# Behavioral response of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) to combinations of dissolved copper and submerged structure in freshwater and seawater.

by

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# ABSTRACT

Behavioral response of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) to combinations of dissolved copper and submerged structure in freshwater and seawater.

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Dissolved copper is one of the more pervasive and toxic constituents of stormwater runoff and is commonly found in stream, estuary, and coastal marine habitats of juvenile salmon. While stormwater runoff does not usually carry copper concentrations high enough to result in acute fish kills, exposure to sublethal concentrations of copper is known to both impair salmon health (e.g. olfactory function) and alter behavior. To evaluate behavior of juvenile Chinook to the presence of sublethal concentrations of dissolved copper, I conducted laboratory studies using a multi-chambered experimental tank. The circular tank was divided into six segments so that water flowed outward from the center of the tank through each of the segments separately, yet fish could move freely between them. The presence of individual fish in each of the segments was recorded at 3-second intervals for one hour. The number of occurrences in each segment was counted before and after introduction of a sublethal and environmentally realistic concentration of dissolved copper (< 20 $\mu$g/L) to one of the segments (exposure segment). Use of exposure segment by each fish was compared before and after introduction of dissolved copper in both freshwater and seawater. To address whether use of preferred habitat is altered by the presence of copper, experiments were also conducted with a submerged structural element. Juvenile Chinook salmon avoided segments with dissolved copper in both freshwater and seawater with no structure present. Fish preferred physical structure in freshwater without copper but that preference disappeared when copper was present. There was no preference for structure in seawater. The presence of sub-lethal levels of dissolved copper altered the behavior of juvenile Chinook salmon in both freshwater and seawater, this could potentially affect behaviors beneficial to growth, survival and reproductive success.

# TABLE OF CONTENTS

## LIST OF FIGURES



## LIST OF TABLES

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# INTRODUCTION

Juvenile Chinook salmon depend heavily on estuarine habitats that provide food resources for rapid growth and shelter from predators (Aitkin et al. 1998, Healy 1982, Thorpe 1994). Loss and degradation of estuarine habitat is considered to be one factor contributing to the decline of wild populations of Chinook salmon and other Pacific salmonids throughout their range.  Estuarine habitat is disproportionately affected by urban and rural development which tends to be more concentrated in coastal areas (POC 2003). Non-point source pollution such as contaminated stormwater runoff is recognized as being a significant and growing threat to aquatic species (Hart-Crowser 2007). Dissolved copper is one of the more pervasive and toxic constituents of stormwater runoff causing behavioral alterations at low levels to death at higher concentrations. Copper is commonly found in streams, estuaries, and nearshore waters receiving stormwater runoff.

Copper enters the aquatic environment from a variety of sources including treated wood products, pesticides and anti-fouling paint from recreational vessels though metal-containing dust shed from trucks and automobiles is the greatest contributor (Davis et al. 2001). Concentrations in stormwater runoff vary greatly depending on time of the year and surrounding land use.  A study on the Governor Albert D. Rosellini Bridge-Evergreen point on SR-520 in Seattle, WA, which isolated stormwater runoff primarily to road deposition on a busy highway found copper levels between 36 and 76.5 µg/L (King

County 2005). Other measurements of stormwater runoff from outputs in and around Sinclaire/Dyes Inlets watersheds' ranged from a median of 1-75 μg/L (Hart-Crowser 2007). A total of more than 100 metric tons of copper are estimated to enter Puget Sound each year from stormwater runoff (Hart-Crowser 2007, King County 2005). With increasing urbanization, it seems likely that animals that inhabit the estuarine and nearshore waters, including listed species of Pacific salmon, will be exposed to copper.

Salmon encounter dissolved copper across a continuum of water conditions between rearing in fresh water until entering the seawater environment. The toxicity of copper varies depending on fish species, life stage and water chemistry, and the mechanism of toxicity appears to be different in freshwater than in seawater (Blanchard and Grosell 2006, Grosell et al. 2007, Hansen et al. 1999). In freshwater, fish must take up sodium to maintain a higher internal ionic concentration than the surrounding environment. Sodium ions are primarily taken up by active transport across the gill epithelium and in the gut. Acute copper exposure causes osmoregulatory dysfunction by interfering with ion transport primarily at the gill epithelium leading to death at relatively low concentrations; exposure to 26 μg/L was found to be the lethal concentration to 50% of test animals (LC-50) over a 96 hour time period for Chinook salmon smolts (Chapman 1978). Dissolved organic matter (DOM), pH, alkalinity, and hardness can reduce toxicity by complexing with free ionic copper to form a variety of copper species, reducing levels of $Cu^{2+}$ and $CuOH^{+}$, the forms of copper most toxic in fresh water or by competing with ionic copper for sodium binding and uptake pathways on the gill. This relationship is modeled with the Biotic Ligand Model (BLM), though the mechanism is complicated and not fully understood (Di Toro et al. 2005, Niyogi and Wood 2004, Santore et al. 2001). Relatively few studies have examined the effects of copper on fish in seawater and the mechanism of toxicity and chemical interactions are less well understood than in freshwater. In seawater as in freshwater, copper toxicity appears to involve ionoregulation but the buildup of nitrogenous products in plasma may also contribute to toxicity along with the disruption of $Na^+$ and $Cl^-$ regulation (Blanchard and Grosell 2006, Grosell et al. 2007, Grosell et al. 2004a, Wilson and Taylor 1993). The abundance of inorganic ligands in seawater is likely to make copper less bioavailable and be protective against acute toxicity (Neff 2002). More importantly, in seawater the osmoregulatory balance is reversed and fish are excreting ions that they would be taking up in freshwater. Fish are more likely to encounter sublethal concentrations of copper in both freshwater and seawater and the sublethal effects of copper are likely to be a more ecologically relevant measure of the effects of copper on fish (Kleerekoper 1976).

