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Hazard/Risk Assessment

Copper Toxicity in Bristol Bay Headwaters: Part 2—Olfactory Inhibition in Low-Hardness Water

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Abstract: We investigated the olfactory toxicity of copper (Cu) to rainbow trout in low-hardness (27 mg/L as $CaCO_3$) water formulated in the laboratory over a 120-h period using a flow-through design. The fish's response to an alarm cue (e.g., reduction in activity) was recorded to determine the exposure concentrations and durations that inhibited olfactory detection of the cue after 3, 24, 48, and 96 h of Cu exposure and after 24 h of clean water recovery following the 96-h exposure period. Exposures were conducted with a range of Cu concentrations from 0.13 (control) to 7.14 μg Cu/L (dissolved Cu). We observed a dose-dependent response in olfactory inhibition with a 20% reduction in the probability of responding to the alarm cue, relative to controls, at 2.7 and 2.4 μg Cu/L after 24 or 96 h of exposure, respectively. Olfactory inhibition manifested between 3 and 24 h of exposure. Our 24- and 96-h 20% olfactory inhibition estimates fell between the criteria derived using the biotic ligand model (BLM; criterion maximum concentration [CMC] and criterion continuous concentration [CCC] values were 0.63 and 0.39 μg Cu/L, respectively) and water hardness–based criteria (CMC and CCC values were 3.9 and 2.9 μg Cu/L, respectively). Therefore, the hardness-based criteria do not appear to be protective and the BLM-derived criteria do appear to be protective against Cu-induced olfactory inhibition given our test water chemistry. Neither the hardness-based criteria nor the BLM-derived criteria appear to be protective against our estimated Cu behavioral avoidance response concentrations at 24- and 96-h exposures (0.54 and 0.50 μg Cu/L, respectively). *Environ Toxicol Chem* 2018;9999:1–12. © 2018 SETAC

Keywords: Copper; Olfactory; Biotic ligand model; Bristol Bay

INTRODUCTION

Our preceding paper (Morris et al. 2019) describes the results of acute bioassays on rainbow trout (*Oncorhynchus mykiss*) and fathead minnows (*Pimephales promelas*) conducted to inform the utility of water hardness-based and biotic ligand model (BLM; Hydroqual 2005) derived acute copper (Cu) water quality criteria (criterion maximum concentration [CMC]) in low-hardness waters in the Bristol Bay watershed in Alaska. As described in Morris et al. (2019), the Bristol Bay watershed is critical spawning and rearing habitat for a world-class salmon fishery, which supports major economic and ecological functions and is critically important to subsistence communities. The results of our research as well as published literature demonstrate that lethal concentrations of Cu to aquatic biota are generally low (in the parts per billion range) but that exposure to even lower (i.e., sublethal) Cu concentrations can result in avoidance behaviors (Hansen et al. 1999a) and adversely affect the olfactory system of salmonids through neurological impairment or inhibition (Hansen et al. 1999b; Baldwin et al. 2003, 2011; McIntyre et al. 2008; Kennedy et al. 2012). Impairment or inhibition of the olfactory system has been shown to adversely affect predator avoidance behavior in juvenile salmonids (McIntyre et al. 2012) as well as recognition of rearing water (Saucier et al. 1991a). Whether the effects of Cu exposure are 1) mortality, 2) avoidance of contaminated waters, 3) inhibition of the olfactory system during imprinting in early life stages, 4) abnormal predator avoidance behaviors, or 5) impacts to the olfactory system during navigation to natal spawning areas, an understanding of the bioavailability and toxicity of Cu to salmonids in the Bristol Bay watershed is critical to the evaluation of the potential environmental consequences of mine development and activity.

In addition to describing the possible use of the BLM in the Pebble Project area, the US Environmental Protection Agency's (USEPA's) Bristol Bay Assessment (chapter 8 in US Environmental Protection Agency 2014) also discusses alternative Cu endpoints, including olfactory sensitivity. In this section they cite Meyer and Adams (2010), who reported that the Cu criteria derived in the BLM for acute effects (i.e., mortality) were also

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protective of olfactory effects such as behavioral avoidance of Cu-contaminated water and olfactory sensory inhibition. Although this assertion may be accurate, the data suitable for such an analysis are sparse; therefore, Meyer and Adams (2010) were only able to utilize a limited data set (4 studies) to calculate their olfactory effect concentrations (20% inhibition concentration [IC20] values in Table 8-14 in US Environmental Protection Agency 2014). Subsequent reports where they applied their model to 133 ambient waters in the western United States (DeForest et al. 2011) included 20 sites with low hardness (<30 mg/L as $CaCO_3$) and low dissolved organic carbon (DOC; <3 mg/L), and 2 of these 20 sites were in Alaska (Kenai River at Soldotna and Johnson River near Tuxedni Bay).

