SUBLETHAL EFFECTS OF THE CARBAMATE INSECTICIDE, CARBARYL, ON COASTAL CUTTHROAT TROUT

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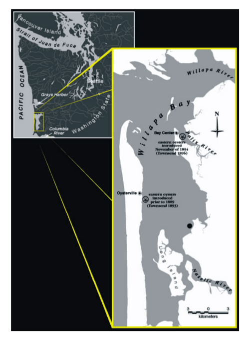
Abstract

Willapa Bay is a coastal estuary in Washington State that provides habitat for cutthroat trout (Onchorhynchus clarki clarki) as well as other salmonids. Cutthroat trout forage throughout the estuary in the summer months when carbaryl, a carbamate insecticide, is applied to oyster beds at low tide to control burrowing shrimp populations. On the day of spray, carbaryl has been measured in the estuarine water column at concentrations >1,000 ppb. Carbaryl is a neurotoxicant that inhibits acetylcholinesterase, an enzyme that hydrolyzes the transmitter acetylcholine at neuronal and neuromuscular synapses. The goals of this study were to determine: 1) if trout can detect carbaryl, as determined by in vivo recordings (electro-olfactograms) from the olfactory epithelium, 2) if trout will avoid dissolved carbaryl in a two-choice behavioral chamber, and 3) the effects of a short-term, environmentally-realistic carbaryl exposure on acetylcholinesterase activity in brain and muscle. We find that carbaryl does not evoke a measurable response from the cutthroat olfactory epithelium, and that animals do not avoid carbaryl-contaminated seawater. A short-term (6 hour) carbaryl exposure was sufficient to depress acetylcholinesterase activity for approximately two days. The onset of acetylcholinesterase inhibition in carbaryl-exposed animals was rapid (< 2 hrs) and enzyme activity was significantly reduced in a dose-dependent manner in brain and muscle (BMC_{20}s of 32 ppb and 23 ppb, respectively). Consequently, cutthroat trout are unlikely to avoid carbaryl that is transported away from oyster beds in Willapa Bay. Moreover, a short-term exposure to carbaryl (i.e., within a single tidal cycle) can be expected to inhibit brain and muscle acetylcholinesterase, and thus may interfere with swimming performance and other behaviors that are critical for predator avoidance and survival.

Introduction



Carbaryl (1-naphthyl–methyl carbamate, CAS# 63-25-2) is a pesticide that is used to control burrowing shrimp populations on oyster beds in Willapa Bay in southwestern Washington State. In the summer months, carbaryl is applied to oyster beds at low tide via helicopter spraying and hand application. In 2002, more than 800 acres were treated at a rate of approximately eight pounds of active ingredient per acre (Tufts et al., 2002). The pesticide is applied as a wetable powder (Sevin® formulation 80 WSP).

Willapa Bay provides estuarine habitat for five species of anadromous salmonids, including coastal cutthroat trout (O. clarki clarki). Cutthroat smolts rely on estuaries for food and protection from predators, and they are vulnerable to the effects of estuarine habitat modification (Johnson et al. 1999). In Washington State, seaward migrations of cutthroat trout start in late spring and peak in mid-May. They spend the summer months foraging in the estuary, and generally migrate back to freshwater in the late fall (Trotter 1989). In estuaries, schools of trout forage along gravel beaches, around oyster beds, and in patches of eel grass, feeding opportunistically on marine crustaceans and small fish (Trotter 1989). In general, they move along the shore in waters that are less than 3 meters deep (Pauley et al. 1989). Consequently, cutthroat trout may be vulnerable to the off-site tidal transport of carbaryl after a spray event in Willapa Bay. On the day of treatment, the concentration of carbaryl in surface waters overlying oyster beds can reach 1,000 ppb or more (Pozarycki 1999). Relatively high levels of carbaryl have also been detected at adjacent sites in and around the main bay. For example, Creekman and Hurlburt (1987) found carbaryl at 2,500 ppb in tidal floodwaters more than 180 meters from a treated bed.