Studies of the sub-lethal effects of contaminants on fish physiology in freshwater have demonstrated copper-linked neurophysiological effects on the olfactory system in salmonids resulting in loss of smell and olfactory-triggered predator avoidance behaviors (Baldwin et al. 2003, City of San Jose 2005, McIntyre et al. 2008, Sandahl et al. 2007, Sandahl et al. 2006) and neurotoxicity in zebrafish (*Danio rerio*) that targets lateral line neurons (Linbo et al. 2009). These studies effectively link copper exposure to physiological damage and related behavioral effects. Behavioral avoidance is a sensitive indicator of exposure to copper and other heavy metals (Scott and Sloman 2004, Woodward et al. 1995) and may manifest before any physiological damage occurs. Many studies have demonstrated avoidance in freshwater (Atchison et al. 1987, Scott and Sloman 2004), but only two have done so in seawater (Koltes 1985, Labenia et al. 2006). Though copper may be acutely toxic to fish in seawater only at high concentrations, studies in seawater have demonstrated avoidance of copper by fish at environmentally relevant levels. The results of copper avoidance studies in seawater are consistent with behavioral responses in freshwater studies; in a two-choice test chamber, cutthroat trout avoided copper at the single test concentration of 40 µg/L in seawater (Labenia et al. 2006); hyperactivity and altered schooling behavior were observed in Atlantic silverside at copper concentrations less than 100 µg/L in seawater, however the test chamber was completely flooded with the exposure solution and did not physically allow avoidance so the altered behavior may have been an avoidance response (Koltes 1985). Because of the lack of studies examining the affects of copper on fish in seawater there exists a gap in information about how copper may affect Chinook salmon in the estuarine and nearshore environment.

Fish function in a complex environment and make choices about how to use that environment such as tradeoffs between avoidance of predators and access to food resources. Avoidance is likely to be the initial response to the presence of copper, and may reduce or prevent further damage that could result from prolonged exposure. Few studies have addressed avoidance in the field in freshwater and none have done so in seawater. Results of field studies in freshwater are consistent with avoidance studies conducted in the lab. In one study, Chinook salmon avoided a metal contaminated tributary compared to one relatively free of metal contamination during spawning migration (Goldstein et al. 1999), potentially reducing available spawning habitat for a portion of the fish population. A related study (Woodward et al. 1997) found cutthroat trout avoided a metals mixture simulating that found in the same system and that avoidance was driven by levels of zinc and copper. Another study illustrates how the avoidance of one stressor may override avoidance of metal contamination. Trout in a Montana stream suffered damage from chronic metal exposure when favoring areas of cooler water contaminated with sub-lethal levels of metals over warmer areas relatively free of metal contamination (Harper et al. 2009). Metals in the cooler areas were above concentrations that have been shown to elicit avoidance behavior but temperatures in the cleaner areas may have reached nearly lethal levels. Behavior affects how fish use their environment and has important ecological implications and avoidance of copper may limit the use of otherwise suitable habitat. Alternately, preference for habitat may increase the risk of exposure to contaminants. Estuaries and nearshore habitats are critical in the life-cycle of many salmon, especially Chinook. Disruption of juvenile Chinook salmon behavior in these environments from dissolved copper may have important implications for health and survival.

To evaluate whether juvenile Chinook salmon may be affected by dissolved copper concentrations typical of urban estuaries, I conducted laboratory experiments using a multi-chambered experimental tank, similar to that used by Scarfe (1985). Distribution of individual fish within the tank was compared in multiple trials before and after introduction of dissolved copper in both freshwater and seawater. To evaluate interactive effects of copper and physical habitat complexity on fish behavior, additional experiments were conducted with a submerged structural element.

# METHODS

*Animals*

Four thousand Fall Chinook salmon were acquired as eyed eggs from the University of Washington's School of Fisheries hatchery, (Seattle, WA, USA) in December 2006 and transported to the NOAA Fisheries Mukilteo Field Facility in Mukilteo, WA for rearing and experiments. Fish were fed standard commercial salmon pellets (Bio-Oregon, Warrenton, OR, USA) and held in a 1.8 m diameter circular fiberglass tank on a recirculating freshwater system supplied by carbon filtered city water (100-300 mg/L total hardness as $CaCO_3$, pH 7.1-7.3, temperature 8-12 °C, oxygen 9-12 mg/L) until smolted over the week of 11 June 2007 (Meador et al. 2006). Forty fish to be used in the freshwater portion of the experiment were transferred to an indoor circular 0.9 m diameter tank on a separate recirculating freshwater system supplied by the same water source on 8 May 2007, prior to smolting. The remaining fish were held on flow-through, sand-filtered seawater, (salinity 30‰, pH 7.8, oxygen 9.5 mg/L, temperature 10-12 °C). The recirculating system for the freshwater trials of the experiment was replenished from city water passed through a 5µm filter and an activated carbon filter and chilled to between 12-14 °C. Seawater for the experiment was sand filtered and passed through a 20 µm cartridge filter and 10 µm bag filter to remove particulates that would otherwise coat the floor of the tank. Temperature was maintained at 10-12 °C.

*Experimental Chamber*

The experimental chamber consisted of a 1.8 m diameter 74 cm tall circular fiberglass tank, divided into six segments with a central distribution manifold (Figure 1). Each segment contained an inner distribution segment, and an outer experimental segment. The distribution segments and dividers were made out of 3.2 mm Plexiglas and attached to the circular tank using silicone glue. The distribution manifold was made from 200 mm diameter PVC pipe to allow water to enter from the top and flow out through 1.3 mm, CPVC pipe fittings into the distribution chamber at the head of each of six segments. Water flow was directed into each experimental segment, to either a center section along the bottom of the tank, or to the sides where sheets of twinwall polycarbonate sheet created a laminar flow effect to help isolate water between segments while allowing fish to swim freely around the tank (Figure 1). Copper solution was pumped into the distribution chamber of the exposure segment (segment 1) so that it mixed with water flowing into the segment, entering at the bottom center at the head of the experimental portion of the exposure segment. Drains were placed at the center of the external wall of each segment, approximately 31 cm above the bottom of the tank providing a water depth of 35 cm. Adjustable 90° elbows leaving the drains on the outside of the tank, allowed outflow of each segment to be regulated separately.  Flows were adjusted so that the exposure segment (segment 1) had negative pressure relative to its neighbors, allowing incoming water to be contained within that segment (Figure 2).

