Olfactory-mediated behavior in juvenile salmonids exposed to aquatic herbicides

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This is to certify that I have examined this copy of a master's thesis by

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and have found that it is complete and satisfactory in all respects, and that any and all revisions required by the final examining committee have been made.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure Number Page

LIST OF TABLES

Table Number Page

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Chapter 1- Background and Justification

Pesticide use has been increasing worldwide with the advent of more intensive agriculture (Laabs et al. 2002), home garden care and maintenance (Frans 2004), and the control of exotic and invasive plants. Herbicides are the most commonly used pesticides, and are the most often detected in surface waters (Frans 2004). In addition to the leaching of herbicides from land, some herbicides are applied directly to water to control aquatic vegetation. While the application rates of chemicals applied to water are often below those levels that are overtly toxic to non-target species, there may be "sublethal" effects on those that are exposed (Wolf and Moore 2002). The biological significance of "sublethal" effects is largely unknown (Grue et al. 2002).

Plants are vital to aquatic systems in that they provide essential habitat for other aquatic organisms. However, an over abundance of plants can degrade water quality, lead to an excess of nutrients, reduce habitat values, block water management structures, interfere with navigation and recreational opportunities, and impair aesthetics (Emmett 2001, 2002, Emmett and Morgan 2004). For regulatory purposes, the Washington Department of Ecology divides aquatic weeds into two types, nuisance weeds, native plants growing in excess, and noxious weeds, plants that are not native to the area. Noxious weeds are considered invasive, and can degrade wildlife habitat, out-competing native species (Emmett 2001, 2002, Emmett and Morgan 2004). In Washington State, there are 28

aquatic, wetland, or riparian species listed on the State Noxious Weed List. Under an Aquatic Weed Grants Program, the Department of Ecology is trying to remove a number of invasive species including: Brazilian Elodea (*Egeria densa*), Eurasian watermilfoil (*Myriophyllum spicatum*), Fanwort (*Cabomba caroliniana*), Fragrant Water Lily (*Nymphaea sp.*), Hydrilla (*Hydrilla verticillata*), Swollen Bladderwort (*Utricularia inflata*), Parrotfeather (*M. aquaticum*), Water Hyacinth (*Eichhornia crassipes*), Water Primrose (*Ludwigia hexapeta*), and Yellow Floating Heart (*Nymphoides peltata*) (www.ecy.wa.gov).

Control of aquatic weeds can be conducted using a variety of methods, each with their own advantages and disadvantages. Options include bottom screening, diver dredging, hand pulling, cutting and raking, rotovation, mechanical cutting and harvesting, biological control, namely grass carp (*Ctenopharygodon idella*), and herbicides.

Bottom screening involves placing a cover over the sediments and plants like a blanket, compressing the plants while reducing or blocking light. Bottom screening is best in small areas and can control plants for 1 to 2 years, possibly up to 10 years if properly maintained. They are non-selective and effects are limited to the treated area. If not secured properly, a difficulty in soft-sediments, they can become navigation hazards and dangerous to swimmers. Bottom screens can also interfere with fish spawning and bottom-dwelling animals, and without regular maintenance, the target plants may quickly re-colonize the bottom screen. (Emmett 2001, 2002, Emmett and Morgan 2004)

In diver dredging, divers clear plants from small areas resulting in 90% removal of plants. Divers are able to be selective in both the area treated and the species removed. Removal of plants can increase turbidity, leading to obscured vision, making the diver less effective, and cause re-suspension of contaminants and nutrients bound to sediments. Diver dredging is expensive and it is often difficult to get the permits required. (Emmett 2001, 2002, Emmett and Morgan 2004)

Manual methods of removal, including hand pulling, cutting and raking, are labor intensive and are best for swimming areas and around docks. They are also good for removing early infestations. The ease and success of this approach depends on the plant type and the sediments the plants are growing in. Plant fragments must be collected to avoid spreading the plants. Hand cutting is done from the water surface, leaves the roots in the sediment, and generates floating plants and fragments that need to be removed. Raking may result in substrate removal and short-term increases in turbidity, making it difficult to see remaining plants. Raking may also disturb benthic organisms. (Emmett 2001, 2002, Emmett and Morgan 2004)

Mechanical options include rotovation, cutting and harvesting. Rotovation uses agricultural tilling machines that have been modified for aquatic use to uproot aquatic plants. Rotovation can cause direct mortality of invertebrates and fish. It disturbs the lake bottom, increasing turbidity, and potentially releasing contaminants and nutrients bound

to the sediments. Rotovation is non-selective, can remove desirable species, and cause plant fragments, which need to be collected for effective control. A number of permits are needed for rotovation. Mechanical cutting and harvesting is good for large scale projects, but regrowth can occur within a month and several treatments may be required per growing season. These methods do not totally eradicate noxious species and can result in significant environmental impacts within the target area. Mechanical cutting and harvesting can disturb sediments if not conducted correctly, are non-selective, and may eliminate valuable fish and wildlife habitat, while causing an accumulation of plant fragments. (Emmett 2001, 2002, Emmett and Morgan 2004)

New methods of control, still being developed, are biological controls. At this time only grass carp (*Otenopharygodon idella*) are widely used, but other methods include plant pathogens, herbivorous insects, competitive plants, and plant growth regulators. Sterile grass carp, which feed on aquatic plants, are generally introduced to ponds and lakes with no inlet or outlet, or the inlet or outlet must be screened. The amount of control provided by grass carp ranges from removal of 20-40% plant cover to complete removal of all submersed plants. Because of this, they are considered an all or none strategy. It can take grass carp 2 to 5 years to control aquatic weeds. Grass carp may not discriminate between plant species and as such may consume threatened and endangered species or other desirable native plants. Once grass carp are stocked, they are nearly impossible to remove short of their 20 year life span. (Emmett 2001, 2002, Emmett and Morgan 2004)

The remaining alternative for aquatic plant removal is the use of chemical control, herbicides. Some advantages of herbicides are they can be less expensive than a number of the other control methods, especially in the case of large infestations. They are easily applied around docks and underwater obstructions. Disadvantages include short-term restrictions for swimming, drinking, fishing, irrigation, and other water uses after application. In addition some slower acting herbicides can take days to weeks before control is achieved, while faster acting herbicides can result in low oxygen levels associated with large scale plant decomposition. Some expertise is required to successfully use herbicides and avoid undesirable impacts. Also, public perception plays a significant role in the application of pesticides to surface waters and some cities and counties may have additional restrictions on use. (Emmett 2001, 2002, Emmett and Morgan 2004, www.ecy.wa.gov)

Another advantage of herbicide use is its potential to provide selective plant control (Sprecher et al 1998), particularly over large areas, through the selection of herbicides that kill only certain types of plants. Selective herbicides can be extremely useful in plant management where native plant species are living among invasive species (Sprecher et al 1998).

There were approximately 200 projects using aquatic herbicides in Washington in 2006 and a similar number is expected in 2007 (K. McLain, personal communication). The most commonly used aquatic herbicides for submersed plant control in Washington State

are DMA® 4 IVM (active ingredient [a.i.] 2,4-D; Dow AgroSciences, Indianapolis, IN), Renovate® 3 (a.i. triclopyr-TEA; SePRO Corporation, Carmel, IN), Reward® (a.i. diquat; Syngenta, Greensboro, NC), and Sonar® A.S. (a.i. fluridone; SePRO Corporation). DMA® 4 IVM and Renovate® 3 are systemic herbicides with modes of action that control growth and target dicot and broadleaf monocot plants (Sprecher et al 1998). DMA® 4 IVM has been shown to be selective for Eurasian watermilfoil at label application rates, leaving native aquatic plants relatively unaffected (Emmett 2001). Renovate® 3 can be effective for spot treatment of Eurasian watermilfoil and is relatively selective for it at label rates, while many native species are unaffected by triclopyr (Emmett and Morgan 2004). Reward® is a non-selective contact herbicide that alters photosynthesis and results in rapid death of the plant, but is dependant on sunlight (Emmett 2002). Reward® is generally used for short-term control of a variety of submerged aquatic plants. Sonar® A.S. is a slow acting systemic herbicide that inhibits carotenoid synthesis and results in the photodestruction of chlorophyll (Netherland and Getsinger 1995). It results in good control of submersed plants where there is little water movement and extended contact time. When used in Washington State, Sonar® A.S. is applied several times during the spring and summer to maintain a low, but consistent concentration in the water. Of the herbicides mentioned above, it is the most expensive (www.ecy.wa.gov).

The use of herbicides in Integrated Pest Management (IPM) plans to control aquatic weeds has been hampered by concerns directed at the non-target toxicity of active herbicidal ingredient. A recent ruling by the 9th Circuit Court of Appeals (*Headwaters,*

Inc. v. Talent Irrigation District, 2001) requires Western states, including Washington, to issue National Pollutant Discharge Elimination System (NPDES) permits for the use of pesticides and adjuvants in aquatic systems (Leintz 2004). Unfortunately, adequate data on the non-target toxicity of aquatic herbicides to aquatic resources are lacking, thereby threatening the permitting process and the success of IPM strategies to control aquatic plants.

Behavioral tests can improve the interpretation and ecological relevance of standardized toxicity test results, such as LC50s (Grue et al. 2002). A number of studies have examined the ability of different fish species to avoid a variety of chemicals, with metals and insecticides being the most frequently tested (e.g., Hansen et al. 1972, Kynard 1974, Folmar 1976, Carr et al. 1990, Morgan et al. 1991, Ishida and Kobayashi 1995, Saglio and Trijasse 1998, Saglio et al. 2001). The ability of animals to detect and avoid toxic concentrations of pesticides in the wild may reduce the hazards associated with their use as long as suitable uncontaminated habitat is accessible elsewhere (Folmar 1976). Olfaction and olfactory-mediated behaviors are also extremely important to fish in finding mates, detecting prey, and avoiding predators, and can be affected by exposure to novel chemicals (Steele et al. 1990, Scholz et al. 2000, Wolf and Moore 2002, Scott et al. 2003).

Pesticide-induced changes in olfactory mediated behaviors in fish can be quantified using a number of different methods, the most common of which are counter current flow

chambers and Y-mazes. In the counter current chamber, water enters from both sides of a square or round chamber and then exits in the middle with little to no mixing. Fish are placed within the chamber, and after acclimation, the position of the fish is documented for a fixed period of time. Chemical is then introduced and the position of the fish is again is determined. The location of fish prior to the introduction of the chemical and after the chemical is introduced are then compared statistically. In the Y-maze, usually a "Y" shaped chamber, water flows down the two sides and out a drain in the base of the Y. Fish are placed at the base of the maze and after acclimation, the chemical is introduced to one arm of the maze while the other side receives clean water. Fish are given a fixed amount of time to swim between the two waters, after which location of fish is recorded and the number of fish in each portion of the chamber is then compared statistically. For all these tests, attraction is defined as the movement of fish into the chemical treated side of the chamber, whereas avoidance is defined as movement to the side of the chamber with clean water, or away from the chemical. "No response" is defined as no change in position following the introduction of chemical.

Salmonids are an important part of the culture of the Pacific Northwest and many stocks are listed as threatened or endangered by the Endangered Species Act (Emmett 2002). Out-migrating smolts depend on olfaction to imprint on their natal stream so they are able to return to it to reproduce (Dittman et al. 1996). Also during this time, juvenile salmonids go through the parr-smolt transformation that alters them behaviorally and physiologically and allows them to adapt to seawater. This is also a period of increased

olfactory sensitivity (Dukes et al. 2004). Salmon out-migration often coincides with the treatment of surface waters with various herbicides to control aquatic weeds (Poovey et al. 2002). The impacts of these chemicals on the olfactory system of fish have not been determined.

Of the aquatic herbicides commonly applied in Washington State to control submersed plants (2,4-D, diquat, fluridone, and triclopyr), juvenile rainbow trout (*Oncoryhnchus mykiss*) were found to avoid 1 ppm 2,4-D a.i. (Folmar 1976), which is below the maximum application rate (i.e., the maximum concentration permitted within the water column) of 4 ppm a.i.. These concentrations are well below the LC50 of 2,4-D for juvenile rainbow trout, which ranges from greater than 100 to 420 ppm (Mayer and Ellersieck 1986). Behavioral changes were observed in juvenile rainbow trout exposed to 88 ppm triclopyr (a.i., as triethylamine salt) as a formulated product (Morgan et al. 1991). The behavioral changes observed by Morgan and colleagues (1991) were loss of equilibrium, erratic swimming, and eventually fish lying on the bottom of test chambers barely breathing. The effects concentration of 88 ppm a.i. is below the reported LC50 of triclopyr for juvenile rainbow trout, greater than 100 ppm (Mayer and Ellersieck 1986), both of which are much higher than the maximum label application rate of 3.49 ppm a.i., or the maximum rate permitted by the Washington State Department of Ecology of 2.5 ppm a.i.. Previous studies indicate juvenile rainbow trout do not avoid diquat at 10 ppm a.i. (Folmar 1976), a concentration close to the LC50 for Reward® of 14.8 ppm (MSDS 2005). Both concentrations are again well above the maximum label application rate of

1.37 ppm a.i.. Behavioral studies with fluridone have not been conducted, but the LC50 of fluridone for juvenile rainbow trout was found to be 4.25-8.4 ppm (Mayer and Ellersieck 1986); the maximum label application rate is 0.15 ppm a.i..

