EVIDENCE FOR BEHAVIORAL PREFERENCE TOWARD ENVIRONMENTAL CONCENTRATIONS OF URBAN-USE HERBICIDES IN A MODEL ADULT FISH

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Abstract—Fish live in waters of contaminant flux. In three urban, fish-bearing waterways of British Columbia, Canada, we found the active ingredients of WeedEx®, KillEx®, and Roundup® herbicide formulations (2,4-D, dicamba, glyphosate, and mecoprop) at low to high ng/L concentrations (0.26 to 309 ng/L) in routine conditions, i.e., no rain for at least one week. Following rain, these concentrations increased by an average of eightfold, suggesting runoff as a major route of herbicide introduction in these waterways. To determine whether fish might be able to limit point-source exposures through sensory-driven behaviors, we introduced pulses of representative herbicide mixtures to individual adult zebrafish (a model species) in flow-through tanks. Fish did the opposite of limit exposure; they chose to spend more time in pulses of herbicide mixtures representative of those that may occur with rain events. This attraction response was not altered by a previous 4-d exposure to lower concentrations of the mixtures, suggesting fish will not learn from previous exposures. However, previous exposures did alter an attraction response to an amino acid prevalent in food (L-alanine). The present study demonstrates that fish living within urban waterways may elect to place themselves in herbicide-contaminated environments and that these exposures may alter their behavioral responses to cues necessary for survival.

Keywords—Zebrafish, Herbicide avoidance, Behavioral attraction

INTRODUCTION

Fishes everywhere live in contaminated water. Even in remote regions free of human activity, water can contain contaminants such as pesticides [1]. In regions with human activity, water may contain complex contaminant mixtures [2]. A recent study of a British Columbia river that passes through a rural area found a complex mixture consisting of at least 40 contaminants, including herbicides and insecticides of agricultural origin [3]. Determining whether contaminant mixtures constitute a risk to fishes and other biota is a challenging task; contaminant concentrations vary in time and space, and some contaminant exposures may cause only subtle alterations [4].

Concentrations of toxic contaminants in the environment are typically far below those that cause death, which indicates most exposures may cause sublethal effects [5,6]. These effects may increase the probability of ecological death, or death by normal ecological processes such as predation, starvation, disease, or myriad other causes [7,8]. Several studies of fishes have determined that numerous pesticides may do just that, by altering locomotory activity [9,10] or by impairing the ability to detect predator scent [11–14]. The complex contaminant mixtures found in aquatic environments may simultaneously cause a diverse array of sublethal effects in fishes; a goal should be to relate such effects to endpoints relevant to survival.

The present study aimed to help determine whether fish can avoid realistic herbicide exposures, and if not, whether the exposures can affect behavior. To accomplish this, representative environmental herbicide concentrations were needed. These were determined by measuring herbicide concentrations in four fish-bearing creeks located within urban settings. In one case, this necessitated developing a new analytical protocol. Based on the herbicide concentrations found in the creeks, mixtures that reflected concentrations lower than, typical of, and greater than the observed average were prepared. We then measured the abilities of fish to avoid or be attracted to these mixtures. We also measured how exposure to the mixtures affected their ability to respond to an amino acid food odor (L-alanine) [15] that evokes attraction [16]. In these experiments, zebrafish (Danio rerio) were used since this species is an emerging model for behavioral toxicity testing [17,18].

MATERIALS AND METHODS

Fish

Zebrafish of both sexes (n = 120, mass 0.47 ± 0.02 g, length 3.89 ± 0.05 cm, condition factor 0.76 ± 0.02) were obtained from a local supplier (Pro-Fish, Canada) and held at 28°C in filtered dechlorinated municipal tap water, dissolved oxygen >90%, under a 12:12-h day:night cycle for at least two weeks prior to experimentation and fed flakes once daily (Wardley Essentials), except 24 h prior to experiments. Experiments were conducted in accordance with the University of Windsor Animal Care Guidelines.