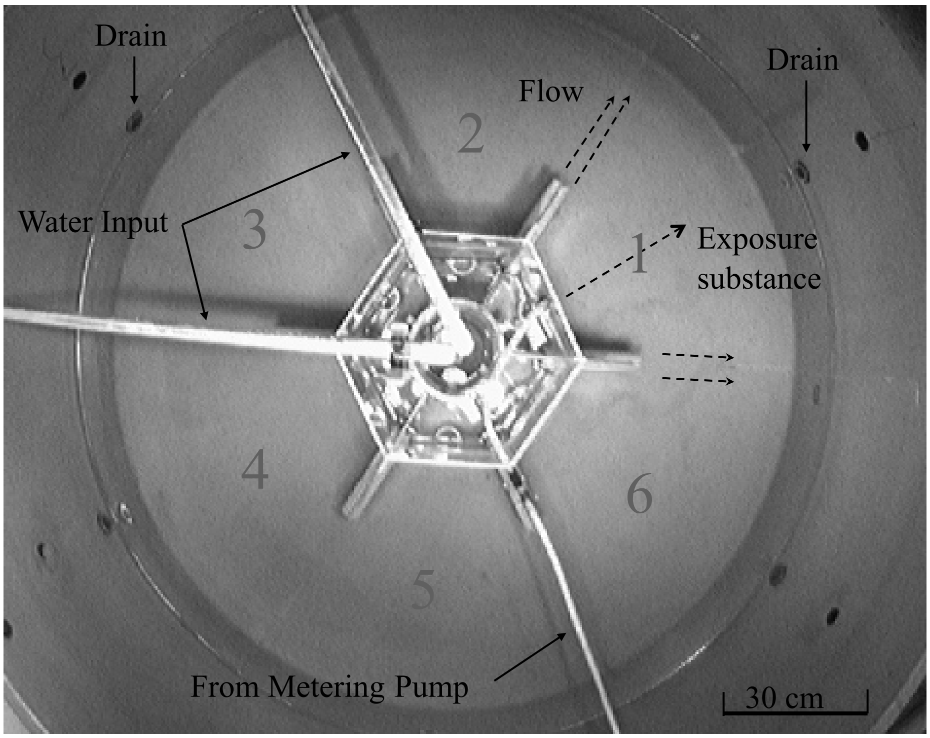


Figure 1. Overhead view of experimental chamber showing segment numbers (1-6). Double-dashed arrows indicate flow from dividers; single-dashed arrow indicates direction of flow of copper solution. Flow and drains are radially symmetrical. Copper solution was introduced in segment 1 only. Water input lines run over the top of the tank on the borders of segment 3.

Isolation of flow within the exposure segment and between it and neighboring segments was verified using a fluorescent Rhodamine water tracing dye (Bright Dyes FTW Red 25liquid, Bright Dyes Miamisburg, OH). Dye was pumped into the exposure tank through the same delivery system used to supply the copper solution and samples collected over time to determine onset, saturation and dispersion. Dye traveled from the input to the far wall of the segment in about 3 minutes and the segment was saturated by 7 minutes. Dye was well contained in the exposure segment.

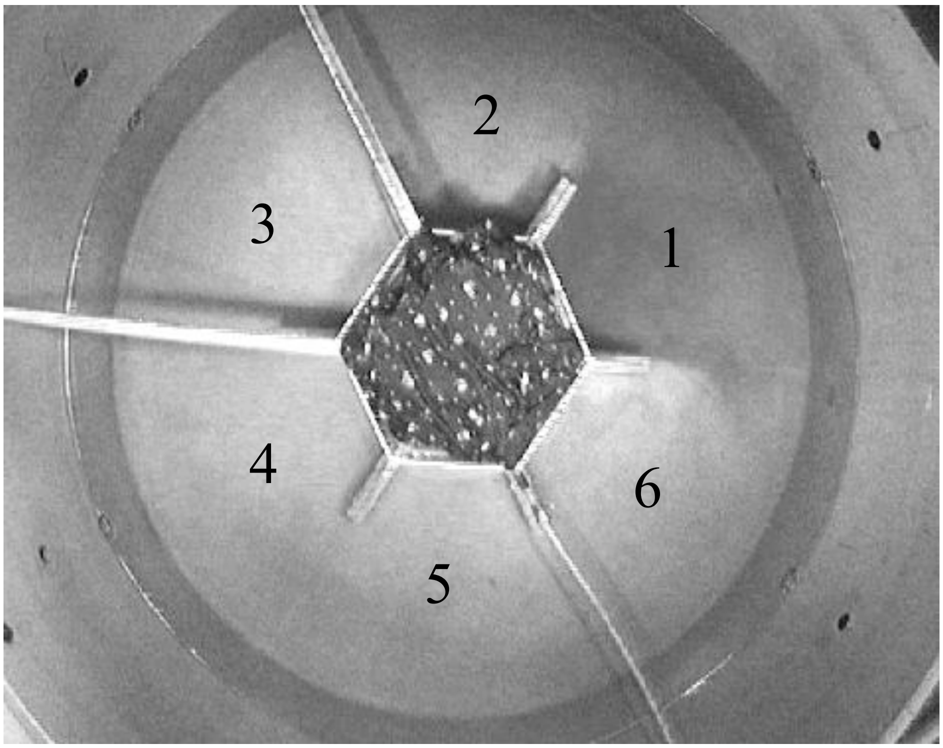


Figure 2. Overhead view of dye test showing containment of treatment water within the exposure segment (1). Distribution chambers are covered with fabric to eliminate reflections. Tank segments are numbered.