Carbaryl is a carbamate insecticide that inhibits the enzyme acetylcholinesterase. The primary function of acetylcholinesterase is the hydrolysis of acetylcholine, a transmitter that is widely used at synapses throughout the central and peripheral nervous system of fish. Since acetylcholine-mediated (or cholinergic) signaling is an integral component of information processing in many areas of the brain, a distributed loss of cholinergic function may result in diverse forms of neurological and behavioral dysfunction. The sublethal biological effects of carbaryl on cutthroat trout and other species of Pacific salmon are largely unknown. To address this uncertainty, we evaluated the effects of short-term, ecologically realistic carbaryl exposures on cutthroat smolts. The study addressed the following key questions:

1. Do cutthroat smolts show an olfactory response to carbaryl – i.e., can they smell it?

2. Do trout avoid carbaryl-contaminated seawater?

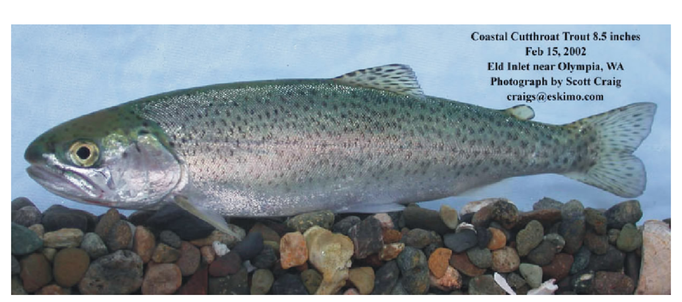
3. What are the effects of short-term carbaryl exposures on acetylcholinesterase activity?

4. What is the time course for carbaryl's effects & how long does it take for exposed fish to recover?

5. Does carbaryl exposure affect the swimming performance of cutthroat trout?

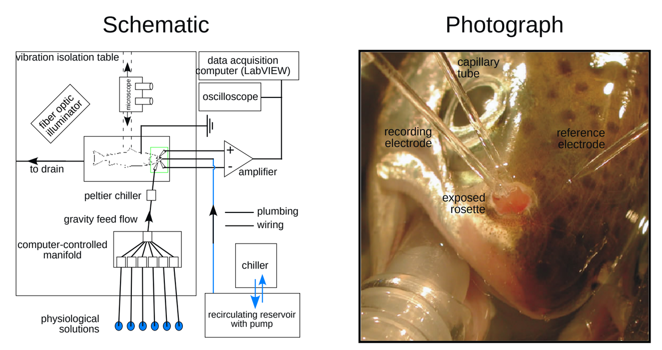
Animals

Cutthroat trout (n≈600) were obtained from the Eells Springs Hatchery in Shelton, Washington. The fish were transported to the Northwest Fisheries Science Center's Mukilteo research facility and were gradually transitioned to seawater over the course of a week. For the olfactory recordings, fish were transported to the Northwest Fisheries Science Center daily. Fish were maintained in filtered seawater throughout the study. All animals were anaesthetized with MS-222 prior to surgical procedures.