Chemicals

All chemicals were purchased from Sigma-Aldrich except for MS222 (tricaine methanesulfonate 99.5%; Argent Laboratories).
Each site was sampled at least six times, and water was analyzed for the presence of herbicides commonly used in urban areas for control of weeds (see Table 2). Nonresidential sample site type Impacted Reference Impacted Impacted Longitude -122.85497 -123.05982 -122.79776 -122.96922 Latitude 49.23666 49.34547 49.26768 49.25921 Sample detection limits are given in analysis purposes all needed to be converted to acids and are arriving to established methods [20]. Acid extractable herbicides capped bottles were shaken vigorously to extract and stabilize samples were spiked with surrogate standards, derivatized with 9-fluorenylmethylchloroformate in borate buffer, acidified to pH 1.5 to 2.5, and extracted on solid-phase extraction (SPE) cartridges (200 mg HLB; Waters). The SPE cartridges were washed with acidified water/methyl tertiary-butyl ether and eluted with basic methanol. Extracts were reduced in volume, spiked with recovery standard, and analyzed using a mass spectrometer (Micromass Quattro Ultima tandem quadrupole) coupled to high-performance liquid chromatography (Waters 2795 Alliance) equipped with a C18 column (SunFire 3.5 mm, 4.6 x 30 mm; Waters). The electrospray ionization source was operated in positive ion mode and mass resolution in the multiple reaction monitoring mode. Glyphosate samples were analyzed within two weeks to avoid the microbial degradation that may occur [19]. For the acid extractable herbicides (AEHs) (dicamba, mecoprop, and 2,4-D), 1 L of water was collected as above in amber glass bottles and 2 ml of concentrated H2SO4 was added. The capped bottles were shaken vigorously to extract and stabilize the herbicides. Quantification of AEHs was carried out according to established methods [20]. Acid extractable herbicides exist as acids, esters, and salts in the environment, but for analysis purposes all needed to be converted to acids and are reported here as such. Sample detection limits are given in Table 2.

Fish exposures and behavior assessment

Three scenarios were used in the present study, each intended to simulate a different type of realistic situation. Two scenarios were used to determine if fish would avoid introduced herbicide mixture plumes, i.e., those mixtures entering a waterway through a point source following a simulated rain event (SRE). The first of these was aimed at determining whether unexposed, naïve fish (those raised in filtered, dechlorinated municipal water) would avoid various mixture concentrations; the second was intended to determine if fish exposed to herbicide mixtures would avoid mixtures at concentrations greater than those of their exposure. A final, third set of experiments was conducted on exposed fish to determine whether exposure affected a behavioral response to L-alanine. To create environmentally realistic herbicide mixtures in laboratory, a consensus mixture was constructed from the average herbicide concentrations of the three impacted creeks (Table 2). The mixture was designed to consist of glyphosate (100 ng/L), dicamba (2.5 ng/L), mecoprop (15 ng/L), and 2,4-D (25 ng/L). The herbicide concentrations were all far below those that would result in lethality (e.g., 96-h median lethal concentration [LC50] values: glyphosate, 620 mg/L, carp [21]; dicamba, 180 mg/L, sheepshead minnow [Cyprinodon variegates] [22]; mecoprop, 92 mg/L, bluegill sunfish [Pomis macrochirus] [22]; and 2,4-D, 5.1 mg/L, carp [Cyprinus carpio] [23]). Immediately before each of the three experiments, 1-L stock solutions of 1,000-fold greater concentration of the consensus mixture were prepared in distilled water. For exposures, dilutions of this were then prepared using tank water. To estimate actual exposure concentrations for the press exposures, water samples were taken randomly from each exposure group (i.e., control, low, medium, and high) once during the experiments and analyzed as above.