There has also been little research on the ability of aquatic species to detect a stimuli following pesticide exposure (Wolf and Moore 2002, Scott et al. 2003). Wolf and Moore (2002) studied the herbicide, metolachlor, by first exposing crayfish (*Orconectes rusticus*) to the herbicide and then testing their ability to detect a stimulus. They determined the crayfish were still be able to detect odors, but did not respond properly. When exposed to the avoidance causing odors, the crayfish moved towards them, instead of away. Scott and colleagues (2003) exposed juvenile rainbow trout to cadmium and then tested their response to an alarm substance (skin extract). They found that cadmium did alter the trout's response to the avoidant, but the response depended on the duration of the exposure to the cadmium.

The overall goal of my research was to determine if aquatic herbicides alter olfactory mediated behavior of salmonids. The objective of my first study, Chapter 2, was to determine if juvenile Chinook salmon (*Oncorhynchus tshawytscha*) avoid formulations of three aquatic herbicides commonly used in Washington State: Renovate® 3 (triclopyr-TEA), Reward® (diquat), and Sonar® A.S. (fluridone). DMA® 4 IVM (2,4-D) was not included in this study as Folmar (1976) had determined that juvenile rainbow trout avoid the herbicide at concentrations less than those associated with maximum label rates,

although he used a different apparatus. The nominal concentrations tested were equal to those associated with the maximum label application rate and 10 times the maximum rate. The objective of my second study, Chapter 3, was to determine if exposure to the four aquatic herbicides (DMA® 4 IVM, Renovate® 3, Reward®, and Sonar® A.S.), at maximum label or field applied application rates, alters olfactory performance in juvenile rainbow trout, used as a surrogate for juvenile salmon. Chapter 4 of my thesis includes a synthesis of my studies a discussion of research needs.

Chapter 2- Do juvenile Chinook salmon (*Oncorhynchus tshawytscha*) avoid Renovate® 3, Reward®, and Sonar® A.S.?

Introduction

The use of herbicides in Integrated Pest Management (IPM) plans to control aquatic weeds has been hampered by concerns directed at the non-target toxicity of active herbicidal ingredients (a.i.). The non-target toxicity of aquatic herbicides needs to be assessed, particularly in light of litigation that has and may continue to force states to adopt new permitting processes that require states to issue National Pollutant Discharge Elimination System (NPDES) permits for the use of pesticides in aquatic systems (Leintz 2004). Unfortunately, adequate data on the toxicity of aquatic herbicides to non-target aquatic resources are lacking, thereby threatening the permitting process and the success of IPM strategies to control nuisance or invasive aquatic plants.

Salmon are an important part of the culture of the Pacific Northwest and many stocks are listed as threatened or endangered under the Endangered Species Act (Emmett 2003). Local, State, and Federal governments and non-governmental organizations are spending millions of dollars annually to protect and enhance salmon populations and their habitats. Many salmon stocks travel through waters that receive chemical inputs (e.g., Collier et al. 1998), and effects of these exposures are not known. For example, during their out-migration to the ocean, juvenile salmon frequently pass through water bodies in which herbicides are used to control nuisance or invasive aquatic plants. Information on how

juvenile salmon respond to aquatic herbicides following operational applications is lacking.

Behavioral tests can improve the interpretation and ecological relevance of standardized toxicity test results, such as LC50s (Grue et al. 2002). Studies have examined the ability of a variety of different fish species to avoid a number of chemicals, with metals and insecticides being the most commonly tested (e.g., Hansen et al. 1972, Kynard 1974, Folmar 1976, Carr et al. 1990, Morgan et al. 1991, Ishida and Kobayashi 1995, Saglio and Trijasse 1998, Saglio et al. 2001). The ability of animals to detect and avoid toxic concentrations of pesticides in the wild can reduce the hazards associated with their use as long as suitable uncontaminated habitat is easily accessible (Folmar 1976).

The aquatic herbicides most commonly applied in Washington State for submersed plant control contain diquat, fluridone, or triclopyr as their active ingredients (K. Hamel, personal communication). Previous studies indicated juvenile rainbow trout do not avoid diquat at 10 ppm a.i. (Folmar 1976), whereas behavioral changes were observed in juvenile rainbow trout exposed to 88 ppm triclopyr a.i. (Morgan et al. 1991); greater than 20X the current maximum label recommendation. The behavioral changes observed by Morgan and colleagues (1991) were loss of equilibrium, erratic swimming, and eventually fish lying on the bottom of test chambers barely breathing. Comparable studies with fluridone are lacking.

My objective was to determine if juvenile Chinook salmon (*Oncorhynchus tshawytscha*) avoid the formulations of three of the aquatic herbicides most commonly used in Washington State for submersed aquatic weed control: Renovate® 3 (triclopyr-TEA, SePRO Corporation, Carmel, IN), Reward® (diquat, Syngenta, Greensboro, NC), and Sonar® A.S. (fluridone, SePRO Corporation). The nominal concentrations I tested were equal to those associated with the maximum label application rate at time of testing (3.49 ppm, 1.37 ppm, and 0.090 ppm a.i. respectively), and 10 times the maximum rate. Herein, I report that juvenile Chinook did not avoid any of the concentrations of the herbicides I tested, but were attracted to the highest concentrations of Renovate® 3 and Reward®. I also describe a new statistical approach for quantifying avoidance and attraction under my test conditions.

Methods

All tests were conducted at the US Geological Survey's Western Fisheries Research Center's, Marrowstone Marine Field Station, in Nordland, WA between 22-29 June 04. The freshwater source for all stages of fish acclimation and testing was the city of Port Townsend Municipal water supply that is degassed upon arrival at the facility. A broad-spectrum analysis of organic and inorganic contaminants in the incoming water by Edge Analytical (Burlington, WA) indicated all values were within daily drinking water tolerances.

Juvenile Chinook (pre-smolts) were obtained from the Soos Creek Hatchery operated by the Washington Department of Fish and Wildlife and were transported to the field station 10 Apr 04 in an stainless steel transport tank equipped with an oxygen supply. Upon arrival, the temperature of the water within the transport tank was allowed to equilibrate to that of the freshwater at the field station. Fish were then distributed to circular holding tanks (568 L) and maintained >70 days prior to testing in flowing aerated freshwater under natural sunlight (temperature 11.2-17.1C; dissolved oxygen >8.0 mg/L; pH 7.3-8.5). Fish were fed to satiation once daily (Bio-Oregon Biodiet Grower 1.5 mm, Warrenton, OR). Mean weight at the time of testing was 13.4 g (SE=2.75, n=120).

Test procedures and apparatus utilized were modified from those described by Exley (2000). Alterations included adaptations for a square chamber, five simultaneous replicate chambers, and a different chemical delivery system. In each chamber, 10 fish were subjected to a directional flow (4.2L/min): inflow at one end and outflow at the opposite end. The test protocol consisted of 30 min of acclimation, 15 min of clean flow, 15 min of chemical flow, and 15 min of clean flow. The flow in the chamber was such that the entire water volume was replaced every 15 min. The flow within each chamber created a chemical front moving across the chamber (confirmed with dye tests, Fig. 2.1) that forced the fish to encounter and respond to the chemical. Chemicals were delivered from a stock concentrate mixed immediately prior to each test into the freshwater flow serving each chamber using a dosing pump (Pulsatron Series D, Puslafeeder, Ponta Gorda, FL). Delivery was monitored by measuring the change in the weight of the stock

bottle during the exposure phase of each test. Potential responses were avoidance, i.e., moving away from the chemical front; attraction, i.e., moving into the chemical toward the inlet; or no response, i.e., no shift in position. One water sample was collected just prior to entering the chamber from each herbicide concentration and was analyzed by Edge Analytical to compare actual vs. nominal concentrations within the test chambers.

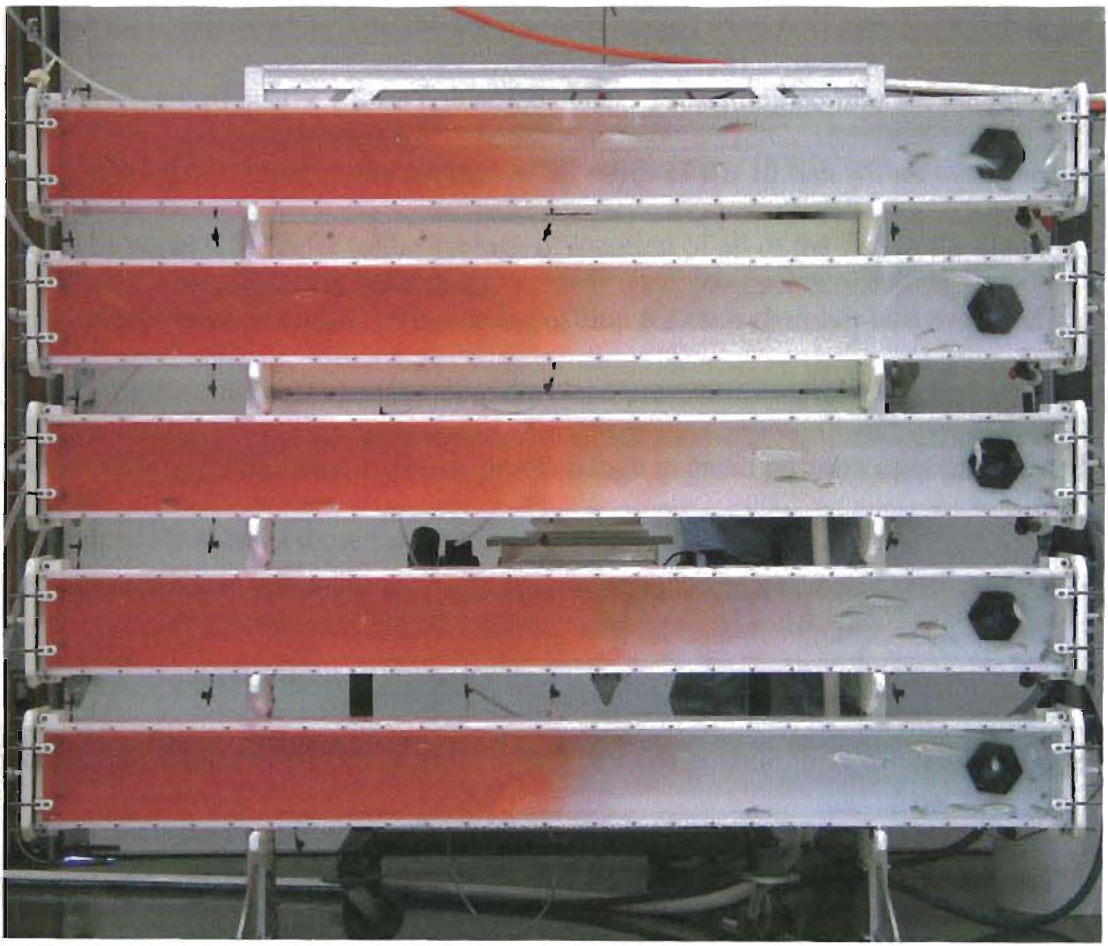

Figure 2.1. Dye test confirming flow of chemical within test chambers. In this photograph water is entering the chambers on the left side of the apparatus and flows across each chamber to an exit on the right side.

Behavior was quantified by photographic image analysis using Image-Pro Plus® 4.5 (Media Cybernetics, Inc. Silver Spring, MD). Digital photos were used to determine the mean position of fish within each replicate test chamber for every minute of each test. Each fish was assigned a position score as a ratio of its distance from the inlet relative to the length of the chamber. The eye of the fish was the exact point scored, or the nose if the fish faced the camera. The resulting scores ranged from near zero for a fish at the inlet, to a score of nearly 1 for a fish at the outlet. Presuming no bias for the inlet or outlet ends of the chamber, the average of all ratios of the 10 fish within each chamber would be about 0.5. I refer to the average of location of all of the fish in the chamber (as a ratio) as the "mean position". The mean position for each chamber was averaged within each of the three different test periods: the clean pre-treatment, the chemical treatment, and the clean post-treatment. The slope of change in mean position over time was also determined for each of these time periods.