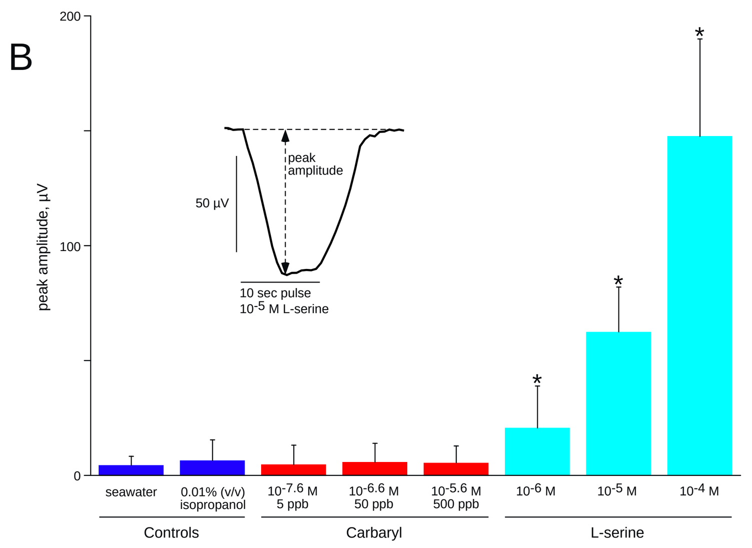
① Olfactory response

A



Stimulus-evoked electro-olfactograms (EOGs) were obtained using a procedure modified from Evans and Hara (1985). Trout were anaesthetized with MS-222 and injected intramuscularly with the paralytic gallamine triethiodide (0.3 mg/kg body mass). The skin overlying the olfactory rosette was removed and the fish was placed in a plexiglass holder on a vibration isolation table. Stock solutions of carbaryl and the model odorant L-serine were prepared in isopropanol, and test solutions were prepared by dilution into filtered seawater. The olfactory rosette was perfused with seawater and 10 second pulses of seawater alone (control), seawater containing isopropanol only (carrier control), seawater containing carbaryl (99% purity; Chem Services), and seawater containing L-serine. Stimulus-evoked EOGs were recorded with a pair of glass microelectrodes, amplified, and acquired using a custom LabVIEW program (National Instruments).

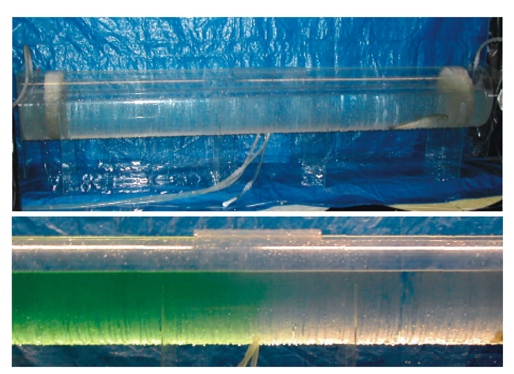
B



The olfactory responses of cutthroat trout were monitored following 10 s pulses of seawater, isopropanol (carrier), carbaryl, and L-serine. The inset is an example of a typical EOG evoked by a 10 s pulse of serine at 10^{-5} M. Data shown are the mean +/- S.D. response for 20 animals. Compared to pulses of seawater alone, there was no significant olfactory response to isopropanol or carbaryl at 5, 50, or 500 ppb. As expected, molar equivalent concentrations of L-serine evoked measurable EOGs that increased in a dose-dependent fashion. Asterisks indicate a significant difference from seawater alone ($p < 0.05$, one-way ANOVA with a Dunnet's post-hoc).

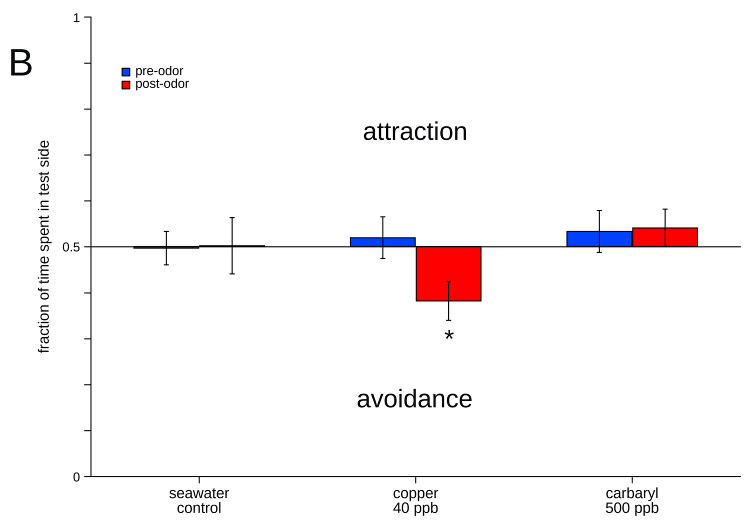
② Behavioral avoidance

A



Avoidance behavior was monitored in an experimental chamber modified from Sprague (1968). The chamber consisted of a six-foot acrylic cylinder with continuous seawater inputs at each end (4 L/min). Seawater from both ends flowed out of the chamber via a drain at the midline (upper photo). A peristaltic pump was used to meter test solutions (seawater, 40 ppb copper, or 500 ppb carbaryl as final concentrations) into one of the two seawater inputs. There was little mixing at the midline between the seawater arm of the chamber and the arm containing the test solutions (dye in lower photo). The pump line was alternated between seawater inputs in successive trials. Individual fish were placed in the chamber and allowed to acclimate. The test solution was then introduced to one arm of the chamber, and the relative position of the fish was monitored for 10 minutes using digital video. Avoidance behavior was measured as the fraction of time the fish spent in the arm containing clean seawater.