A solution of 0.01 g/L, dissolved copper was added to the exposure segment at a rate of 12.6 mL/min via a metering pump (Liquid Metronics Incorporated, model LMIA11). Delivery of the copper solution at this rate provided a nominal concentration of 17 µg/L for one-sixth of the total flow of the tank. Total flow into the experimental tank was 45 L/min and flow out of the exposure segment was a minimum of 8 L/min. The dissolved copper solution was prepared before each experimental trial by adding 10 mL/L to $dH_2O$ of a stock solution of 1 g/L Cu (2.68 g/L $CuCl_2 \cdot 2H_2O$), which was made up weekly. The delivery line from the copper solution source was purged daily and fresh solution run through the line.

*Copper Concentration*

Samples of exposure solutions were drawn from the exposure tank at 0, 10, and 30 minutes. Samples were taken from the center of each segment and the border between the exposure segment (segment 1) and adjacent segments 2 and 6. Single samples were taken at each time point. Samples were submitted to an outside laboratory (Frontier Geosciences, Seattle, WA, USA) for analysis of total dissolved copper by inductively coupled plasma mass spectrometry (detection limit 0.1µg/L in freshwater and 0.02 µg/L in seawater). In freshwater, total dissolved copper ranged from 2.6-3.6 µg/L at the various sampling locations at time zero. At 30 minutes, the exposure segment (segment 1) reached a concentration of 7.9 µg/L while surrounding segments remained low. In seawater, segment one had 0.4 µg/L dissolved copper at time zero and 16.9 µg/L at 30 minutes (Table 1). The higher time zero levels of copper in the freshwater portion of the experiment may be due to the recirculating system that provided water for the experiment as well as fish held in a separate tank for use in the experiment. Trace amounts of copper from food as well as chillers and pumps may have caused the elevated time zero values. Levels of copper at the 10 and 30 minute sampling points in freshwater were roughly half of those in seawater. This may be due to insufficient priming of the pump delivering the copper solution at the time of the sampling.

Table 1. Results of Pre-experiment water samples

| | | Copper Concentration (µg/L) by Segment | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Medium** | **Time** | **1** | **1-2** | **1-6** | **2** | **3** | **4** | **5** | **6** |
| Freshwater | 0 | 2.6 | NT | NT | 2.6 | 3.0 | 2.8 | 2.7 | 2.7 |
| | 10 | 3.5 | 5.9 | 5.2 | 2.5 | 2.6 | 2.7 | 2.6 | 2.7 |
| | 30 | 7.9 | 7.9 | 4.1 | 3.9 | 2.7 | 2.7 | 2.7 | 3.2 |
| Seawater | 0 | 0.41 | NT | NT | NT | NT | NT | NT | NT |
| | 10 | 7.32 | 0.88 | 2.91 | 0.42 | 0.39 | 0.38 | 0.38 | 0.95 |
| | 30 | 16.9 | 5.75 | 3.74 | 1.15 | 0.43 | 0.42 | 0.49 | 1.68 |

NT indicates samples not taken. 1-2 and 1-6 indicates samples taken at the borders of adjacent segments.

The freshwater portion of the experiment was conducted between 14 August 2007, and 6 September 2007. Each replicate trial was run using a single fish placed into the experimental chamber. The fish was given an hour to acclimate before beginning the trial. Fish were evaluated after acclimation (see Evaluation of Acclimation below) before beginning the experiment. If fish behavior was acceptable, frame capture began. At 20 minutes, the metering pump was started and dissolved copper delivered to the exposure chamber. The run was concluded after 60 minutes. The fish was euthanized and the tank was flushed twice before the next run.

For trials where fish were kept on a freshwater recirculating system, a separate drain system was constructed so that the drain was switched immediately before adding copper to the experimental chamber, so that copper contaminated water would not return to the recirculating system. After each run, the system was replenished with filtered, de-chlorinated city water and the drain was switched back to return to the recirculating system. Seawater trials were conducted using a flow-through system.

For trials with added physical structure, a bundle of 12.7 mm PVC pipes, forming a loose teepee shape was placed in the exposure segment. The structure took up most of the segment but did not extend beyond the segment (Figure 3). Otherwise, trials were run identically to those without structure. Seven trials were completed with and without structure.

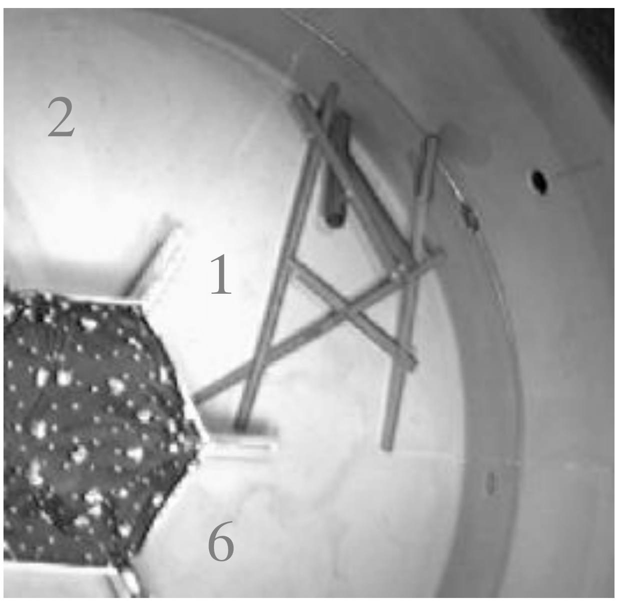

Figure 3. Overhead view of the exposure segment with added physical structure.