When fish respond to the test chemical with a quick and sustained shift away from the inlet during the chemical flow period, a shift in mean position between the clean period and the chemical period will be the most sensitive response endpoint (Fig. 2.2). If fish respond slowly to the presence of the chemical, resulting in a gradual and continuous shift away from the chemical front a comparison of the mean position for each time period will not be a very sensitive endpoint (Fig. 2.3). The alternative is to examine the slope of the line that fits the gradual shift in position over time (Fig. 2.4). It is important to note that neither statistical approach will identify both the quick/sustained and the

slow/gradual models. In order to detect which response might exist, both methods were used in the data analysis. To detect statistically significant shifts, two-tailed paired t-tests between the pre-chemical and chemical time periods for both their mean position and slopes were conducted. Due to the more variable nature of behavioral responses and small sample sizes (n=5), I *a priori* chose an alpha level of 0.10 for all hypothesis testing.

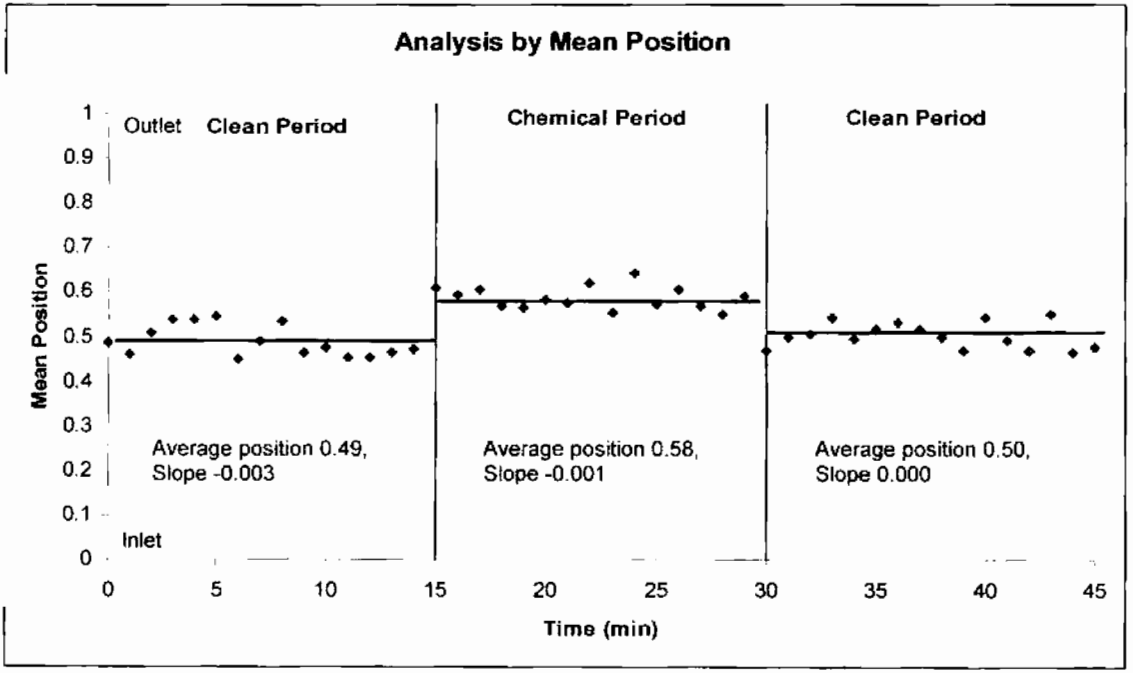

Figure 2.2. Theoretical response of fish moving rapidly away from the chemical as detected by a shift in mean position. Data points are the mean position of fish across all chambers.

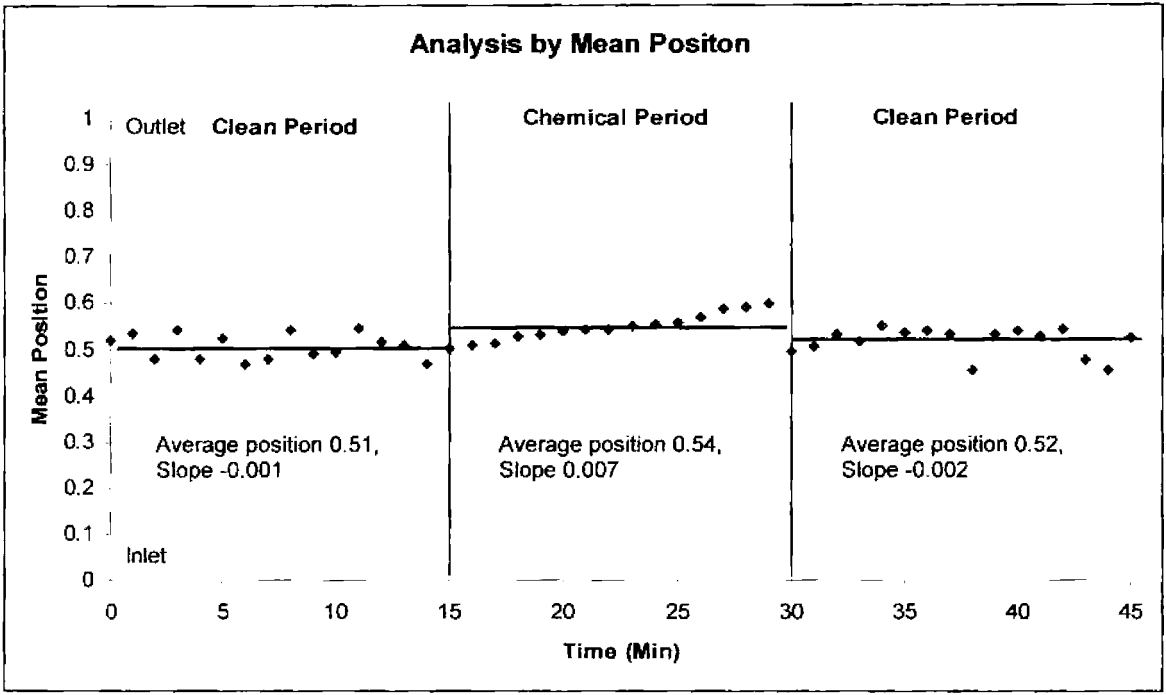

Figure 2.3. Theoretical response of fish moving slowly away from the chemical such that a difference in mean position is not detected. Data points are the mean position of fish across all chambers.

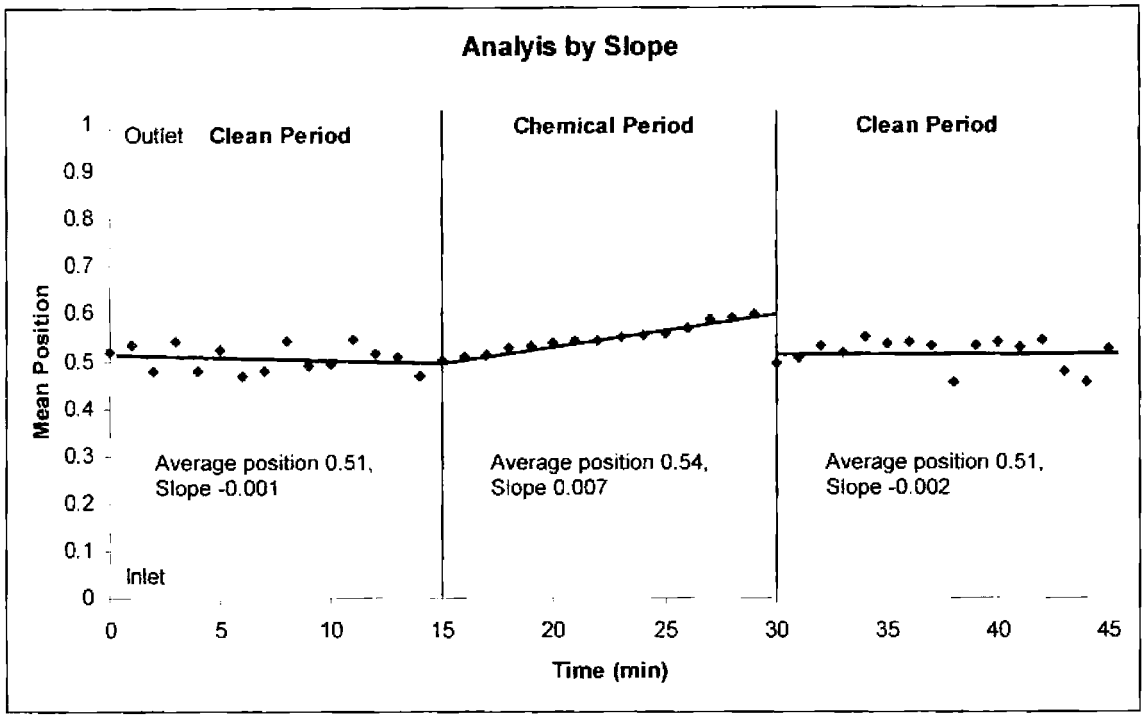

Figure 2.4. Theoretical response of fish moving slowly away from the chemical detected by different slopes for each time period. Data points are the mean position of fish across all chambers.

Results

Water quality parameters within the test chambers during the avoidance trials (temperature 16.4-16.6 C; pH 7.6-7.7; dissolved oxygen [DO] 8.6-9.4 mg/L) were either within or close to those recommended for toxicity tests with salmonids (temperature 10-14 C; pH 6-8; DO > 5 mg/L; USEPA 1996). Although the ambient temperature of the incoming freshwater to the facility was slightly greater than that recommended by the US EPA for standardized toxicity tests, it was within the range of temperatures juvenile salmon would experience within water bodies in Washington State to which herbicides are applied (Tamayo et al. 2000). Actual herbicide concentrations within the water flow

in each test (Table 2.1) were lower than targeted for all but the 10X concentration of Renovate® 3 (95-116%), Reward® (82-87%), and Sonar® A.S. (62-77%).

Table 2.1. Concentrations (ppm) of the herbicides used to test for avoidance by Chinook salmon smolts. Actual concentrations are corrected for percent recovery.

Formulated Product	Active Ingredient	Nominal Concentration	Actual Concentration	% Recovery	% Target
Renovate® 3	Triclopyr	3.49	3.31	100	95
		34.9	40.4	88	116
Reward®	Diquat	1.37	1.125	94	82
		13.7	12.0	94	87
Sonar® A.S.	Fluridone	0.090	0.069	95	77
		0.900	0.554	95	62

I used calcium hypochlorite (1.6 ppm, Fig. 2.5) as a positive control to verify the effectiveness of the apparatus and new statistical methods. There was a significant shift ($p=0.10$) of the juvenile Chinook away from the chemical when examining the slopes of the change in position over time between the first 15 min of clean water and the 15 min of chemical exposure. The difference in the mean positions within these two periods was also nearly significant ($p=0.13$). The data also showed an attraction to the subsequent flow of clean water as indicated by the negative slope.

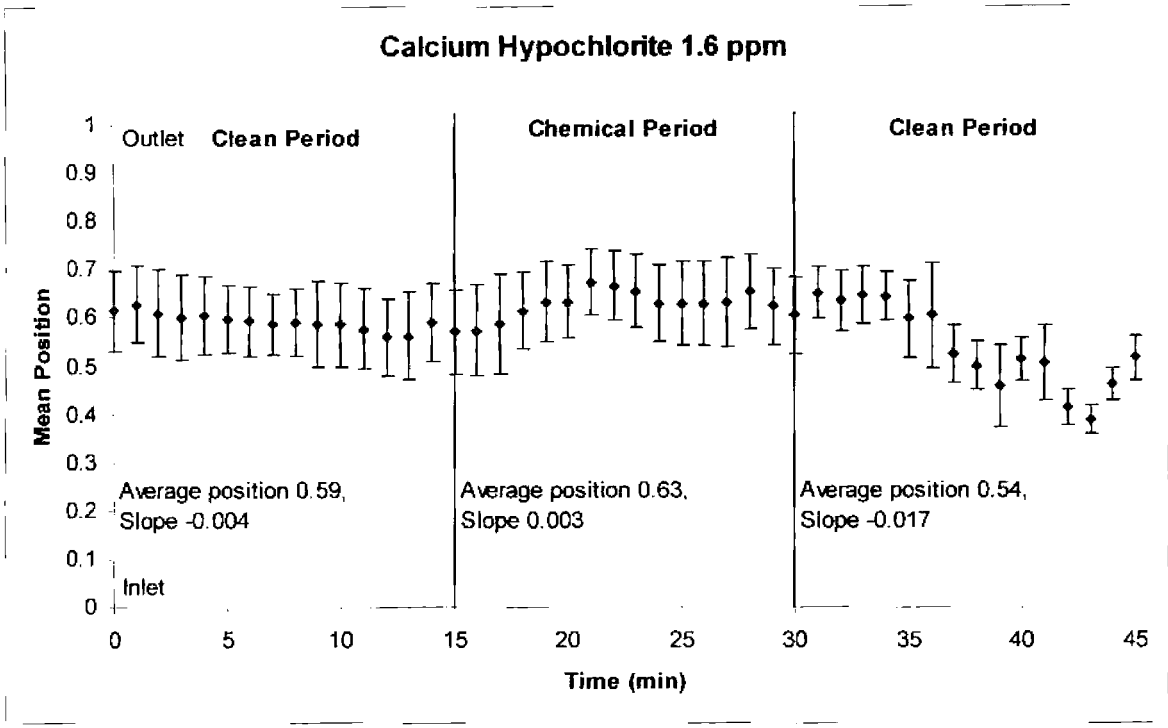

Figure 2.5. Response of Chinook salmon to calcium hypochlorite. Data points are the mean position of fish across all chambers and the bars represent standard errors. The change in slope of the mean position through time during the chemical exposure was statistically significant (p=0.10). The corresponding change in mean position was nearly significant (p=0.13).