B



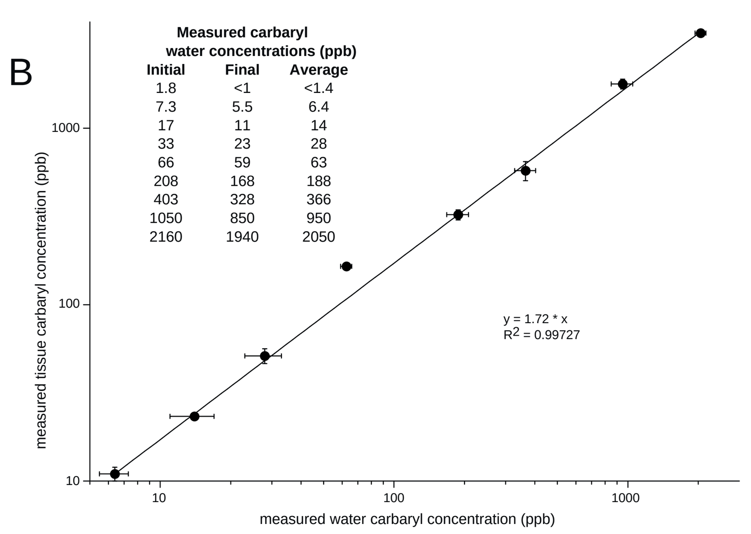
Behavioral avoidance of seawater, copper, and carbaryl. Since salmonids are known to avoid copper (e.g., Hansen et al., 1999), we used copper (copper chloride; Sigma) as a positive control in this experiment. Eight fish were tested in each of the three treatment groups and the mean +/- S.D. shown. Fish in all three groups did not show a significant preference for, or avoidance of, the stimulus arm of the chamber during the acclimation period (blue bars). Similarly, there was no significant avoidance (red bars) of seawater during the 10 min test interval. As expected, cutthrout trout significantly avoided copper ($p < 0.05$, paired t-tests). No significant avoidance of carbaryl was observed.

③ Acetylcholinesterase inhibition

A

Cutthroat trout were exposed to carbaryl in seawater, and carbaryl accumulation and acetylcholinesterase inhibition were measured in muscle and brain. Carbaryl is typically applied to oyster beds in Willapa Bay at low tide. Therefore, we exposed animals to carbaryl for six hours to approximate an exposure from off-site transport during a single flood tide on the day of spray. The range of exposure concentrations was based on previous field measurements of carbaryl in the water column following a spray event (e.g., Creekman and Hurlburt, 1987). Exposures were static with 15 fish per treatment group. A seawater control, a carrier (isopropanol) control, and nine carbaryl exposure concentrations were analyzed (n = 165 fish total). Seawater and tissue samples were collected and shipped to the Mississippi State Chemical Laboratory for carbaryl analyses according to standard procedures. For acetylcholinesterase activity, brain and muscle tissues were removed, flash frozen in liquid nitrogen, and subsequently homogenized. Enzyme activity was assayed on an Optimax plate reader according to the methods of Ellman (1961), as modified by Sandahl and Jenkins (2002). Substrate and tissue blanks were included. Values were normalized to the protein content of each sample, and are expressed relative to acetylcholinesterase activity in the control (seawater) exposure group.