Methods were essentially the same in seawater as in freshwater except that acclimation time was increased to 2 hours, the number of trials was increased to 15 for trials with structure and 16 without structure, and the experimental tank was drained and refilled once between trials.

*Evaluation of Acclimation*

When placed into the experimental chamber, fish adopted one of three behaviors: hiding, rapid swimming, or exploration. When hiding, fish dropped to the bottom of the tank usually near a wall and stopped moving, sometimes for hours. Some fish would begin rapidly swimming around the tank, usually around the perimeter and at the surface. This appeared to be a panic swim as the fish paid no attention to structure or the presence of copper and seemed to ignore their surroundings. When exploring, fish would move around the tank, often in mid-water but varying between the bottom and the surface between sections occasionally reversing direction. With structure present, some fish would immediately dart into structure while others would approach slowly before entering structure. Often fish would initially display hiding or rapid swimming behavior before exploring. Hiding and rapid swimming responses were deemed unsuitable for the experiment because while displaying these behaviors, fish did not appear to be interacting with their environment and no useful information could be gained from their inclusion. Fish which were not displaying exploratory behavior by the end of the acclimation period were rejected.

Acceptance criteria in trials without structure:

1. Fish must complete at least one full circuit of the chamber.
2. Fish must be moving enough to cross border between segments at least once every five minutes (except when structure was present).

3. Fish must spend at least half of the time in mid water column.
4. Fish must have been displaying this behavior during the last ten minutes of the acclimation period.

In trials with structure, the criteria needed to be different because of the attraction of structure and criterion 2 was not used. Not all fish exhibited behavior that was considered acceptable for use in the experiment. In fresh water, 11 fish were rejected out of 25 attempts. In seawater 55 fish were rejected out of 86 total attempts. In the seawater portion, acclimation time was increased to 2 hours. This allowed more time for fish to settle into exploratory behavior and many of the fish that were accepted would have been rejected if the acclimation time were 1 hour. Published avoidance studies with fish are inconsistent with their reporting of rejection criteria and rates but this study is comparable to those published in Labenia, 2007. Additionally, it is difficult to compare the rejection rates in this study with other published avoidance studies because of the variation in equipment, species and rejection criteria.

*Data Collection and Analysis*

Fish movement in the experimental chamber was recorded using a digital camera with a vari-focal lens (DFK 21F04, Unibrain Inc., San Ramon, CA, USA). Images of the fish were captured using time-lapse software on a laptop computer. Overhead lighting was provided by seven 60-watt floodlights. Images were analyzed for presence or absence of fish in each segment at each frame capture, using image analysis software (Image-J, NIH) and manually verified. Individual trials had a one-hour duration and images were captured at 3-second intervals producing 1200 images for each trial. These intervals were frequent enough to capture the movement of fish in the experimental chamber and provided sufficient duration to capture overall fish behavior based on initial observations of fish performance. Each trial was analyzed in two periods: the 20 min before copper was introduced into the exposure segment, and the final 30 minutes, after introduction of the copper solution, which was counted as the exposure portion. The first ten minutes after exposure began were not analyzed while copper permeated the exposure segment. Frames were captured at 400 time points in the pre-copper portion and 600 in the exposure portion.

The percentage of time spent in the exposure segment was calculated for both pre-copper and post-copper portions of individual trials by dividing the number of frames where a fish appeared in the exposure segment by the total number of frames for that portion. For all analyses, percentages were arcsin transformed before statistical analysis (Zar 1984). Baseline use of all segments in the experimental tank was analyzed by comparing all non-structure, pre-copper trials to a theoretical even distribution of 16% in each segment by one-sample t-test. The attraction to structure was analyzed by two methods: First, by comparing pre-copper use of the structure segment (segment 1) between trials using structure and those not using structure by unpaired t-test. Second, percentage of time spent in the test segment was compared to a hypothesized even distribution of 16% with a one-sample t-test. Response to copper was similarly analyzed by two methods: First, using paired t-tests for percentage of time spent in the exposure segment before and during copper exposure. Second, percentage of time spent in the test segment was compared to a hypothesized even distribution of 16% with a one-sample t-test. Data were analyzed using Statview statistical software (Abacus Concepts, Inc. Berkeley, CA).

# RESULTS

On average, fish did not display bias in the mean percent time spent in the exposure segment without structure and without copper when compared to a hypothesized even distribution between all segments of 16% (one sample t-test, $n = 22$, $p = 0.14$).

*Structure*

In freshwater without copper, fish were present in the exposure segment (segment 1) over four times as often (76% versus 14%, $p = 0.016$) when structure was present than when structure was not present (Figure 4a). Without structure, presence in the exposure segment was not different ($p = 0.34$) from a hypothesized mean of 16% but was greater ($p = 0.02$) when structure was present (Figure 4a). In seawater, the presence of structure did not affect the likelihood that fish would be found in the exposure segment (12% with structure, 15% without, $p = 0.37$, Figure 4b). Presence in the exposure segment was not different from a hypothesized mean of 16% whether structure was present or not (no structure $p = 0.30$, with structure $p = 0.15$).