No significant differences in mean position or the slope for change in mean position over time were detected for any of the herbicides at their maximum (1X) label rates. At 10X the maximum rate, fish were attracted to Renovate® 3 (Fig. 2.6) and Reward® (Fig. 2.7) based on changes in mean position and the slope of the change in mean position over time, respectively. All other comparisons were not statistically significant (Table 2.2).

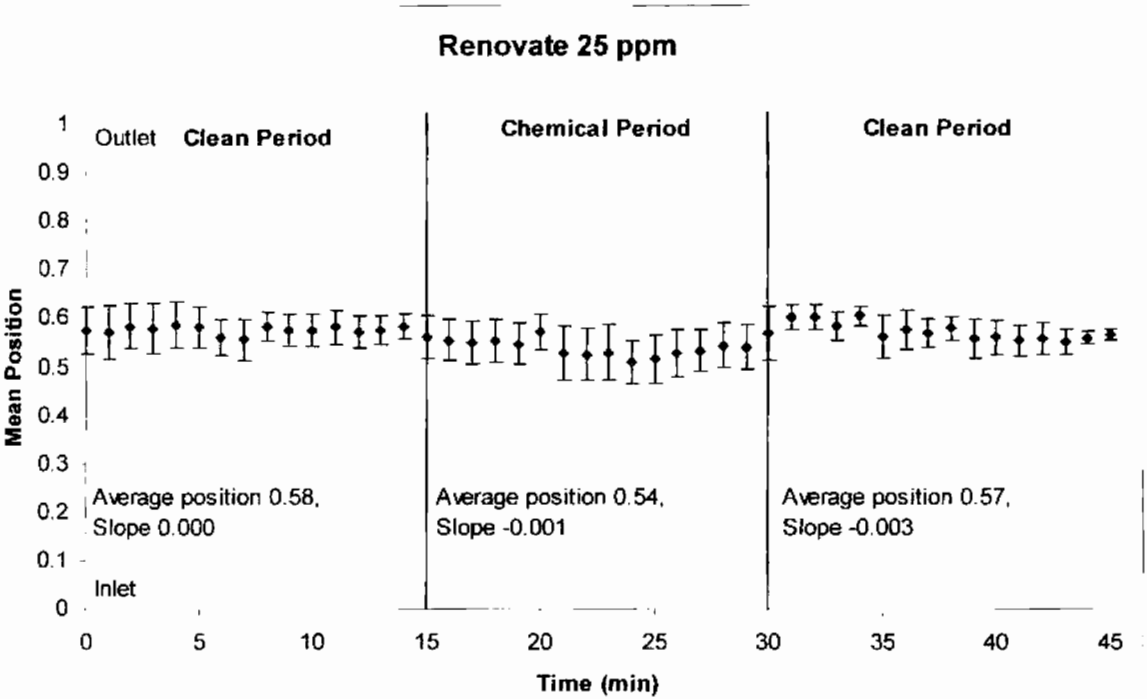

Figure 2.6. Response of Chinook salmon smolts to the herbicide Renovate® 3 (a.i. triclopyr). Data points are the mean position of fish across all chambers and the bars represent standard errors. The change in mean position during the chemical exposure was statistically significant (p=0.08).

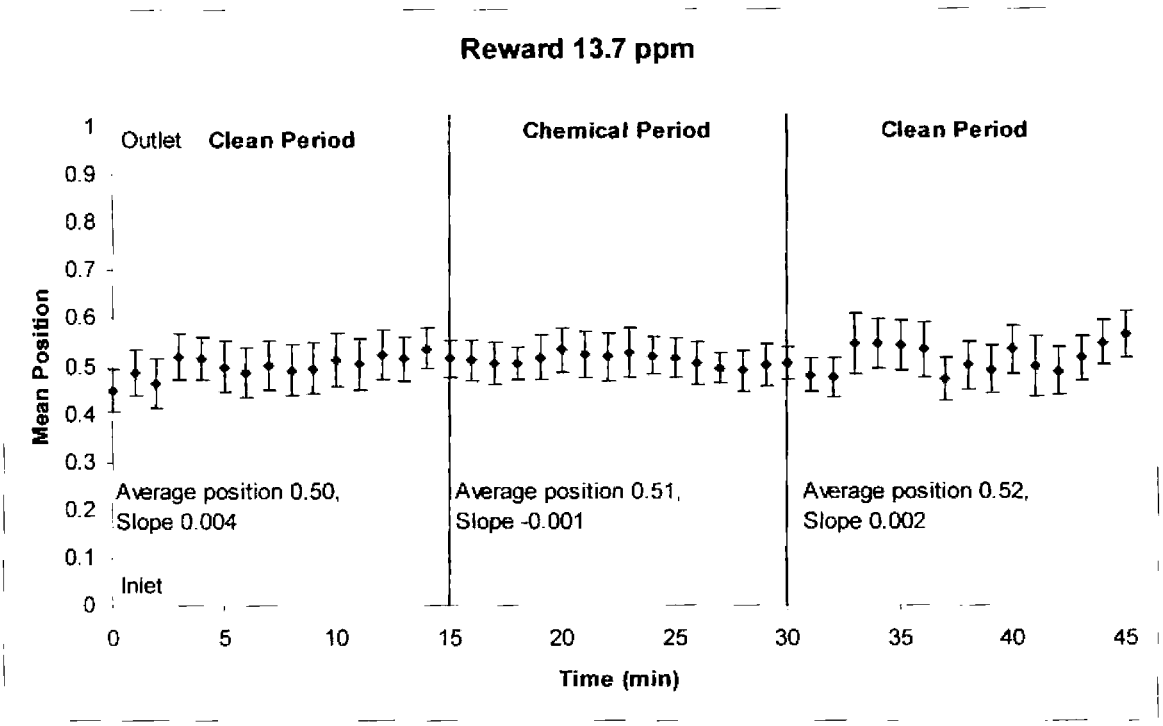

Figure 2.7. Response of Chinook salmon smolts to the herbicide Reward® (a.i. diquat). Data points are the mean position of fish across all chambers and the bars represent standard errors. The change in slope of associated with mean position during the chemical exposure was statistically significant (p=0.08).

Table 2.2. Results of all avoidance/attraction tests conducted with Chinook salmon smolts. Prior to analysis, an alpha level of 0.10 was selected due to the inherent variability in behavioral data. Concentrations represent nominal concentrations (ppm).

Chemical	Concentration	Analysis	Significance	P-value	Interpretation
$Ca(OCl)_2$	1.6	Slope	Yes	0.10	Avoidance
	1.6	Position	Nearly	0.13	Avoidance
Renovate® 3	2.5	Slope	No	0.50	No Effect
	2.5	Position	No	0.50	No Effect
	25	Slope	No	0.78	No Effect
	25	Position	Yes	0.08	Attraction
Reward®	1.37	Slope	No	0.25	No Effect
	1.37	Position	No	0.40	No Effect
	13.7	Slope	Yes	0.08	Attraction
	13.7	Position	No	0.52	No Effect
Sonar® A.S.	0.090	Slope	No	0.40	No Effect
	0.090	Position	No	0.96	No Effect
	0.90	Slope	No	0.46	No Effect
	0.90	Position	No	0.35	No Effect

Discussion

Juvenile Chinook did not avoid concentrations of the herbicides tested equal to those that would occur following application of the maximum rate on the label. However, they were slightly attracted to 10X the maximum label application rate of both Reward® and Renovate® 3 (diquat and triclopyr, respectively). These results suggest that, if present, fish would not actively avoid and might actually be slightly attracted to a potentially toxic environment. The median lethal concentration (LC50) of diquat (active ingredient only) for 96 hour static test to juvenile rainbow trout (*O. mykiss*) is greater than 100 ppm (Mayer and Ellersieck 1986). My fish were attracted to a concentration well below that at 13.7 ppm (nominal concentration), suggesting that concentrations of this magnitude should not result in overt toxicity. The nominal and actual concentrations to which the fish responded, however, are an order of magnitude higher than the maximum application rate on the label. Whether or not concentration gradients of this magnitude exist following operational applications according to the label is not known. The LC50 of triclopyr for juvenile rainbow trout is greater than 100 ppm (Mayer and Ellersieck 1986), which is well above my nominal highest concentration of 34.9 ppm. Similarly, the LC50 of fluridone for juvenile rainbow trout is 4.25-8.4 ppm (Mayer and Ellersieck 1986); my highest nominal concentration tested (0.90 ppm) is well below lethal levels. Overt toxic effects would not be expected in juvenile salmon occupying ponds and lakes in which any of the three herbicides were applied according to the label. No fish mortalities have been reported in Washington State due to use of herbicides in surface waters (K. Hamel, personal communication.)

Folmar (1976) studied the response of rainbow trout fry to a number of herbicides and found that the trout did not avoid nor were attracted to 10 ppm of diquat a.i.. Based on his results, I would not have expected juvenile Chinook to be attracted to my nominal concentration of 13.7 ppm of diquat. There are, however, a number of differences between my tests and those of Folmar including different testing apparatus, species, chemical formulations, and length of exposure to the chemical. In Folmar's study, tests were conducted using a Y-maze, in which the fish were exposed to the chemical for 60 min and were then allowed equal access to either clean or contaminated water. They would need to be moving around the chamber to detect an alternative type of water. In my uni-directional chambers, fish have the opportunity to respond immediately to the presence of the herbicide. Recent studies in our laboratory indicate that olfactory-mediated behavior in juvenile rainbow trout is altered by exposure to 1.37 ppm of diquat (as Reward®; Curran et al., unpublished manuscript), ca. one-tenth of the concentration used in Folmar's study. An herbicide-induced reduction in olfactory ability may explain the absence of a response by the trout fry in the study by Folmar. In addition, the fact that Folmar used the active ingredient alone and I used a formulated product, could explain the difference in response. Having not examined the individual constituents of the formulated product, it could be one or more of the other ingredients alone or in combination with the active ingredient that resulted in the attraction I observed.

Morgan and his colleagues (1991) examined the avoidance behavior of rainbow trout to triclopyr as Garlon® 3A (44.4% a.i.). They reported avoidance to Garlon® 3A at ≥ 800 ppm (~355 ppm a.i.); however, in their tests fish preferred one arm of the Y-maze. Preference to a location can cause fish to tolerate a higher level of contamination than when there is no preference (Morgan et al 1991, Scherer and McNicol 1998). The lowest concentration tested by Morgan and his colleagues was 44 ppm a.i. triclopyr, which is higher than the concentration used in my study (34.9 ppm a.i.) and at which I observed attraction. It has been found that fish are attracted to some chemicals at one concentration, but avoid the chemical at a different one (Giattina et al. 1982, Smith and Bailey 1989). Again, differences in test apparatus, species, and formulated products may also have been important.

Despite the differences among these studies, results suggest that juvenile salmonids will not avoid the concentration gradients associated with operational applications of three herbicide formulations most often used to control aquatic plants in Washington State and elsewhere in the Pacific Northwest. The absence of an avoidance response also suggests that in the case of partial water body applications, juvenile salmonids may not move to suitable untreated habitats when exposed to the herbicides. However, avoidance behavior might force young fish out of plant beds exposing them to predators, whereas the absence of avoidance of a chemical at non-lethal levels may be a "safer" alternative.