B

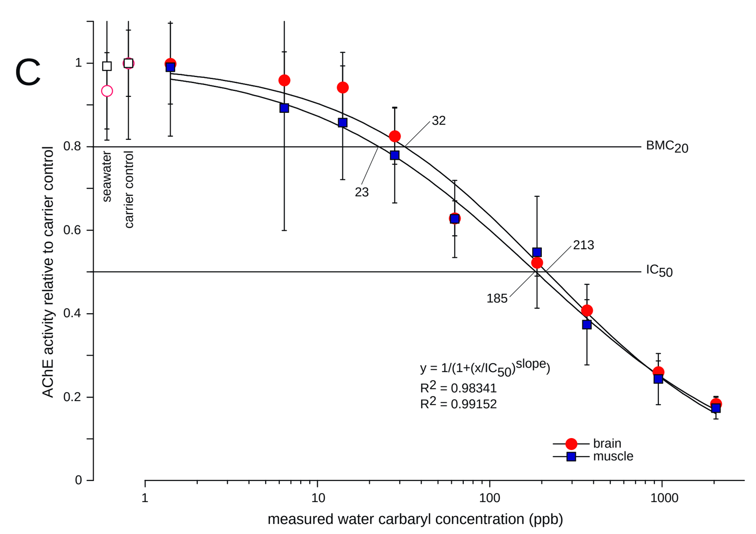


Measured carbaryl water concentrations (ppb)

Initial	Final	Average
1.8	<1	<1.4
7.3	5.5	6.4
17	11	14
33	23	28
66	59	63
208	168	188
403	328	366
1050	850	950
2160	1940	2050

Carbaryl was measured in seawater (inset table) and in muscle tissue from animals exposed at concentrations ranging from ~ 1 ppb to ~ 2,000 ppb for six hours. Triplicate samples were analyzed. Each value represents the mean +/- S.D. Horizontal error bars indicate the range of measured carbaryl concentrations in seawater (initial to final) for each exposure group. No carbaryl was detected in seawater from the two control exposures. Carbaryl accumulated in the muscle tissue of cutthroat trout and was approximately 1.7-fold higher in muscle than in seawater at the end of the exposure interval. Non-detections of carbaryl in tissue are not plotted.

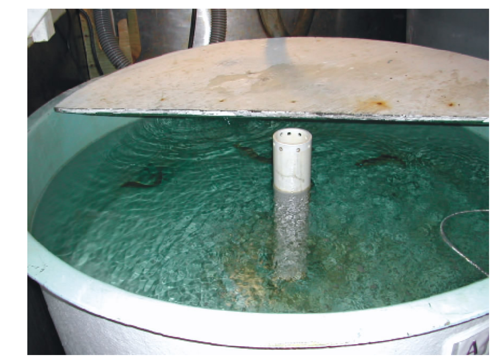
C



Acetylcholinesterase activity was reduced in the muscle (blue symbols) and brains (red symbols) of fish exposed to carbaryl for six hours. Dose-response curves for both tissues are shown. Each value represents the mean +/- S.D. of 15 fish. The 50% inhibition concentration (IC_{50}) for muscle and brain was 185 and 213 ppb, respectively, and the benchmark concentrations for 20% acetylcholinesterase inhibition (BMC_{20}) were 23 ppb for muscle and 32 ppb for brain. No significant acetylcholinesterase inhibition was observed in fish from the carrier control group.

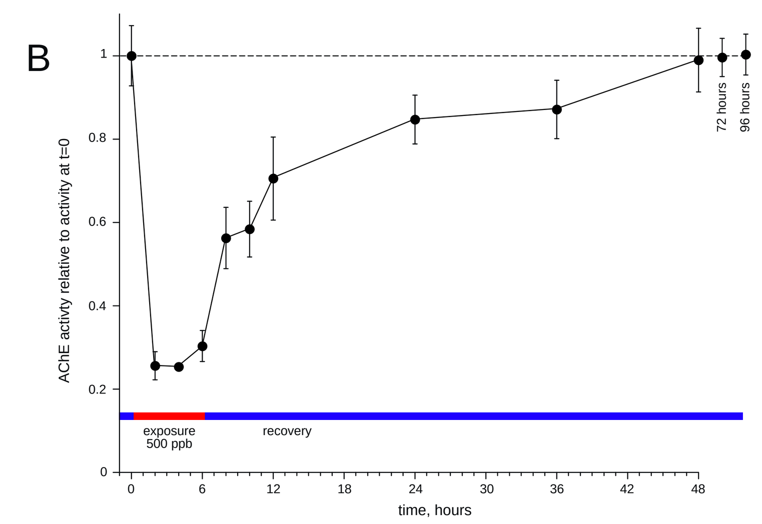
④ Time-to-effect and recovery

A



To determine the time course for carbaryl's inhibitory effects on brain acetylcholinesterase, cutthroat trout were exposed to 500 ppb carbaryl (nominal) for six hours and then allowed to recover for four days. A total of 84 fish were used in this experiment. All 84 animals were exposed to carbaryl at the outset, and groups of seven fish were subsampled at t = 0 and at two-hour intervals during the exposure period. The remaining fish were then transferred to clean seawater. Additional groups of fish were sampled every two hours for six hours and then over longer intervals for the next few days. Brain acetylcholinesterase activity was determined according to the same procedures described in 3A.