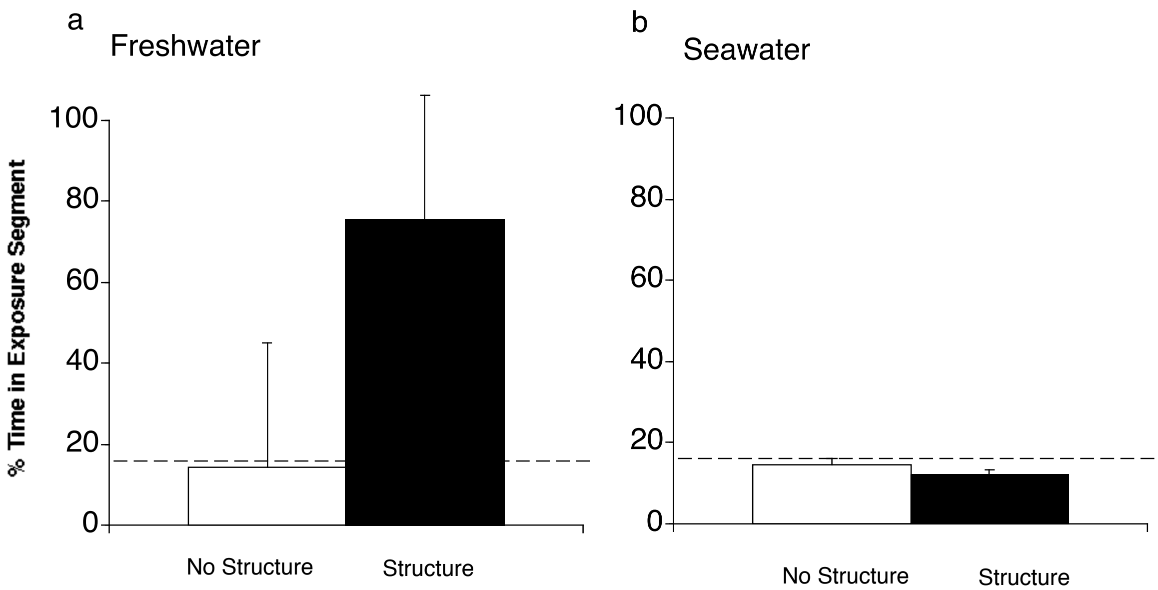


Figure 4. Mean percent time spent (± se) by individual fish in the exposure segment before exposure. Individual treatments were compared to a theoretical even distribution between all segments of 16% (dashed line).

*Copper exposure without structure*

In freshwater without structure, fish spent less time in the exposure segment (12% versus 14%) when copper was present than before copper was present (Figure 5a, paired t-test, $n = 6$, $p = 0.05$). Time spent in the exposure segment was similar to a hypothesized mean of 16% before copper was introduced ($p = 0.34$) but less than 16% ($p = 0.05$) while copper was present.

In seawater without structure, fish were present in the exposure segment 15% of the time before the introduction of copper and 11% when copper was present (Figure 5b, paired t-test, $n = 16$, $p = 0.02$). Time spent before copper was introduced was not different from a hypothesized mean of 16% ($p = 0.30$) but declined to less than 16% when copper was present ($p = 0.0004$).

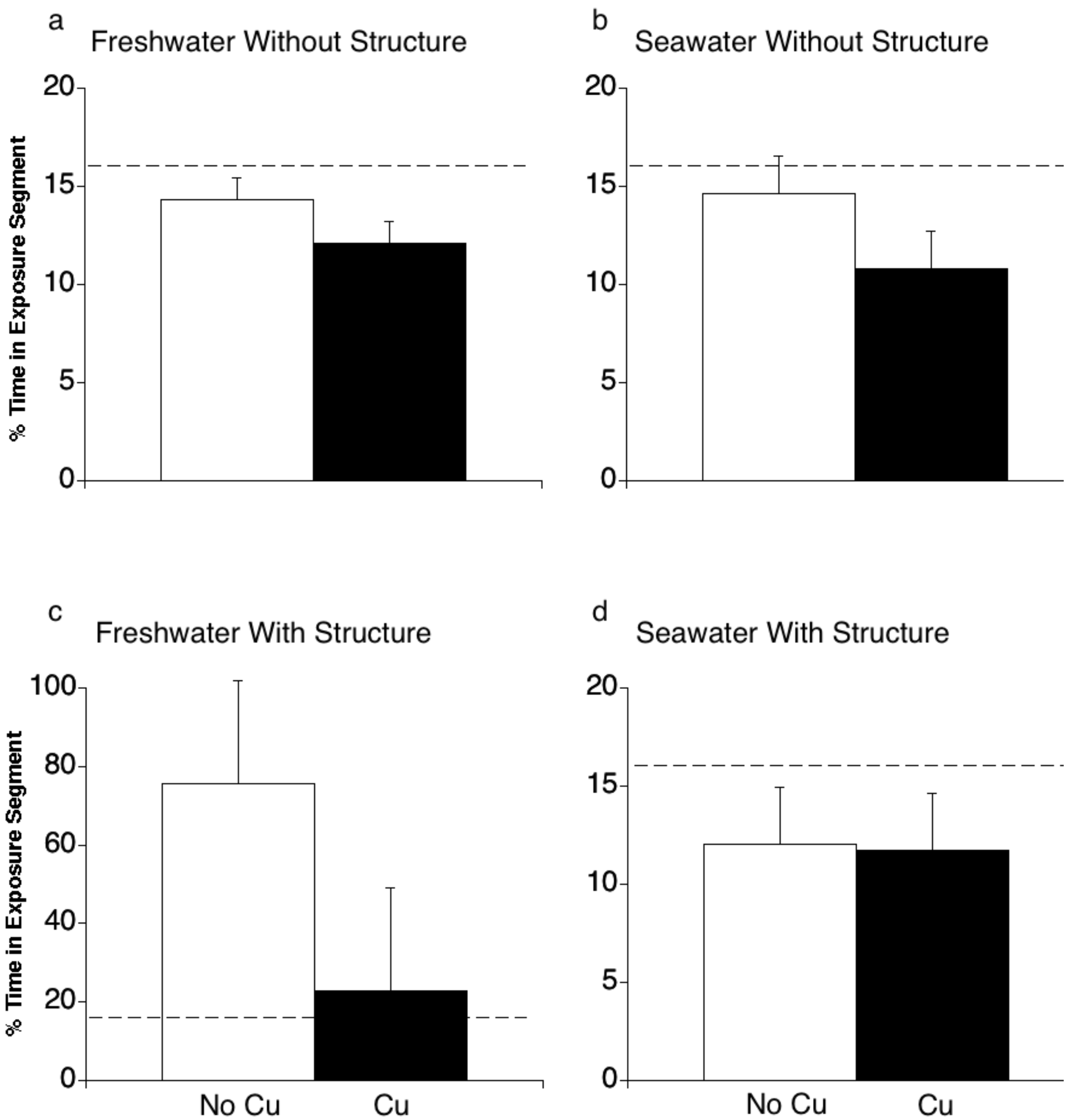


Figure 5. Mean percent time (± se) spent in exposure segments before and during exposure. Individual treatments were compared to a theoretical even distribution between all segments of 16% (dashed line). Note different scale on y axis of panel b.