The statistical approaches I applied to the response data in my study appear to be more sensitive than those used in previous studies. I examined the response of the fish within the entire tube and not just a portion, as in Exley (2000) where only the inlet section was used for data analysis. With my procedure fish need to be continually moving away from the chemical front, while other methods do not examine the continued response of the fish. My methods also allow for a slow response to the chemical to be detected statistically, which facilitates the interpretation of results and the identification of effects.

Chapter 3- Olfactory performance in salmonids exposed to aquatic herbicides

Introduction

Pesticide use has been increasing worldwide with the advent of more intensive agriculture (Laabs et al. 2002), home garden care and maintenance (Frans 2004), and the control of exotic and invasive plants. Herbicides are the most commonly used pesticides, and are the most frequently detected in surface waters (Frans 2004). In addition to the leaching of herbicides from land, some herbicides are applied directly to the water to control aquatic vegetation. While the application rates of chemicals applied to water are often below lethal levels to non-target species, there can still be sublethal effects on aquatic organisms living in the ecosystem (Wolf and Moore 2002).

The use of herbicides in Integrated Pest Management (IPM) plans to control aquatic weeds has been hampered by concerns directed at the non-target toxicity of active herbicidal ingredients (a.i.). The non-target toxicity of aquatic herbicides needs to be assessed, particularly in light of new permitting processes that require the 14 western states, including Washington, to issue National Pollutant Discharge Elimination System (NPDES) permits for the use of pesticides and adjuvants in aquatic systems (Leintz 2004). Unfortunately, adequate data on the non-target toxicity of aquatic herbicides to aquatic resources are lacking, thereby threatening the permitting process and the success of IPM strategies to control aquatic weeds.

Olfaction is extremely important to fish in finding mates, detecting prey, and avoiding predators. Olfaction can be affected by exposure to chemicals (Scott et al. 2003, Wolf and Moore 2002). Most studies on olfaction have examined the ability of fish to detect and avoid novel chemicals (e.g., Hansen et al. 1972, Kynard 1974, Folmar 1976, Carr et al. 1990, Morgan et al. 1991, Ishida and Kobayashi 1995, Saglio and Trijasse 1998, Saglio et al. 2001, Curran et al. unpublished manuscript), with herbicides and insecticides being the most frequently tested. Of the herbicides commonly applied in Washington State to control submersed plants (2,4-D, diquat, fluridone, and triclopyr), juvenile rainbow trout (*Oncorhynchus mykiss*) avoided 1 ppm 2,4-D as the a.i. (Folmar 1976), a concentration below the maximum application rate, whereas Chinook smolts (*O. tshawytscha*) were slightly attracted to 13.7 ppm diquat a.i. (as Reward®) and 34.9 ppm triclopyr-TEA a.i. (as Renovate® 3), ten times the maximum application rate (Curran et al. unpublished manuscript). Behavioral changes were observed in juvenile rainbow trout exposed to 88 ppm triclopyr (a.i.) as a formulated product (Morgan et al. 1991). The behavioral changes observed by Morgan and colleagues (1991) were a loss of equilibrium, erratic swimming, and eventually fish lying on the bottom of test chambers barely breathing. Juvenile Chinook smolts did not show any behavioral changes when exposed to 0.09 or 0.90 ppm a.i. of fluridone (as Sonar® A.S.). There has also been little work on the ability of aquatic species to detect a stimulus following pesticide exposure (Wolf and Moore 2002, Scott et al. 2003). Wolf and Moore (2002) studied the herbicide, metolachlor, by first exposing crayfish (*Orconectes rusticus*) to the herbicide and then

testing their ability to detect a stimulus. They determined that crayfish were still be able to detect odors, but did not respond appropriately. When exposed to an odor that normally elicited aversion, the crayfish moved towards the odor, instead of away from it.

Salmonids are an important part of the culture of the Pacific Northwest and many salmon runs or stocks are listed as threatened or endangered under the Endangered Species Act (Emmett 2003). Many of these stocks travel through waters that receive chemical inputs (Collier et al. 1998). Effects of these exposures are not known (Scholz et al. 2000). For example, it is during out-migration that a number of herbicides are applied to surface waters for aquatic weed control. Out-migrating smolts depend on olfaction to imprint on their natal stream so they are able to return to it to reproduce (Dittman et al. 1996). The effects of aquatic herbicides on the olfactory system of fish have not been determined. The objective of my study was to determine if exposure to four commonly used aquatic herbicides (DMA® 4 IVM, Renovate® 3, Reward®, and Sonar® A.S.), at maximum label or field applied application rates, alters olfactory performance of juvenile rainbow trout, used as a surrogate for salmon smolts.

Methods

All tests were conducted at the School of Aquatic and Fishery Sciences at the University of Washington in Seattle, WA. Juvenile rainbow trout were purchased from Nisqually Trout Farm and were transported to the University in a stainless steel transport tank

equipped with an oxygen supply. Fish were held in 375 L (100 gal) acclimation tanks with flowing freshwater from the City of Seattle (3.78 L/min (1 gal/min), temperature=12.0-13.4 °C, dissolved oxygen [DO]=7-9 mg/L). The City water is dechlorinated within the University's laboratory facilities. Fish were fed daily to satiation with a commercial diet (BioDiet Grower, Bio-Oregon, Warrenton, OR) until 2 days before exposure to the herbicides. A subsample of 30 fish were anesthetized with MS-222 (100 ppm + buffer) and weighed prior to testing to ensure the correct fish to water loading rate (1 g fish/1.25 L water).

EPA protocols for 96 hour static toxicity tests were used to expose the fish to the maximum label, or maximum permitted concentrations of each of the herbicides (Table 3.1). Ten fish per replicate were used for all tests (10.33 ± 2.48 g, 9.78 ± 0.80 cm). Fish were not fed during herbicide exposure. Water quality measurements (temperature, dissolved oxygen, pH, and conductivity) were measured daily in a randomly selected subset of the tanks within each treatment such that measurements were taken on each tank at least once during the exposure period. Fish were visually inspected for mortality and changes in behavior at 0, 24, 48, 72, and 96 hours. In previous studies, no mortality or overt behavioral changes were observed at the concentrations tested. A water sample was collected from two tanks within each herbicide treatment at 0 and 96 hours for chemical analyses (Edge Analytical Inc., Burlington, WA) to compare nominal vs. actual concentrations.

Table 3.1. Concentrations (ppm a.i.) of the herbicides tested, based on maximum label or operational application rates. Actual concentrations are corrected for percent recovery.

Formulated Product	Active Ingredient	Nominal Concentration	Actual Concentration	% Recovery	% Target
DMA® 4 IVM	2,4-D	4.0	2.6/2.7	130	65/68
Renovate® 3	Triclopyr	2.5	2.1/2.4	113	83/96
Reward®	Diquat	1.37	1.32/1.72	93	96/125
Sonar® A.S.	Fluridone	0.150	0.235/0.164	97	157/109

Olfactory performance was tested using the behavioral response of the fish to a known stimulus. The test apparatus consisted of five replicate counter current chambers. In this design, water enters from both sides of the chamber at equal flows, meeting in the middle at a common drain (Fig. 3.1). An attractant or avoidant is introduced into one side of the chamber per replicate with the other side receiving clean water. There is little mixing between the two flows (chemical vs clean water). Dye tests using food coloring were conducted prior to any testing to confirm the desired flow pattern was achieved (Fig. 3.1).

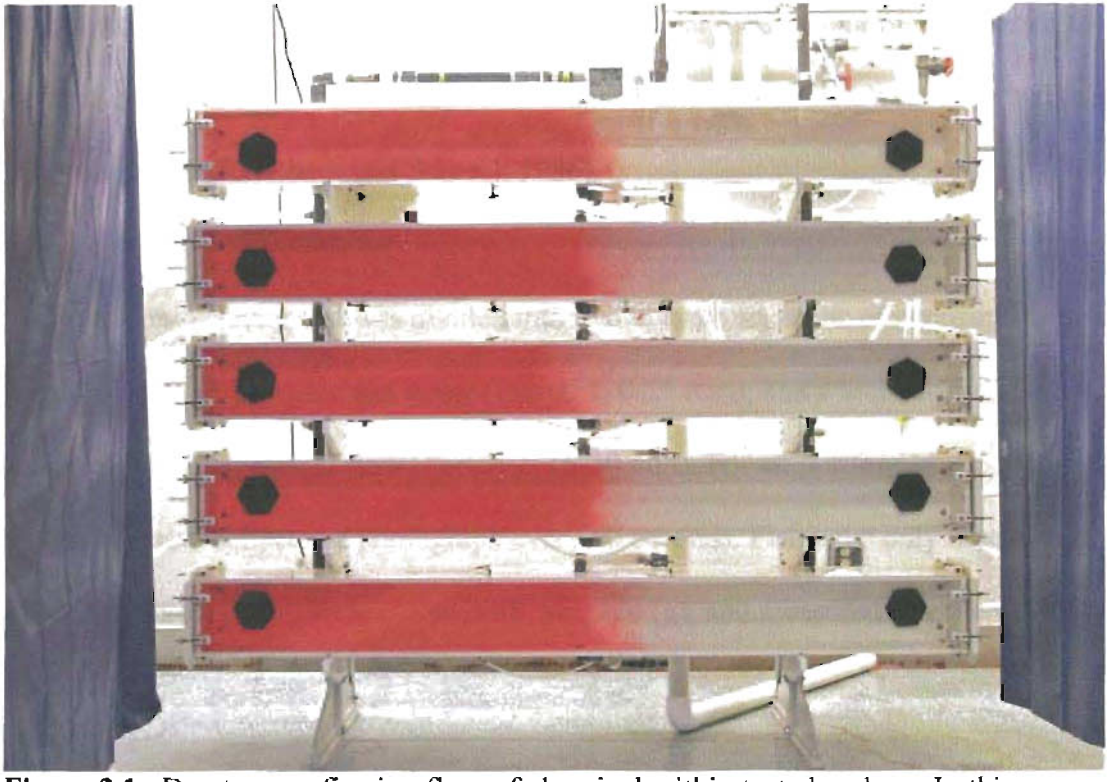

Figure 3.1. Dye test confirming flow of chemical within test chambers. In this photograph water is entering the chambers on the both the right and left side of the apparatus and flows across each chamber to an exit in the middle.

Attraction is defined as the movement of fish into the chemically treated side of the chamber, whereas avoidance is defined as the movement of fish to the side of the chamber with clean water, or away from the chemical. "No response" is defined as no change in position following the introduction of chemical. Digital photography was used to document the position of all fish in the chambers. For my stimulus, I wanted to find a chemical/extract similar to what fish would experience in the wild during out-migration. I tested two food or attraction responses and two predator-like avoidance responses with juvenile rainbow trout prior to herbicide exposures. Only chemical stimuli that elicited a strong response were used and included the amino acids, alanine and serine, a food

extract, and rainbow trout skin extract. Alanine and the food extract were expected to elicit attraction (Steele et al. 1990), whereas serine and skin extract were expected to elicit avoidance (Rehnberg and Schreck 1986).

The olfaction/behavior test was divided into two distinct segments of exposure, the initial flow of 15 min clean water (Period 1, 0-15 min), and a second 15-minute period of chemical flow (Period 2, 15-30 min). For all portions of the test a digital camera collected a photograph every 60 seconds. Fish were only used once. After testing, fish were euthanized, weighed (g), and measured (fork length, mm).

Behavior was quantified by photographic image analysis using Image-Pro Plus® 4.5 (Media Cybernetics, Inc. Silver Spring, MD). Digital photos were used to determine the mean position of fish within each replicate test chamber for each minute of each test. Each fish was assigned a position score as a ratio of its distance from the chemical inlet relative to the length of the chamber. The eye of the fish was the exact point scored, or the nose if the fish faced the camera. The resulting scores ranged from near zero for a fish on the left side (chemical) inlet, to a score of nearly 1 for a fish on the right side (clean) inlet. Presuming no bias for either inlet/side of the chamber, the average of all ratios of the 10 fish within each chamber would be about 0.5, or the outlet/middle of the chamber. It is this average of locations of all of the fish in the chamber (as a ratio) that I refer to as the "mean position". The mean position for each chamber was then averaged

within each test period: the clean pre-treatment, and the chemical treatment. The differences between the mean positions during the clean period were compared with the chemical period using paired t-tests. The comparison of mean position is best suited to detect quick and sustained shifts away from or toward the chemical flow inlet during the chemical period of the test (Curran et al., unpublished manuscript). It was expected that variability would decrease within the chambers from the clean pre-treatment period to the chemical period. To confirm this we did a paired t-test on the coefficient of variation (CV). Due to the more variable nature of behavioral responses and small sample sizes (n=5), I *a priori* chose an alpha level of 0.10 for all hypothesis testing. In addition, the magnitude to shift between the clean time period and the chemical period was examined between control and herbicide exposed fish using a one-way ANOVA followed by Dunnett's test to determine where differences occurred. Again, an alpha level of 0.1 was used to indicate statistical significance. Avoidance replicates were only included when at least one fish was detected in the chemical portion of the chamber during chemical exposure.