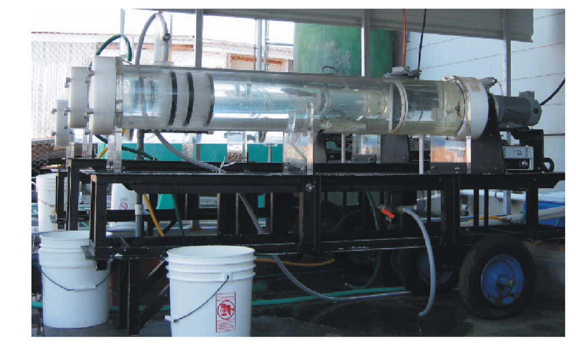
B



Brain acetylcholinesterase activity is rapidly inhibited by carbaryl. Inhibition appears near-maximal after two hours of exposure. Enzyme activity showed some initial recovery when animals were restored to clean seawater, and gradually recovered to pre-exposure levels after 42 hours. Values shown are the mean ± S.D. for n = 7 fish.

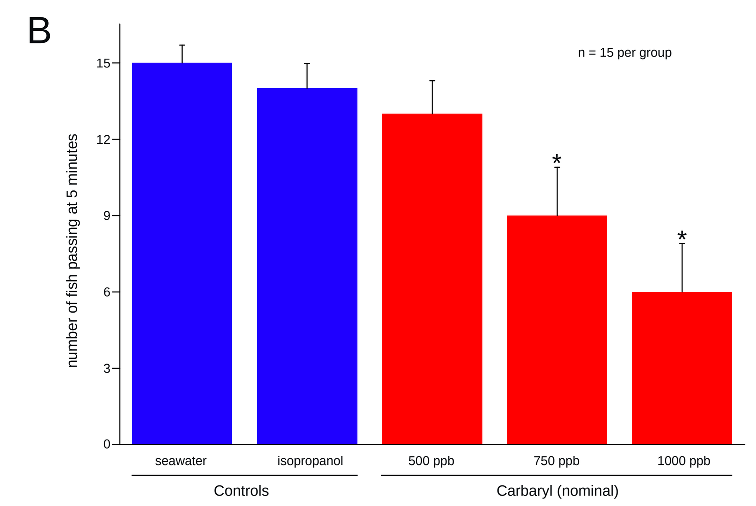
⑤ Swimming performance

A



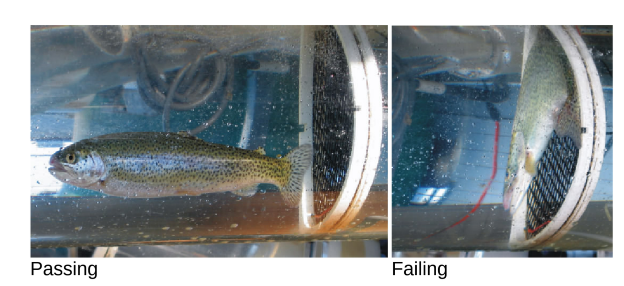
The effect of carbaryl on the swimming performance of cutthroat trout was assessed using Blazka-style swimming chambers (reviewed in Beamish, 1978). Each chamber consists of two concentric plexiglass cylinders (see photo above). A propeller pulled water through the inner cylinder (to the right in the photo) at a controlled flow rate with the water returning between the inner and outer cylinders. Six hour exposures to control or carbaryl solutions were performed as described in 3A. A fish was then placed into the inner cylinder (the end cap on the left is removable) and allowed to acclimate for 20 minutes. The motor was then started and a 45 cm/s flow established within 10 seconds. Swimming performance was based upon the fish's ability to react and orient to the sudden change in flow (a test of sensori-motor integration). Fish were scored as passing this performance test if they were swimming free and clear of the rear screen after 5 minutes. Muscle acetylcholinesterase activity was then determined according to the procedures described in 3A.