*Copper exposure with structure*

While fish were more likely to be in the exposure segment when structure was present in freshwater (76%), the preference for structure was not apparent when copper was present (23%, n = 7, p = 0.06, figure 5c). Presence in the exposure segment differed from the hypothesized mean of 16% (One sample t-test n = 7, p = 0.02) before copper, and did not differ (One sample t-test n = 7, p = 0.44) when copper was present.

In seawater with structure present, fish were no more likely to be found in the segment with structure than in segments without and were no less likely to be in the exposure segment with copper present than without (12% with copper, 12% without, paired t-test, n = 15, p = 0.86, Figure 5d). Presence in the exposure segment was not different from a hypothesized mean of 16% whether copper was present or not (p = 0.15 with copper, p = 0.23 without).

## DISCUSSION

The findings of this study indicate that sublethal concentrations of copper altered the behavior of juvenile Chinook salmon in seawater and freshwater at environmentally realistic levels at a size and age when fish enter the estuarine environment and transition from freshwater to seawater. Also, the use of physical structure in freshwater was altered by the presence of copper. The use of submerged structure by pre-smolt salmon has been well documented primarily as the use of large woody debris (Quinn and Peterson 1996, Roni et al. 2002). In this study, pre-smolt juvenile fish also showed a preference for submerged structure in freshwater in the laboratory but that preference disappeared when copper was elevated. Fish were not attracted to submerged structure in seawater which may indicate behavioral difference between pre- and post-smolt life stages.

Water chemistry can be protective against acute toxicity but results of this study suggest that avoidance behavior is less affected by water chemistry than acute toxicity. There is a great deal of experimental evidence supporting the general effectiveness of the biotic ligand model which predicts the effect of water chemistry on acute toxicity in freshwater (Niyogi and Wood 2004, Paquin et al. 2002). The model predicts the chemistry of seawater to be highly protective against lethal copper toxicity and experimental evidence supports that prediction. The total copper LC50 for coho smolts is 63 $\mu$g/L in freshwater and 601 $\mu$g/L in seawater (EPA 2007). Whether the mechanism for this difference lies in the competition for binding sites on the gill by other ligands as in the biotic ligand model, the difference in osmotic physiology between freshwater and seawater, or some other mechanism is not clear. In both freshwater and seawater however, dissolved copper will elicit an avoidance response at concentrations below those reported for acute toxicity in a variety of fish species (Atchison et al. 1987, Blanchard and Grosell 2006, Grosell et al. 2007, Grosell et al. 2004b). In freshwater, the Lowest Observed Effect Concentration (LOEC) for copper avoidance was reported for rainbow trout at 0.1 $\mu$g/L (Folmar 1976), though most studies report LOECs for salmonids in the 2-7 $\mu$g/L range (Atchison et al. 1987). Threshold avoidance levels have not been explicitly studied in seawater and at 18 $\mu$g/L this study finds avoidance at a level lower than found in previous studies (Koltes 1985, Labenia et al. 2006). While water chemistry is protective against acute copper toxicity, it may have less of an effect on sensory function and avoidance.

The two endpoints are dissimilar in terms of the physical mechanism of copper toxicity; acute toxicity, binding of copper to sites on the gill and attenuation of toxicity by ligands described by water chemistry and dissolved organic matter (DOM); and sensory toxicity as seen in studies of the effects of copper on olfactory cells and sensory cells of the lateral line. Although avoidance behavior has not been mechanistically linked to physiological toxicity, it seems likely to be linked to sensory input like olfaction or the lateral line. The specific sensory input that elicits avoidance has not yet been identified but the loss of alarm response behavior has been shown to correspond to the loss of olfactory response to alarm chemicals (Sandahl et al. 2006). Additionally, the effect of water chemistry on avoidance behavior appears to be mechanistically more similar to sensory toxicity than it is to acute toxicity in that water chemistry is only minimally protective against sensory toxicity (Linbo et al. 2009, McIntyre et al. 2008), though the effects of water chemistry on avoidance behavior have not been studied specifically. While the consequences of acute toxicity are obvious, those of avoidance behavior are less so. The alteration in the use of submerged structure in this study may have implications for the effect of copper avoidance on the utilization of estuarine critical habitat.

The relationship between submerged structure primarily as large woody debris (LWD) and juvenile salmon has been extensively studied in freshwater and the preference for structure in this study corroborates this work. Few studies have focused on the use of submerged structure by juvenile salmon in the estuary and nearshore after the transition to seawater but the importance of large woody debris appears to weaken as fish enter brackish water and ocean-type Chinook in particular make extensive use of shallow shoreline, mudflats, and seagrass beds (Simenstad 1982, Wick 2002). In this study, the loss of attraction to submerged structure between freshwater and seawater seems to corroborate this. While this study was unable to determine whether the presence of dissolved copper would cause fish to abandon attractive habitat in seawater using submerged structure, it is still possible that the presence of copper would cause fish to avoid estuarine and nearshore habitats that they would otherwise use. The modification of behavior shown by individual fish in this study could impair their ability to use their environment, effectively resulting in a loss of habitat. Additional research is needed to understand how the presence of copper affects fish in the field as well as at what levels the effects become apparent.