Results

Water quality parameters within the test chambers were within those recommended for toxicity tests with salmonids (Table 3.2; USEPA 1996). Actual herbicide concentrations within the exposure portion in each test (Table 3.1) were 35% lower than targeted for

DMA® 4 IVM, and 57% higher for one Sonar® A.S. replicate. All other concentrations were close to nominal.

Table 3.2. Water quality during the 96-hour herbicide exposures prior the testing of olfactory-mediated behavior in juvenile rainbow trout. Data are the mean plus or minus the standard deviation with minimum and maximum below.

	Control	DMA® 4 IVM	Renovate® 3	Reward®	Sonar® A.S.
Temperature (°C)	13.2 ± 0.3 12.5-13.8	13.1 ± 0.5 12.3-14.0	13.2 ± 0.3 12.6-13.9	13.0 ± 0.3 12.4-14.0	13.2 ± 0.3 12.5-13.8
Dissolved Oxygen (mg/L)	9.86 ± 0.29 9.15-10.63	9.55 ± 0.39 8.69-10.01	9.80 ± 0.27 9.06-10.18	9.67 ± 0.35 8.83-10.60	9.85 ± 0.30 8.60-10.22
pH	6.9 ± 0.3 6.4-7.2	6.6 ± 0.2 6.3-6.9	6.9 ± 0.2 6.4-7.2	6.9 ± 0.2 6.3-7.2	6.9 ± 0.3 6.4-7.2
Conductivity (uS)	74.0 ± 2.6 67.4-78.6	71.1 ± 1.6 69.0-73.9	74.3 ± 3.3 67.4-80.4	75.2 ± 2.7 68.3-80.2	73.8 ± 3.2 67.1-78.9

Initial stimulus testing found skin extract to elicit the most statistically repeatable response. A concentration of alanine at 10^{-3}M did not elicit any response in my rainbow trout (Fig 3.2). Food extract resulted in statistically significant attraction (Fig 3.3), but I felt food was not a strong motivator for out-migrating salmon and as such was a less important response for this type of testing. Serine at a concentration of 10^{-3}M only occasionally resulted in a statistically significant avoidance response (Fig 3.4). Lower concentrations did not elicit any response, and higher concentrations were not possible due to limitations of the test apparatus. Rehnberg and Schreck (1986) found that coho salmon (*O. kisutch*) avoided serine concentrations as low as 10^{-7}M, but they used a different test method and species. Using skin extract, created from conspecifics, I was able to create a repeatable and marked response (Fig 3.5).

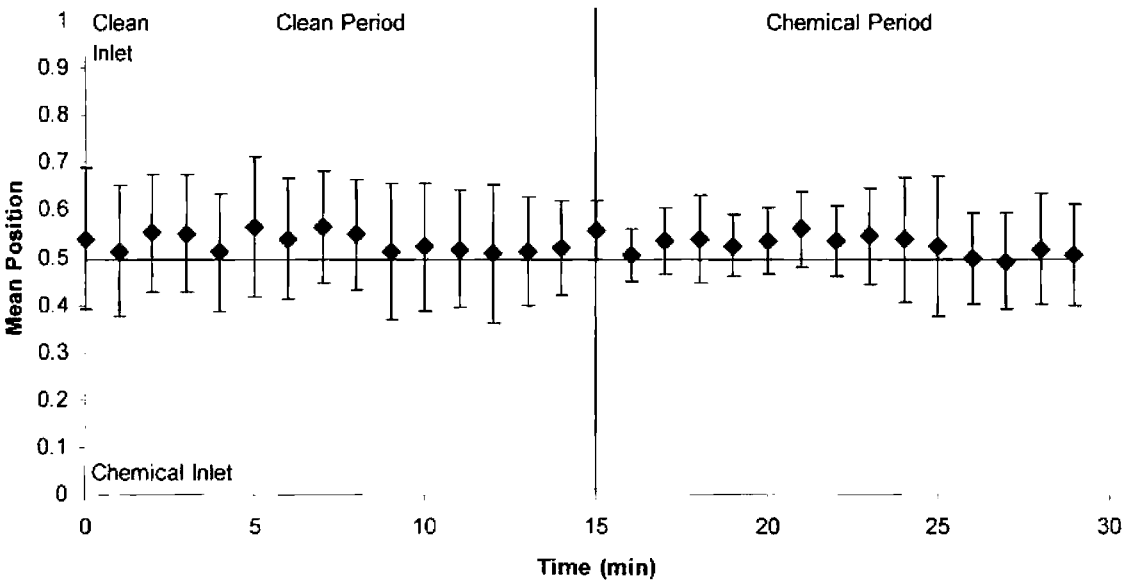

Figure 3.2. Response of juvenile rainbow trout to 10^{-3}M alanine. Data points are the mean position of fish across all chambers and the bars represent standard errors. There was no statistically significant response to the stimulus.

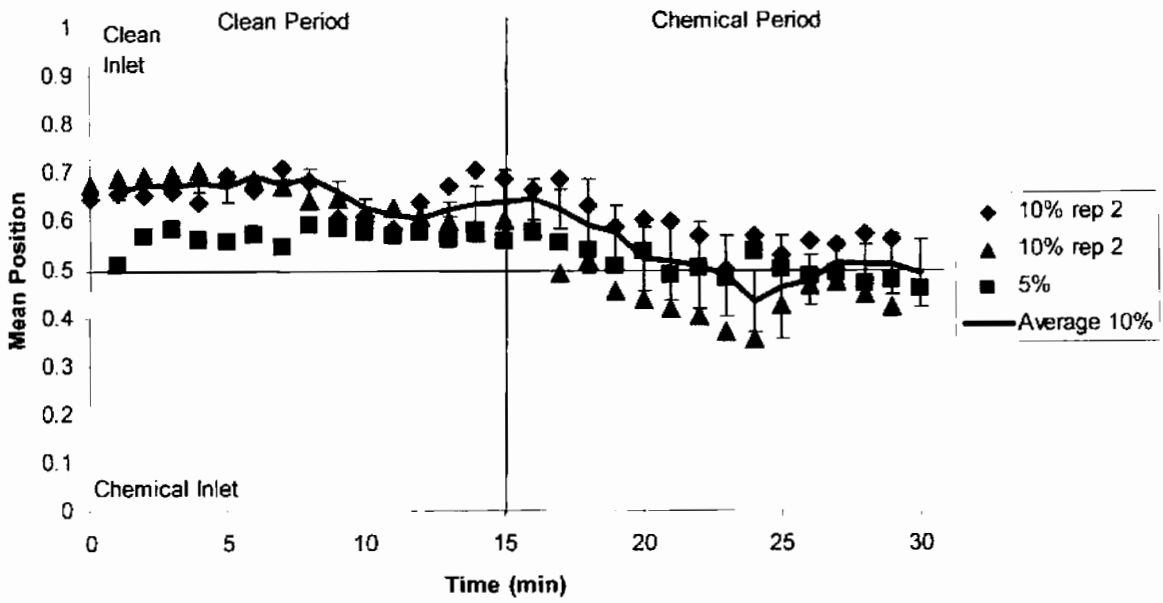

Figure 3.3. Response of juvenile rainbow trout to food extract at various concentrations. The data points are the mean position of fish across all chambers. The 10% concentration resulted in a statistically significant attraction response ($p=0.02$).

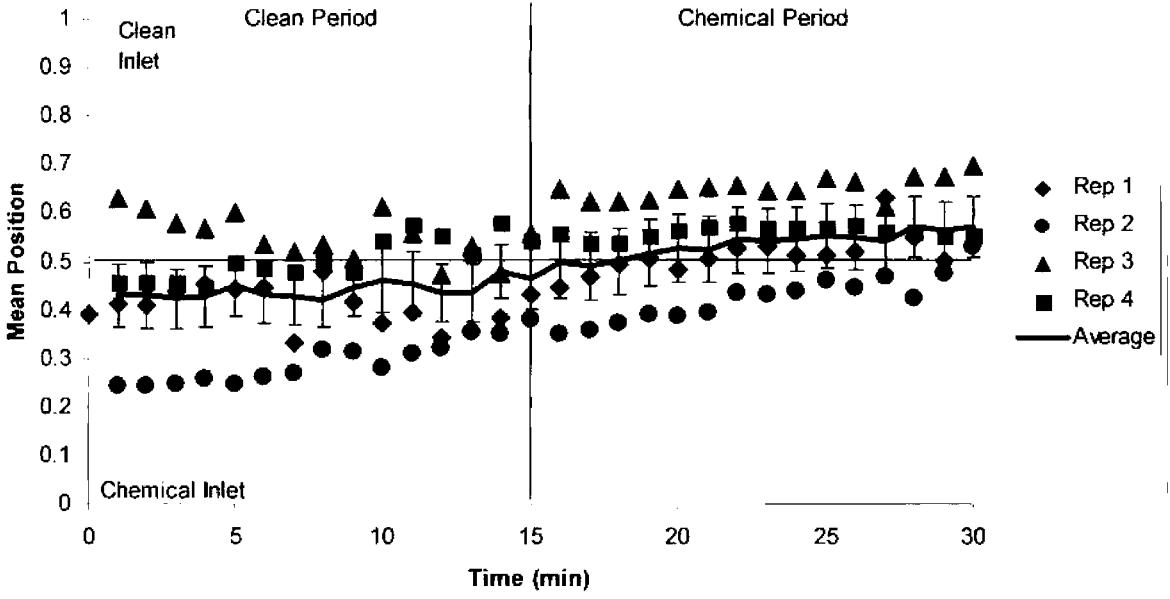

Figure 3.4. Response of juvenile rainbow trout to 10^{-3} M serine. The data points are the mean position of fish across all chambers. Only some replicates resulted in a statistically significant avoidance ($p \leq 0.10$).

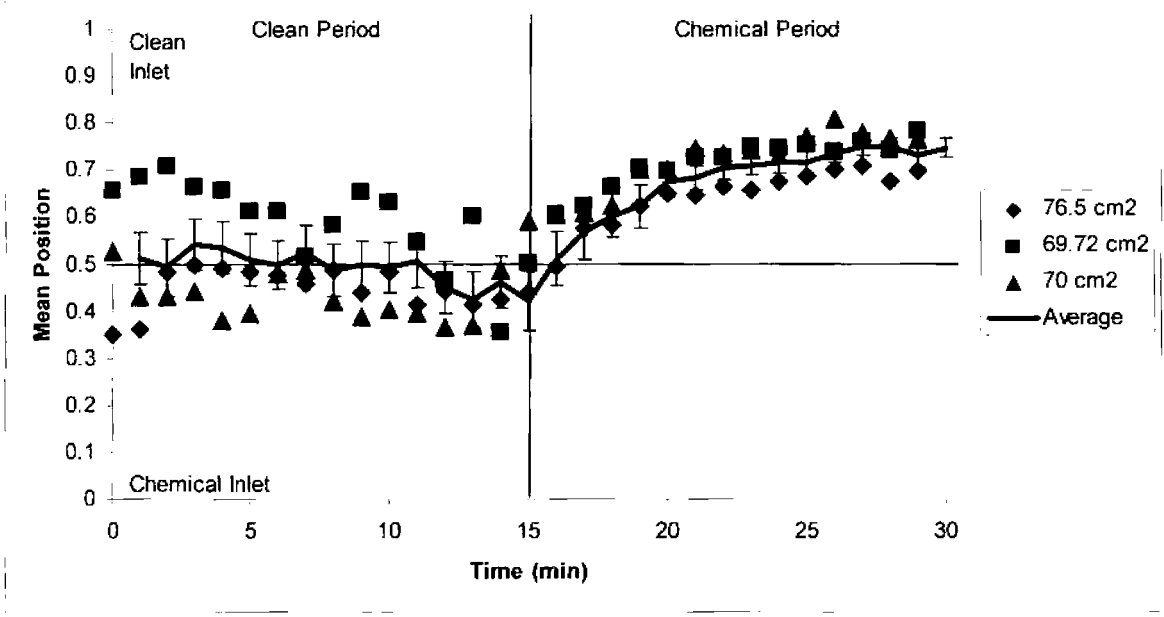

Figure 3.5. Response of juvenile rainbow trout to skin extract created from conspecifics. The data points are the mean position of fish across all chambers. Responses to each concentration were statistically significant ($p \leq 0.10$).

