after a 30-day exposure (Hamilton *et al.* 1986). If the predation observed among sublethally exposed prey reflects impurities in formulation, the use of predation measures in assessing complex mixtures is further justified.

Prey activity patterns may influence predation; hyperactive prey are most visible and vulnerable to predation (Farr 1977), and carbaryl (100 μg/L) was found to impair schooling, an important anti-predator defense in Atlantic silversides, *Menidia menidia* (Weis and Weis 1974). During the present study, however, predation rates in exposed prey did not always correspond with their activity levels. Fish exposed to high and intermediate concentrations of pentachlorophenol were similarly inactive, yet predation was significantly increased only in fish exposed to the high concentration. Factors such as the alertness of prey and their tendency to elude predators by hiding in the substrate or remaining motionless in the water column may contribute to their vulnerability to predation. The separate exposure of predator and prey allowed an individual evaluation of the toxicological endpoints—prey capture and predator avoidance. Combined exposures of predators and prey would be beneficial in predicting interspecific responses to exposure in the field. Such tests could be incorporated in hazard assessment study designs since several aquatic species, including fish and invertebrates, are often evaluated.

In summary, behavioral responses are valid indicators of sublethal toxicity and should be incorporated in routine toxicity assessments. The measurement of fish behavior after short-term exposure to a toxicant can yield a more comprehensive assessment of toxicity than would be provided by mortality alone. Relatively brief behavioral tests may realistically estimate toxic effects that would only become evident after longer periods of exposure. Thus behavioral measurements used in acute range finding tests could predict chronic toxicity of single chemicals or complex effluents. Behavioral responses can also predict effects that might occur in the field. Diminished feeding, for example would enable one to anticipate decreases in the size classes within a population. As a test protocol, measurements of several behavioral endpoints, especially feeding and swimming responses, should provide an effective assessment of hazards posed by toxic substances.

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