Increasing urbanization in the Puget Sound region makes it likely that copper will continue to be a concern. The evidence from the few published studies that exist indicate that the effects of copper in the estuary and nearshore may cause sublethal effects in fish and deserves further study. Future work should include the determination of copper avoidance LOECs for salmon at a range of salinities including full strength seawater. The results of this study show effects at the lowest level of copper in seawater yet published and would likely find effects at lower concentrations. Damage to the olfactory sensory system is another sublethal effect of copper demonstrated in freshwater that may also occur in seawater. The ability to smell is disrupted after exposure to dissolved copper in freshwater (Baldwin et al. 2003, McIntyre et al. 2008, Sandahl et al. 2007, Sandahl et al. 2006, Scott and Sloman 2004). Loss of smell can affect imprinting on home streams

(Wisby WJ and AD 1954) as well as a variety of olfactory-mediated behavior such as predator avoidance (Brown 2003) and cohort identification (Quinn and Busack 1985).

The methods used in this study, though based on techniques used in other behavioral experiments, are unique to this experiment. These methods proved effective by providing results in freshwater that are comparable to other studies of copper avoidance in freshwater. There are however areas where the method used could be improved upon. At 16% the theoretical likelihood of presence in a given segment provides a low baseline for detecting avoidance and is better suited to detecting preference for a given segment. Flooding two or three segments with the test substance would provide a greater baseline usage (i.e. 50% rather than 16%) and increase the ability to detect a reduction in usage. Additionally, quantifying additional behavioral changes might increase the ability to detect avoidance. Fish entering the exposure segment before sensing the copper and either reversing direction to avoid the copper or accelerating through the segment, are counted as being in the segment while actively leaving it. Measuring reversal of direction or change in speed would make the experiment more sensitive to behavioral changes from the presence of copper though would make the process of collecting data considerably more complicated and laborious. Also, a relatively high number of fish were rejected from the experiment because of behavior that may have been caused by the stress of the experimental chamber. Experimental evidence exists that suggests that the presence of multiple fish may reduce some of this stress (Grand and Dill 1999). The use of multiple test subjects may reduce the rejection rate and behavior may become more natural. Finally, field studies of the effects of copper on fish are almost nonexistent due most likely to the amount of effort and the logistical difficulties of performing field studies with toxic substances but efforts should be made to verify laboratory findings in the field.

In conclusion, the results of the present study contribute to a sparse body of work focusing on the behavioral effects of copper on fish in seawater. Dissolved copper can elicit avoidance behavior in salmonids in seawater at levels below those commonly found in urban streams feeding into Puget Sound. Further, the use of preferred habitat can be disrupted at a life stage where estuarine habitat is likely critical to healthy salmon populations. All Puget Sound estuaries and coastal marshes have been classified as critical habitat to threatened Puget Sound salmon species. With growing urbanization in the Puget Sound region, toxics like copper will continue to enter streams flowing into estuarine habitat at a time when coastal development has reduced the area of estuaries and salt marshes to 40% of historic levels (Collins BD 2005, Hart-Crowser 2007).

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# APPENDIX 1

Percentage time spent by individual fish in exposure segment 1.

| | No Structure | | | Structure Present | |
|---|---|---|---|---|---|
| **Date** | **No Copper** | **Copper** | **Date** | **No Copper** | **Copper** |
| **Freshwater** | | | | | |
| 8/28/07 | 10.0 | 7.5 | 8/14/07 | 49.0 | 17.7 |
| 8/30/07 | 16.0 | 14.0 | 8/16/07 | 32.8 | 6.0 |
| 9/1/07 | 18.8 | 18.2 | 8/17/07 | 12.5 | 16.8 |
| 9/4/07 | 17.5 | 12.8 | 8/20/07 | 91.8 | 29.8 |
| 9/5/07 | 9.0 | 9.8 | 8/24/07 | 88.5 | 21.8 |
| 9/6/07 | 14.0 | 9.8 | 8/25/07 | 47.3 | 62.2 |
| | | | 8/26/07 | 100.0 | 0.0 |
| **Seawater** | | | | | |
| 10/1/07 | 12.0 | 12.7 | 10/18/07 | 13.3 | 0.2 |
| 10/3/07 | 12.3 | 6.5 | 10/19/07a | 6.3 | 13.2 |
| 10/11/07 | 19.0 | 7.2 | 10/19/07b | 16.0 | 18.2 |
| 10/12/07 | 5.5 | 1.2 | 10/22/070 | 1.3 | 5.2 |
| 10/17/07a | 11.5 | 17.2 | 10/24/07 | 41.5 | 51.7 |
| 10/17/07b | 8.5 | 15.0 | 10/25/07 | 12.5 | 15.7 |
| 10/30/07 | 15.0 | 10.0 | 10/26/07 | 4.8 | 0.8 |
| 10/31/07 | 22.5 | 14.0 | 11/5/07 | 9.0 | 11.2 |
| 11/1/07a | 17.3 | 14.5 | 11/8/07 | 15.5 | 13.5 |
| 11/1/07b | 16.3 | 15.5 | 11/9/07a | 6.5 | 6.7 |
| 11/2/07 | 14.8 | 10.2 | 11/9/07b | 19.8 | 14.8 |
| 11/29/07 | 18.3 | 9.0 | 11/14/07 | 3.8 | 2.8 |
| 11/30/07a | 9.8 | 5.8 | 11/16/07 | 15.0 | 10.8 |
| 11/30/07b | 18.8 | 16.5 | 11/20/07 | 4.3 | 2.3 |
| 12/4/07 | 8.3 | 7.8 | 11/26/07 | 8.8 | 5.7 |
| 12/5/07 | 23.0 | 10.0 | | | |