The only mortality observed occurred in two separate replicates of Reward®, in which, one fish died per replicate.

During the first set of exposures (A and B), there was a slight change in procedure between exposures, and because of the change I was unable to combine replicates from both weeks for statistical analysis. In an attempt to eliminate bias associated with the end of the chambers to which fish were added, I changed the location between the 2 weeks of the test. However, because of the low sample size as a result of the split, only DMA® 4 IVM (2,4-D) and the controls showed repeatable responses (DMA® 4 IVM, A: n=3,

p=0.07; B: n=3, p=0.03; Controls, A: n=2, p=0.08; B: n=3, p=0.05; Figs. 3.6 and 3.7). There were only two successful replicates for controls during Exposure A because in the third replicate, run late in the day, the fish behaved differently from all other previous controls. This replicate was removed from the analysis. Both controls and DMA® 4 IVM exposed fish showed marked avoidance of the skin extract. There were no significant differences in the magnitude of the shift in position between fish previously exposed to DMA® 4 IVM for 96 hours or clean water.

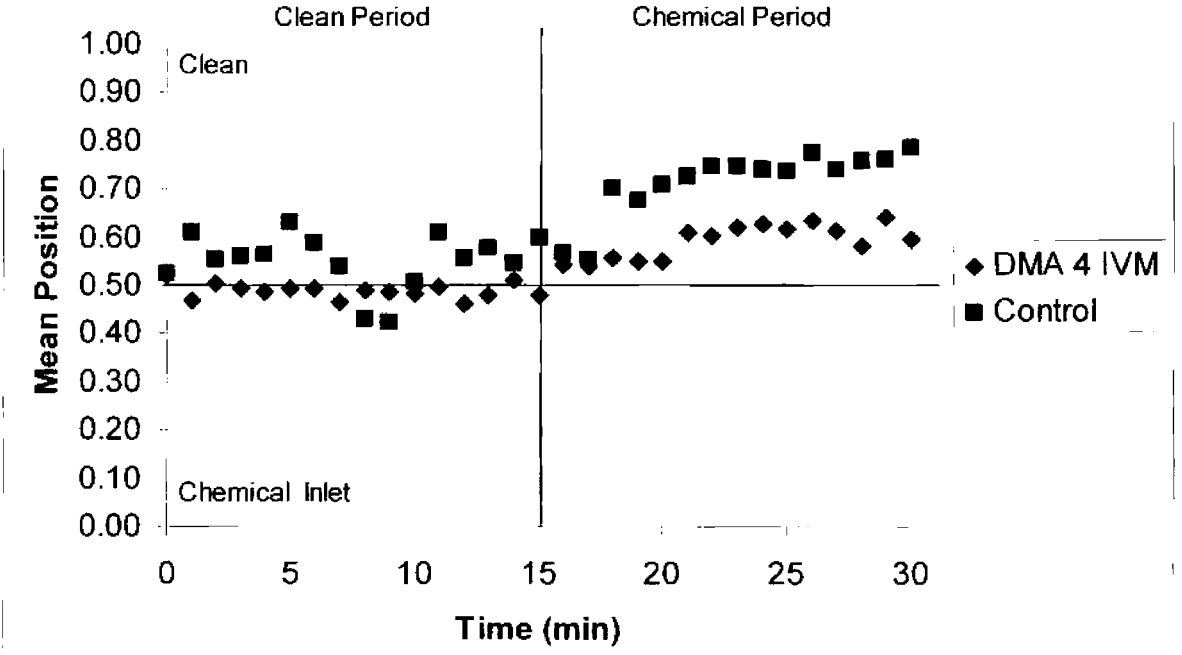

Figure 3.6. Response of juvenile rainbow trout to skin extract following exposure for 96 hours to either the herbicide, DMA® 4 IVM or clean water (controls). Data points are the mean position of fish across all chambers. Avoidance responses were statistically significant (alpha=0.10) in fish exposed to DMA® 4 IVM (n=3, p=0.07) or clean water (n=2, p=0.08).

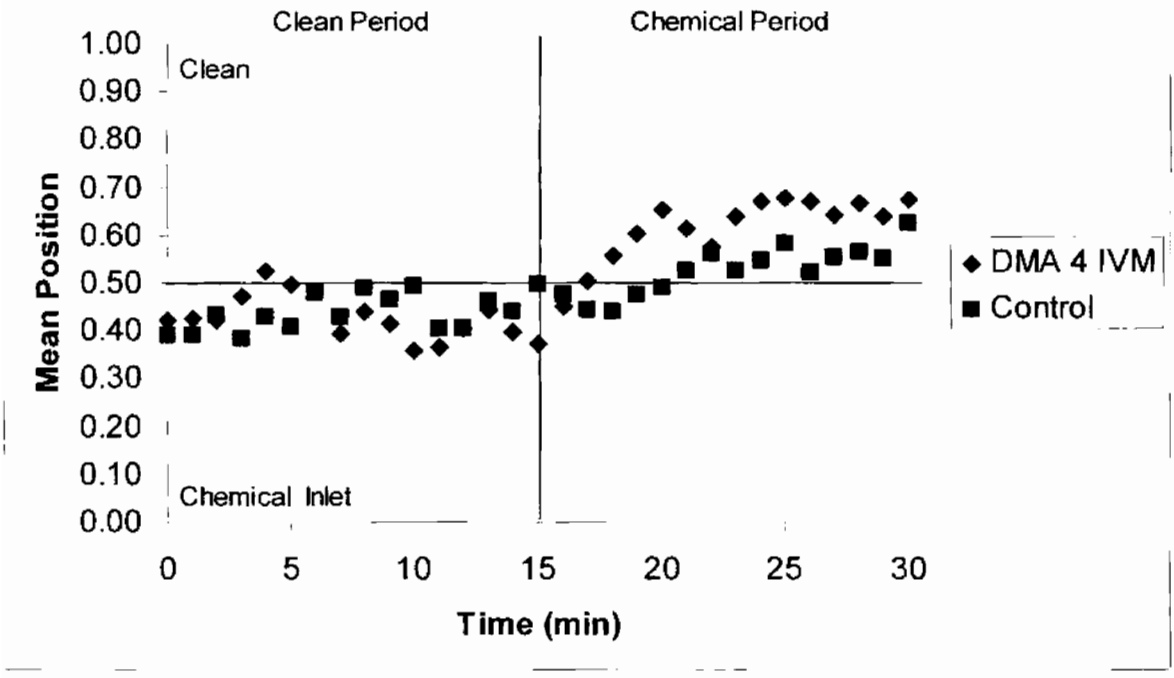

Figure 3.7. Response of juvenile rainbow trout to skin extract following exposure for 96 hours to either the herbicide, DMA® 4 IVM or clean water controls. Data points are the mean position of fish across all chambers. Avoidance responses were statistically significant (alpha=0.10) in fish exposed to DMA® 4 IVM (n=3, p=0.03) or clean water (n=3, p=0.05).

Additional exposures (C-F) were completed with the herbicides, Renovate® 3 (triclopyr), Reward® (diquat), and Sonar® A.S. (fluridone), with the fish loading location varied within weeks, so all replicates could be combined. Also, only two avoidance tests were run per day, so that the problem with the last replicate in the previous tests could be avoided. For exposures C-F, Renovate® 3, Sonar® A.S., and the controls all resulted in statistically significant avoidance responses to the skin extract (control, n=5, p=0.02, Renovate® 3, n=5, p=0.04, Sonar® A.S., n=5, p=0.08; Fig. 3.8). In addition, there were no significant differences in the magnitude of the shift in mean position between fish exposed to Renovate® and Sonar® compared to controls (p=0.54 and p=0.97,

respectively). Rainbow trout exposed to Reward®, however, did not respond to the skin extract, indicating impacts to their olfactory system (n=5, p=0.83; Fig 3.8). This non-response was also detected with the ANOVA, where there was a significant difference between the magnitude of shift between control fish and those exposed to Reward® (p=0.03). There was a slight change in flows during exposure D that caused the mixing zone between clean and chemical side to be wider than for other replicates. However, the response of controls to the skin extract was not affected. Due to this change, I repeated the Reward® exposure to confirm the effects observed. For exposures G and H, only controls and Reward® were used, and again controls showed significant avoidance (n=5, p=0.04), whereas fish exposed to Reward® did not respond to the skin extract (n=4, p=0.81, Fig 3.9). There were only four viable replicates for Reward®, because in one replicate, the fish were not detected in the skin extract side of the chamber.

As expected, the average CV for controls (p=0.01) and fish exposed to DMA® 4 IVM (a, p=0.02, b, p=0.10), Renovate® (p=0.01), and Sonar® (p=0.03) decreased within the second time period, as a result of movement into the clean side of the chamber due to avoidance of the skin extract. The average CV for fish exposed to Reward also decreased (C-F p=0.07, G and H p=0.06) indicating that they moved closer together but failed to shift position into the clean water flow. This change in behavior suggests the fish detected the stimulus but were unsure how to respond to it.

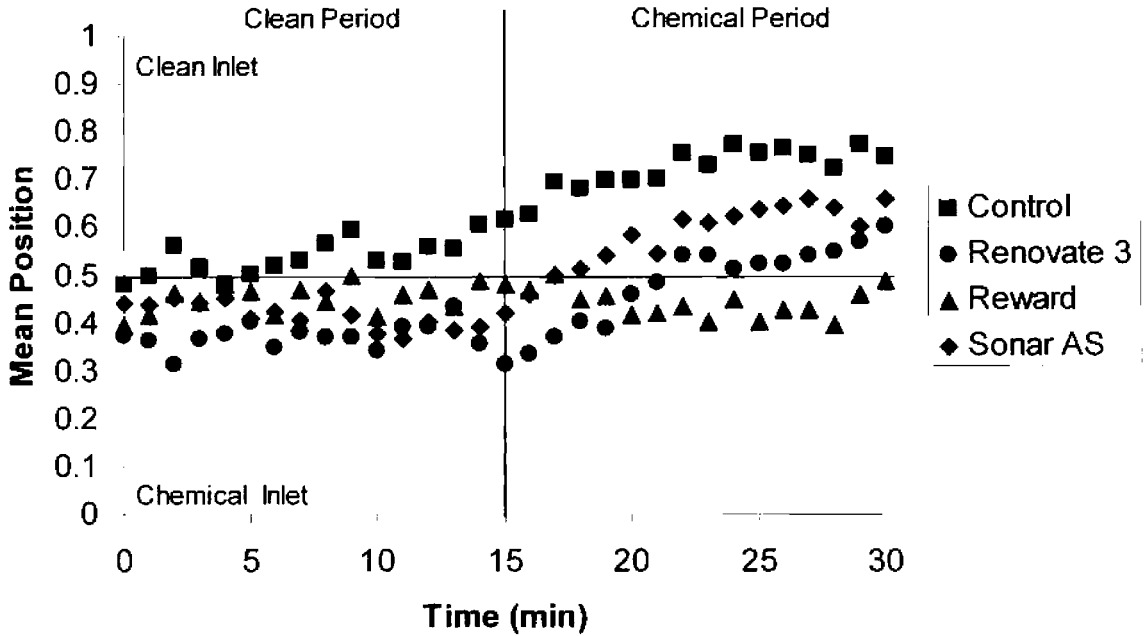

Figure 3.8. Response of juvenile rainbow trout to skin extract following exposure for 96 hours to the herbicides, Renovate® 3, Reward®, or Sonar® A.S., or clean water controls. Data points are the mean position of fish across all chambers. Avoidance responses were statistically significant (alpha=0.10) in fish exposed to Renovate® 3 (n=5, p=0.04), Sonar® (n=5, p=0.08) and clean water (n=5, p=0.02), but not Reward® (n=5, 0.83).