Behavioral responses of individual fish were determined in an array of five, 500-ml circular flow-through tanks (10 cm Ø, 6 cm water depth), each with their own fluid delivery system and overhead closed circuit digital video camera (Matco). The fluid delivery system consisted of a multichannel peristaltic pump (Masterflex) and polytetrafluoroethylene (PTFE) tubing (0.318 cm OD, 0.159 cm ID; Clean Air Engineering) that replaced 1% of the tank volume in 2 min (i.e., flow rate was 2.5 ml/min; calibrated before each test). Video from all 90-min trials was recorded by a computer running surveillance software (EverSecure; Matco). Fish position in the horizontal plane was determined every 30th of a second using EthoVision XT 5.1 (Noldus). Fish swimming speed was also determined throughout the trials. To determine whether the herbicide mixtures and L-alanine evoked attraction, the amount of time fish spent in an area around the inflow was determined. This area was demarcated by an arc centered about the inflow with a radius equal to one half of the tank radius, an area of 10.6% of the total tank area (Fig. 1). Preliminary trials indicated that this area would permit capturing fish activity when their head was either pointed downstream of of the water flow. To create environmentally realistic herbicide mixtures in laboratory, a consensus mixture was constructed from the average herbicide concentrations of the three impacted creeks (Table 2).
directly placed on or within approximately 5 mm of the inflow. Dye tests indicated that compounds introduced into the tank inflow would form a plume of high odor concentration that would have largely left the inflow area within 5 min of introduction (Fig. 2). For this reason, we consider attraction responses to be evident as any increase in time spent in this area during the 5 min following introduction as compared to the previous 5 min. Average swimming speed following herbicide mixtures and L-alanine introduction was used as a metric of activity.

Simulated rain event responses

To determine behavioral responses of unexposed fish to herbicide pulses, individual fish were placed in the tanks, left to acclimate for 30 min, after which the inflow was switched to either vehicle (tank water) or herbicide mixtures at medium and tenfold lower or higher concentrations for a period of 2 min (values in Table 2). This means the average dilution factor in the tank was 100-fold (5 ml into 500 ml). In the present article we report concentrations that reflect this approximate final dilution concentration, not the delivery concentration. Following 2 min of delivery, the inflow was returned to tank water. At 60 min following the initial inflow change, fish were removed and sacrificed using over-anesthesia (0.5 mg/L of MS222 buffered 1:1 with NaHCO3) (total time in the behavior tanks: 90 min). Two replicates of five fish (one per tank) were tested at each mixture concentration (control, low, medium, high; n = 40).

To determine if fish previously exposed to herbicide mixtures were capable of limiting further exposure by avoiding water with a higher herbicide concentration, fish were given four-day (96 h) exposures to the same mixtures as above (Table 2), placed in the behavior tanks, left to acclimate for 30 min, then given an inflow of herbicide mixture twofold greater than the highest exposure concentration for 2 min (n = 40). Fish were removed and sacrificed after 90 min as above. The 96-h exposures were carried out in 10-L glass tanks using a 24-h static and renewal regimen with five fish (one replicate) per tank.

Food odor responses

To determine whether herbicide exposure altered attraction to food odor, fish were given 96-h exposures as above, then placed in the behavior tanks to test their attraction to L-alanine. A decrease in attraction to L-alanine suggests impaired food
searching ability. The 2-min pulses were carried out as above, only in place of water/herbicide pulses a pulse of L-alanine (10^{-7} M) was used. As above, fish were sacrificed after 90 min and two replicates were tested at each concentration (n = 40).