B



Nominal carbaryl exposures produced a dose-dependent response with higher concentrations causing a decrease in the number of fish passing the performance test. Swimming performance (as measured by this test) was significantly reduced (relative to isopropanol controls, binomial test) at carbaryl concentrations ≥750 ppb. All three carbaryl concentrations produced significant reductions in muscle acetylcholinesterase activity (similar to 3C). Error bars represent 1 S.D. of the corresponding binomial distribution.

C



Passing Failing

Photo on the left shows a fish (scored as passing) swimming clear of the screen (which is not electrified) after 5 minutes at 45 cm/s. Photo on the right shows a fish (scored as failing) impinged upon the screen and unable to orient or react to the suddenly established flow. Important to note, when the flow rate was gradually raised to 45 cm/s (over several minutes) most exposed fish were able to maintain themselves in the flow.

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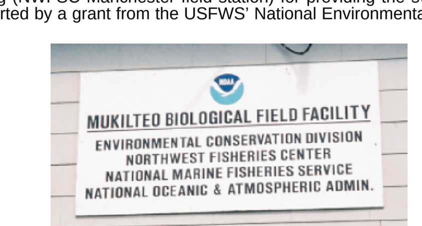

Conclusions

1. Do cutthroat smolts show an olfactory response to carbaryl – i.e., can they smell it?

Cutthroat trout show no measurable olfactory response to carbaryl at nominal concentrations ranging up to 500 ppb. Responses to carbaryl were not significantly different from seawater or carrier controls. By contrast, animals show a dose-dependent response to the amino acid L-serine at similar molar concentrations.

2. Do trout avoid carbaryl-contaminated seawater?

Cutthroat smolts do not avoid carbaryl-contaminated seawater in a two-choice behavioral test. As expected, animals did avoid seawater contaminated with copper.

3. What are the effects of short-term carbaryl exposures on acetylcholinesterase activity in brain and muscle?

A short-term (six hour) exposure leads to the uptake of carbaryl into trout tissues and the dose-dependent inhibition of brain and muscle acetylcholinesterase. The IC_{50} values for enzyme inhibition in brain and muscle are 213 and 185 ppb, respectively.

4. What is the time course for carbaryl's effects, and how long does it take for exposed fish to recover?

Carbaryl's inhibitory effects on brain acetylcholinesterase occur rapidly (< 2 hours). Recovery from a six hour sublethal exposure takes place gradually, with acetylcholinesterase activity returning to control (pre-exposure) levels in approximately 2 days.

5. Does carbaryl exposure affect the swimming performance of cutthroat trout?

Exposure to nominal carbaryl concentrations ≥750 ppb did affect the swimming performance of cutthroat trout as measured in the swimming start bioassay.

Summary and future directions

Foraging habitat for coastal cutthroat trout in Willapa Bay is periodically contaminated with carbaryl that is transported off-site from oyster beds via tidal activity. As surface water monitoring studies have previously shown, concentrations of carbaryl in the water column can reach 1,000 ppb or more on the day of spray. As we have shown, smolts do not show an olfactory response to carbaryl at ecologically representative concentrations, and they do not avoid carbaryl-contaminated seawater. Since cutthroat trout forage in shallow waters (e.g., over oyster beds) during the summer months, it is very likely that wild fish will be exposed to carbaryl on the day(s) of insecticide application. Even a brief exposure to carbaryl can be expected to significantly depress acetylcholinesterase activity for approximately two days and affect the swimming performance of cutthroat trout.

An increase in predation is the primary concern for cutthroat trout exposed to carbaryl. Previous studies have shown that short-term, sublethal pesticide exposures can significantly increase rates of predation on salmonid smolts (ecological death; e.g., Kryzinski and Birtwell, 1994). Moreover, anticholinesterase insecticides have previously been shown to interfere with the swimming performance of salmonids (e.g., Brewer et al., 2001). A key question is whether acetylcholinesterase inhibition of 20% or more among carbaryl-exposed fish results in behavioral impairments that significantly increase predation risk. The specific relationships between enzyme inhibition, motor function, and predator avoidance will be addressed in future studies.

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