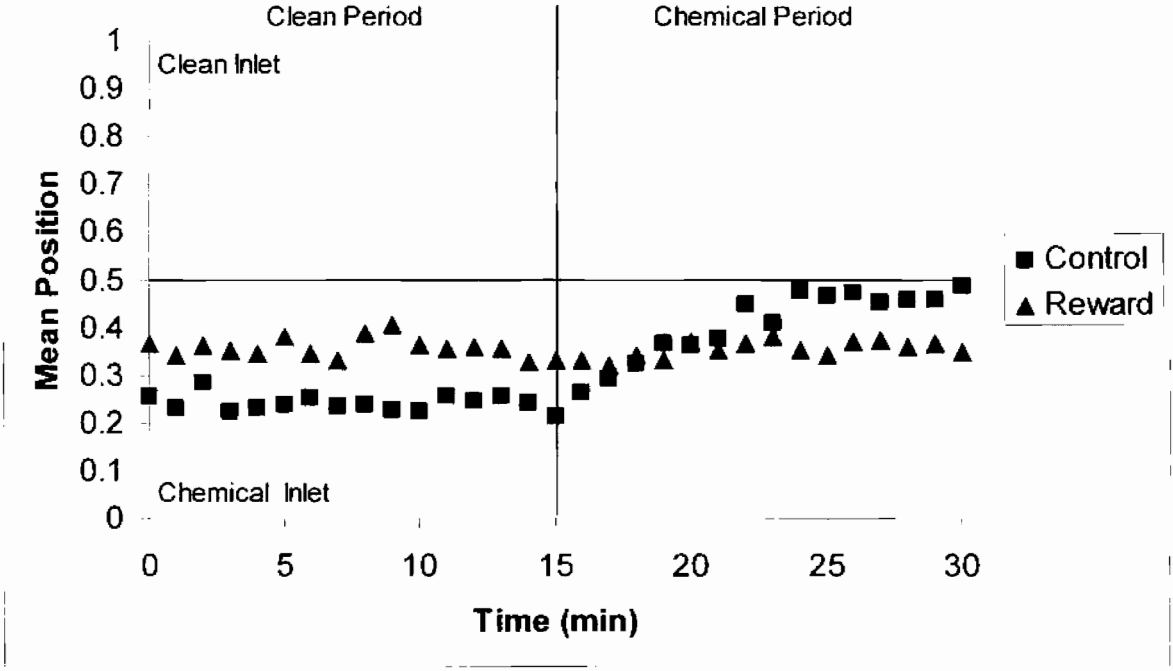

Figure 3.9. Response of juvenile rainbow trout to skin extract following exposure for 96 hours to the herbicide, Reward®, or clean water (controls). Data points are the mean position of fish across all chambers. The avoidance response (alpha=0.10) in control fish was statistically significant (n=5, p=0.04). Avoidance was not detected in the fish exposed to Reward® (n=4, 0.81).

Discussion

Exposure to the herbicides at their maximum application rates DMA® 4 IVM, Sonar® A.S., and Renovate® 3 (2,4-D, fluridone, and triclopyr respectively) did not alter the ability of juvenile rainbow trout to avoid skin extract; exposure to Reward® (diquat) did alter olfactory-mediated behavior.

The response of Reward® exposed fish, as detected with a comparison of CV, indicates the fish may still be able to smell, but have lost the ability to process the odor. Fish exposed to the herbicide did not move out of the skin extract, as did control fish, but did move closer together during the stimulus portion of the tests. Wolf and Moore (2002) found that crayfish were still be able to detect odors after exposure to the herbicide, metolachlor, but did not respond appropriately. When exposed to an odor that normally elicited aversion, the crayfish moved towards the odor, instead of away from it. Because the mechanisms underlying the lack of response were not determined in this study, it is difficult to say exactly what is occurring within the olfactory system of exposed fish. However, results clearly indicate the ability of Reward® exposed fish to respond correctly to a predatory cue is significantly impaired.

The different results in the controls during Exposure A could be due to the fact that few fish moved around the chamber during the 15 minutes prior to stimulus introduction. Therefore when 3 fish became more active during the chemical period, the group as a whole appeared to move into the stimulus. Unfortunately, the amount of time actually spent on that side of the chamber could not be determined due to the testing protocol. In all previous tests, control fish were always active during the entire test period, but never moved into the chemical side of the chambers while skin extract was flowing. Additionally, the inactivity in Reward®-exposed fish during exposure H that resulted in none of the fish experiencing the skin extract is similar to the inactivity in the control fish

observed in Exposure A, and both likely reflect the natural variation in behavior among different groups of fish.

The mortality observed in two Reward® tanks is interesting because the MSDS for Reward® reports an LC50 of trout as 14.8 ppm a.i., which would suggest that the concentration I tested is likely to be within the lower bounds of the effects range. This could explain the occasional mortality I observed in the Reward® exposed fish. The LC50 of triclopyr for juvenile rainbow trout is greater than 100 ppm a.i. (Mayer and Ellersieck 1986), which is well above my highest nominal concentration of 2.5 ppm a.i.. Similarly, the LC50 of fluridone for juvenile rainbow trout is 4.25-8.4 ppm a.i. (Mayer and Ellersieck 1986); suggesting that the nominal concentration I tested (0.150 ppm) was well below lethal levels. Mayer and Ellersieck (1986) report LC50 values for 2,4-D for rainbow trout of greater than 100 to 420 ppm a.i., again well above the tested concentration of 4 ppm a.i.. Overt toxic effects would not be expected in juvenile salmon occupying ponds and lakes in which any of the herbicides were applied according to the label. No fish mortalities have been reported in Washington State due to use of these herbicides in surface waters (K. Hamel, personal communication). Chinook smolts were slightly attracted to 10X the concentrations of Renovate® 3 and Reward® I tested, suggesting fish might move into a potentially toxic environment (Curran et al, unpublished manuscript), and in the case of Reward®, that exposure has the potential to impact olfactory performance. However, the concentrations I tested have not been

associated with adverse effects in juvenile coho or Chinook smolts (King et al, unpublished manuscripts).

After I initiated my studies, the manufacturer changed the label rate for Reward® and the maximum application rate is now half of the concentration I tested. Because I did not test lower concentrations, I cannot say whether applications at the lower concentration would impair olfactory-mediated behavior. However, a 2X exposure following operational applications at the new rate may not be unrealistic. Additional studies are needed. If effects occur at the new rate, minimum effective exposures and recovery times should also be determined. An examination of the timing and location of applications relative to out-migrating salmon smolts would also help in determining the actual hazards posed by the herbicide.

The Renovate® 3 label has a maximum target water concentration of 2.5 ppm triclopyr as acid equivalents, which when converted to active ingredient is 3.49 ppm triclopyr. Due to an error interpreting the label, I tested 2.5 ppm a.i. of the active ingredient, ca. 28% less than the legal maximum. However, operationally no more than 2.5 ppm triclopyr is permitted in Washington State. Additional studies are needed to determine the threshold for effects.

Chapter 4- Synthesis of studies and research needs

The overall goal of my research was to determine if aquatic herbicides used in Washington State have adverse impacts on the olfactory mediated behavior of salmonids. The objective of my first study, Chapter 2, was to determine if juvenile Chinook salmon (*Oncorhynchus tshawytscha*) avoid the formulations of three herbicides: Renovate® 3 (triclopyr-TEA), Reward® (diquat), and Sonar® A.S. (fluridone). The objective of my second study, Chapter 3, was to determine if exposure to the three herbicides noted above and DMA® 4 IVM (2,4-D), alter olfactory performance in juvenile rainbow trout (*O. mykiss*), used as a surrogate for juvenile salmon.

Juvenile Chinook smolts were attracted to concentrations of triclopyr (34.9 ppm a.i.) and diquat (13.7 ppm a.i.), both as formulated products, Renovate® 3 and Reward®, respectively. According to the labels for these products, concentrations eliciting attraction were 10 times greater than maximums associated with field applications. My work did not include 2,4-D (as DMA® 4 IVM) because previous work by Folmar (1976) determined fish would avoid 1 and 10 ppm of the herbicide as a.i.. DMA® 4 IVM is applied at rates of 2-4 ppm a.i., suggesting that fish would avoid application rates of the herbicide.

Knowledge of the characteristics of the pesticide plumes created by applicators of the herbicides would be helpful in evaluating fish response. Should concentrations be greater than those I tested, additional avoidance/attraction tests would be warranted. Applicators often note that fish swim away from the treatment area during their applications (K. Hamel personal communication), but my data suggest this response may be caused by the disturbance associated with the application and not the herbicide itself.

A concentration of 1.37 ppm a.i. of diquat, as the aquatic herbicide Reward®, resulted in juvenile rainbow trout being unable to properly respond to skin extract, a known deterrent. This effect was observed after fish were exposed to the herbicide for 96 hours. However, whether or not the same effects would occur following shorter exposures is not known. Diquat (Reward®) has been shown to have a half life of 1-4 days (Emmett 2002). The other herbicides tested have shown similar half lives. 2,4-D (DMA® 4 IVM) has been shown to break down in the environment in as little as 23 hours, and as long as 7 days (Emmett 2001), while triclopyr has a typical half life of 3.5-7.5 days, but it can be as little as 12 hours (Emmett and Morgan 2004). Fluridone (Sonar® A.S.) is the most variable with a half life of 2-60 days depending on environmental conditions (Emmett 2001). These data suggest that the 4 day exposure I used, in most cases, probably represents the maximum fish would receive in natural waters.

I tested my fish immediately after they were removed from the chemical. It is possible that fish would be able to recover their olfactory ability once exposed to uncontaminated

water. Given that whole lake treatments are rare, except for some fluridone treatments (K. Hamel, personal communication), there frequently may be clean water within the system for fish to move to. Although my research (Chapter 2) suggests they will not move away due to the chemical presence, fish may naturally move within the system. In addition to out-migrating smolts there are juvenile Chinook and coho populations that overwinter in lakes. These populations may experience more frequent exposure to aquatic herbicides and of longer duration. An additional study quantifying recovery times would provide a more complete assessment of possible impacts the herbicide may have on olfaction in juvenile salmonids.

I used formulated products in my tests that are available for use by pesticide applicators. Formulated products, however, contain more than just the active (herbicidal) ingredient. Manufacturers do not need to report what those other ingredients are on the label; they just have to report what percentage of the product is the active ingredient. It is possible that it is not the diquat or triclopyr itself that is causing attraction or, in the case of diquat, that which is altering the olfactory system, but instead one of the "other" ingredients. For aquatic herbicides, it has been found that the additional ingredients in end products are actually the most toxic component (Smith et al. 2004). A test comparable to mine, but using technical grade diquat and triclopyr would determine whether it is actually the herbicide, or one of the "other" ingredients in the formulated products, or an interaction between the herbicides and other components of the formulation that is causing the

response I observed in juvenile salmonids. It is the end products, however, that ultimately enter the water.

Rainbow trout appear to be a good surrogate for salmon in toxicological studies (Teather and Parrott, unpublished manuscript). However, it would be useful to examine out-migrating smolts because non-anadromous trout do not go through the parr-smolt transition, which is a critical stage in the development of the olfactory system. For my olfactory study, I tried to use Chinook smolts, but there was significant morality due to infections of branchial ichthyobodiasis, and secondary bacterial septicemia that we could not cure with medicated feed and formalin treatments as prescribed by the University Animal Care Veterinarian. It was a problem throughout Washington State hatcheries that year. The second year I tried to use Chinook again, but they were schooling and did not respond well to the exposure to serine. This has been seen in other fish species, where fish will tolerate higher levels of contaminants when there are other motivating factors to remain in the area, such as shade, and prey availably (Scherer and McNicol 1998). These Chinook, however, were likely at a physiologically younger age than their hatchery counterparts due to reduced food rations and that could have impacted results. As Chinook get older they tend not to school as much as their younger counterparts (D. Beauchamp personal communication). Chinook used in the avoidance study were of an older age and did not school at all. It might be possible to repeat my olfaction tests using Chinook, or another salmonid species that do not have a tendency to school, to better access effects at the critical stage of smoltification and olfactory development. To further

examine the biological significance of olfactory effects with salmon, it would be interesting to expose smolts prior to release and see if there are any differences in survival during outmigration and return rates compared to unexposed controls.

A variety of different apparatuses and methods have been used to assess avoidance/attraction in fishes exposed to contaminants (Chapter 1). The development of standardized methods for behavior testing, increasing comparability among studies, would be a significant contribution to the field of behavioral toxicology. A standardized method would also allow future researchers to begin to test for sublethal effects more efficiently. As it stands now, most researchers have to familiarize themselves with the available methods and protocols and then choose the most appropriate for their research question, undoubtedly involving many trials and failures before perfecting their system. In my avoidance study, I made a number of improvements to the methods described by Exley, including replicate tubes, tube shape, and chemical delivery. Statistical methods for analyzing the data were also improved. In addition, skin extract proved to elicit a highly significant and reproducible avoidance response.

Although none of the herbicides at the maximum label rates resulted in avoidance or attraction, however, Chinook were attracted to Renovate® 3 and Reward® at 10X the maximum label. Exposure to DMA® 4 IVM, Renovate® 3, and Sonar® A.S. did not alter the olfactory ability of rainbow trout. Reward®, however, did impact olfaction. A full hazard assessment with salmonids and aquatic herbicides is needed. Information on the

factors governing exposure, including the intersection between the habitats treated and the presence of fish, and the magnitude and duration of effects (Grue et al. 2002) are necessary to place my results in a broader ecological context.

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