Statistics

To detect behavioral responses in each of the three experiments, the amount of time (s) fish spent in the inflow area in each of the 5-min after odor/mixture introduction was compared.
to the average time spent in the inflow area during the 5-min prerelase period. In order to compare responses across fish and treatments, the post-odor/mixture times were ordered by their magnitude. This was necessary as fish do not necessarily respond immediately to an introduced odor (i.e., their smallest response may have occurred in the first minute; their largest in the last). By ordering the responses the comparison isolates the size of the responses, and not their timing. Ordering also helps eliminate the influence of fish position in the tank at the time of odor/mixture delivery on the timing of a behavioral response. After ordering, fish were compared across treatments using a two-way (pre- versus postdelivery; min), repeated measures analysis of variance (ANOVA), followed by a Tukey honestly significant difference multiple comparisons test. Behavioral attraction was considered to occur if the number of minutes in which exposed fish spent more time in the inflow increased (versus control); avoidance, the opposite. For clarity of presentation, boxplots of the difference between pre- and postdelivery are shown. Differences in postdelivery swimming speed across the treatment groups were compared using ANOVA followed by a Holm-Sidak multiple comparisons test against control. The limit of significance for all tests was set at \( p < 0.05 \). SigmaPlot 11 (Systat) was used for graphing and statistics.

RESULTS

Herbicide concentrations in field and laboratory

Over the five months spanning the seven sampling events, only mecoprop and 2,4-D were detectable in the reference creek (Hastings), and then on one day only (May 14, 2007), and at very low concentrations (0.795 and 0.557 ng/L, respectively). In contrast, all four herbicides were detectable in the impacted creeks (Como, Scott, and Still) (Table 2). Dicamba concentrations were, on average, the lowest, ranging from 0.105 to 22.5 ng/L. Mecoprop and 2,4-D were typically similar in concentrations, ranging from 1.10 to 187 and 2.04 to 309 ng/L, respectively. Glyphosate concentrations were the greatest, ranging from 22.8 to 455 ng/L. Rain increased all herbicide concentrations the overwhelming majority (86.7%) of the time (Fig. 3). For example, in Como Creek, following a rain event in July, herbicide concentrations increased by 7.8, 12, 16, and 23-fold for glyphosate, mecoprop, 2,4-D, and dicamba, respectively (Fig. 3). On average, rain increased herbicide concentrations by 8.0-fold.

In laboratory exposures, concentrations were quantifiable for all herbicides except for control and low glyphosate concentrations and the control 2,4-D concentration (Table 2). This was likely due to comparatively high sample detection limits (SDLs) for these two herbicides (19.9 ± 0.3 and 0.53 ± 0.03 ng/L, respectively), compared to 0.05 ± 0.00 and 0.13 ± 0.03 ng/L for dicamba and mecoprop. In the control (tank) water of the laboratory, trace concentrations of dicamba and mecoprop were detected. However, these concentrations (0.117 and 0.114 ng/L) were far lower than the averages of the impacted creeks (2 and 0.38%). The prepared mixture exposure concentrations captured the range of values observed in field. For example, the high concentrations were within 3.6% (on average) of the high concentrations observed in field. The average (medium) and low concentrations typically fell within the bulk of field observations. The measured values did vary from the intended (nominal) values. Specifically, nominal concentrations for the medium exposures intended to be 100, 2.5, 15, 25 ng/L of glyphosate, dicamba, mecoprop, and 2,4-D, respectively, were measured at 65.9, 6.10, 5.03, and 48.4. However, the total exposure (135 ng/L of herbicides) was close to the intended (142 ng/L). The total high concentration exposure was lower than intended (875 vs. 1425 ng/L), while the low total concentration was greater (by 32.9 ng/L).

Behavioral responses

Responses to simulated rain events. The introduction of water did not evoke attraction in control fish (Figs. 4A, 5A). In fact, controls spent a greater proportion of 2 min away from the inflow area \( (p < 0.001) \). This general pattern did not change with 2-min pulses of low and medium concentrations of herbicides (Fig. 5B,C), but was altered by a high concentration pulse (Figs. 4A, 5D). Specifically, fish spent 8.79 s more in the inflow area during 1 min of the 5 min following herbicide introduction \( (p < 0.001) \), suggesting that the herbicide mixture caused an attraction response. Swimming speed was also affected by exposure \( (F_{3,38} = 5.159) \), with the high concentration pulse increasing speed 2.00 cm/s above control \( (p < 0.001) \) (Fig. 6A).

Previous herbicide exposure did not alter an attraction response to a higher mixture concentration (Figs. 4B, 5E–H). Specifically, whether fish were exposed to low or high mixture concentrations the attraction to a very high concentration pulse remained \( (p \text{ values for Control, Low, Medium, and High: } 0.037, 0.044, 0.007, \text{ and } 0.012, \text{ respectively}) \). With swimming speed, in contrast to unexposed fish, mixture exposure tended to reduce the speeds \( (F_{3,38} = 3.289) \), with fish given a low concentration exposure swimming 1.97 cm/s slower than control \( (p = 0.004) \) (Fig. 6B).
Responses to food odor following mixture exposure. In the 5 min following L-alanine presentation, searching behavior of control fish was characterized by 1 min in which fish spent significantly more time in the inflow area, and 1 min in which they spent less time (Fig. 5I). In contrast, fish exposed to herbicides spent significantly more time in the inflow area over 3 to 4 min, and did not have a minute in which they spent less time (Figs. 4C, 5J–L) (\(P\) values for Control: 0.007 [min 1], 0.138 [min 2], 0.393 [min 3], 0.057 [min 4], 0.001 [min 5]; Low: 0.001 [min 1], 0.001 [min 2], 0.016 [min 3], 0.154 [min 4], 0.55 [min 5]; Medium: 0.001 [min 1], 0.003 [min 2], 0.008 [min 3], 0.04 [min 4], 0.976 [min 5]; High: 0.001 [min 1], 0.018 [min 2], 0.039 [min 3], 0.61 [min 4], 0.119 [min 5]). Swimming speed was significantly altered with L-alanine exposure (\(F_{3,35} = 3.269\)), but post-hoc analysis did not identify any groups differing from control (Fig. 6C). However, the mean difference between control and low was greatest (1.67 cm/s) and approached significance (\(p = 0.055\)).

DISCUSSION

Behavioral responses of organisms are intended to place them in conditions that favor their survival. Improving access to resources, such as food, while avoiding injurious situations, contaminant exposure, helps achieve this. In the present study, fish chose to move into a plume of herbicide contaminants representative of those found in example urban aquatic environments. The implication for this unexpected decision is that fish with the ability to select between environments differing in contamination, such as upstream pristine conditions and downstream impacted conditions, may choose to reside in contaminated, potentially harmful areas.

Herbicides in field and laboratory

Not all of the globally used pesticides reach only their targets. In the United States, pesticides were detected in 97% of urban and agricultural waterways [24]. Typically, concentrations were in the ng/L range, although they spiked well into the \(\mu\)g/L range following rain events. In the present study of four creeks within a Canadian urban setting, the active ingredients from Weedex, Killex (2,4-D, dicamba, and mecoprop), and Roundup (glyphosate) were found in mixtures, with some concentrations up to 455 ng/L (glyphosate). The herbicides can be attributed to human activity directly adjacent to the creeks since herbicides were largely absent in the reference creek. In the impacted creeks, herbicide concentrations were consistent, except following rain events. After rain, herbicide concentrations increased on average by a factor of 8. Given that the rainfall in this region is considerable (yearly average of 1.3 m; www.climate.weatheroffice.ec.gc.ca), runoff clearly plays a substantial role in the unintentional deposition of herbicides in urban aquatic ecosystems.

Behavioral responses to and following herbicide exposure

It is generally accepted that as contaminant concentrations become toxic, fish will move to less contaminated areas [25]. For this to be true, contaminants need to be perceived as noxious, which is not always the case; fish can be attracted to some herbicides, including dalapon [26], nicosulfuron, and...
In the present study, fish exhibited attraction to a mixture containing four herbicides, none of which have been previously associated with attraction. In fact, two of the herbicides, 2,4-D and glyphosate in its Roundup formulation, evoked avoidance (2,4-D: [27,28]; Roundup [14]). However, the avoided concentrations in these studies were typically 1,000-fold greater than the total mixture concentration tested here (e.g., 1 μg/L vs. 1 mg/L). The mixture attraction response of the present study may have been related to concentration, as studies have found that attraction can switch to avoidance with increasing concentration. For example, rainbow trout were attracted to 6 μg/L of NiCl₂ but avoided 24 μg/L [29]. Alternately, the combination of the four herbicides, two of which are known to evoke olfactory sensory neuron responses (2,4-D and glyphosate; [13]), may have created an interesting odor bouquet. Whether the attraction was a concentration and/or
mixture effect, fish chose to expose themselves to herbicide mixtures.

Sublethal contaminant exposures may have associated energetic costs. To provide a proxy for energy acquisition, studies have included behavioral endpoints to food odors. For example, responses to food extract by goldfish were reduced by 12-h exposure to 100 mg/L carbofuran [30] and 24-h exposure to 330 mg/L parathion [31]. In the present study, zebrafish exposed to herbicide mixtures similar to those in the environment (i.e., in low concentration and through surrounding water) spent a greater amount of time in a plume of an amino acid (L-alanine) food odorant. Olfactory studies have typically found short duration herbicide exposures impair olfactory sensory neuron responses to amino acids [5,32]. However, these studies generally used concentrations greater than in the present study and were of short duration, which limits physiological adaptation of the olfactory tissues. The concentrations in the present study may have been too low to impair olfactory responses, or perhaps the fish had time to adapt. In either case, fish may have experienced systemic effects that increased their willingness to search for a perceived food. Herbicide exposure may have cost energy in terms of biotransformation and excretion, and so these fish may have been hungrier, although this remains for future study.

Fish activity level may also have energetic ramifications and can be affected by synthetic contaminant exposure. Some clear examples are agents that alter motor neuron functionality, such as acetylcholinesterase (AChE)-impairing insecticides. These

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**Fig. 6.** Average swimming speeds of zebrafish over 60 min following the introduction of tank water (control) or simulated rain events (i.e., herbicide mixture pulses), both without previous mixture exposure (A) and with previous (96 h) exposure (B), as well as following the introduction of a food odorant (10⁻⁷ M L-alanine) (C). Fish in (A) were naïve fish (held in filtered water prior to behavioral testing), whereas fish in (B,C) received 96-h exposures to various herbicide mixture concentrations (for exposure concentrations see Table 2; all controls received tank water). Asterisk indicates significant difference versus control.
agents can evoke hyperactivity with low levels of AChE impairment [9] and hypoactivity as impairment increases [33]. Other agents that do not have mechanisms of action that so directly relate to swimming activity have been observed to alter swimming. The herbicides atrazine and Roundup, for example, evoke hyperactivity and hypoactivity, respectively, in rainbow trout [14]. In the present study the activity of unexposed fish was increased in the 60 min following exposure to a mixture of herbicides, at a concentration below those of the above study (i.e., ng/L vs. ≥μg/L) (Fig. 6A). This response was reversed in at least one group of fish that had been given a previous (96 h) exposure to the mixture (Fig. 6B). While we will not speculate on the mechanisms by which these changes in fish activity occurred, it is worth noting that should any such alterations occur with fish in environmental situations, predator/prey dynamics could be affected. Both hyper- and hypoactivity may make fish more conspicuous.

CONCLUSION

Behavioral alterations such as found in the present study generally occur with exposures one to two orders of magnitude lower than those that cause lethality [22]. We noted a change in food odor attraction with herbicide exposures at approximately 1 μg/L; lethality values for all of the herbicides in the mixture are in the mg/L range. The data of this study suggest that significant behavioral changes can occur at greater than three orders of magnitude below lethality. We also noted that environmentally relevant concentrations of herbicide mixtures may attract fish. A goal for future studies should be to explore how the individual constituents of the mixture contribute to attraction, perhaps by herbicide class. An important second goal for future studies would be to determine how these subtle behavioral changes, both to food odor and synthetic contaminants, affect organism fitness.

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