However, notwithstanding the ongoing efforts of Meyer and Adams (2010) and Meyer and DeForest (2018) to model the effects of water chemistry on potential olfactory effects in salmonids (and other species), given the limited amount of data and research on this topic as it pertains to watersheds with chemistry similar to the Bristol Bay watershed (low hardness and DOC), we proceeded to conduct the research described in the present study. The present study was conducted as a follow-on to a preceding 96-h bioassay using rainbow trout exposed to laboratory water with water chemistry adjusted to values that were similar to site water collected from the Bristol Bay watershed as described in our companion paper (Morris et al. 2019). The purpose of this experiment was to determine the effects of Cu exposure over time on the olfactory system of juvenile rainbow trout using a chemical alarm cue as a behavioral stimulus. This experiment is relevant to watersheds with low-hardness waters, and it is unique compared to other olfactory bioassays because we quantified the response of the same fish to an alarm cue at multiple time points over the course of the 96-h Cu exposure and subsequent 24-h recovery periods.

METHODS

Laboratory and exposure water

As with the preceding 96-h bioassay to assess survival (Morris et al. 2019), we also conducted this 120-h bioassay in the aquatic toxicity laboratory at the Colorado Parks and Wildlife office in Fort Collins, Colorado, USA. The laboratory water we used was the same blend of dechlorinated tap water and dechlorinated tap water treated with a cation exchange column (Siemens tank W5TDICAT0045FSP; Table 1) used in the preceding bioassay.

Rainbow trout olfactory bioassay

We conducted this bioassay using the same cohort of rainbow trout we obtained from within the Colorado Parks and Wildlife hatchery system to conduct the preceding 96-h bioassay. We also conducted this bioassay using the same flow-through system, in which 2-L exposure aquaria received 30 mL/min of laboratory water, which resulted in a 99% theoretical volume replacement every 5 h (calculated from figure 2 in Weber 1993). We added 2 fish (approximately 6.2 g, 7.8 cm) to each of 4 replicate aquaria over 6 exposure treatment levels. We stopped Cu addition to the diluter system after 96 h of exposure and continued running the flow-through system with uncontaminated water for an additional 24 h to all treatments to determine if there was recovery of any olfactory inhibition caused during the first 96 h of the bioassay. Our aquaria were arranged in a single water bath in a randomized block design, and fish were not fed during the 120-h bioassay. Dividers were placed between all tanks so that fish could not see fish moving in other tanks during the 120-h bioassay. We introduced alarm cues into exposure tanks at 3, 24, 48, 96, and 120 h and recorded behavior using cameras mounted above the tanks connected to a computer. We also hung a plastic tarp around the water bath and aquaria so that fish behavior was not influenced by people administering alarm cues or working near the experiment. We monitored all aquaria daily and recorded and removed all mortalities.

TABLE 1: Water quality parameters measured during rainbow trout 120-h olfactory bioassay in laboratory water

Parameter	Units	Average	SD	*n*
Temperature	°C	12.0[a]	—	—
pH	s.u.	6.47	0.13	12
Dissolved oxygen	mg/L	8.1	0.2	6
Hardness	mg/L as $CaCO_3$	27	0.6	2
Alkalinity	mg/L as $CaCO_3$	9	0.0	2
Dissolved constituents (0.45-μm filter)				
Organic carbon	mg/L	0.98	—	1
Calcium	mg/L	9.31	—	1
Magnesium	mg/L	0.88	—	1
Sodium	mg/L	1.97	—	1
Potassium	mg/L	0.46	—	1
Sulfate	mg/L	12.50	—	1
Chloride	mg/L	6.83	—	1

[a]Nominal value.
s.u. = standard units.

Water chemistry

We collected water samples from each treatment at the beginning of the rainbow trout bioassay. We collected filtered (0.45 μm pore size) and unfiltered water samples and acidified them (pH <2) for cation, Cu, and organic carbon analyses. The water samples we collected for organic carbon analyses were stored in amber bottles. We also collected water samples for anion analysis, which we filtered (0.45 μm pore size) and stored with no preservative. We refrigerated all water samples after collection/preservation and shipped them on ice overnight to Columbia Analytical Services (now ALS Environmental) for analysis. Water samples were analyzed for cations (calcium, magnesium, potassium, and sodium; USEPA method 6010C, inductively coupled plasma-atomic emission spectrometry), Cu (USEPA method 6020A, inductively coupled plasma-mass spectrometry), anions (sulfate and chloride; USEPA method 300.0, (ion chromatography), and organic carbon (standard method 5310 C).

We measured water quality parameters including temperature, pH, dissolved oxygen, hardness, and alkalinity in the laboratory during testing. In addition, we monitored the water bath temperature using a temperature logger that was

FIGURE 1: Example top-down view of exposure tanks from camera used to record pre- and post cue behavioral video. This image was recorded 3 h after test initiation.

placed in one of the exposure tanks as well as with a handheld thermometer. We monitored dissolved oxygen using an optical probe and pH using a meter that was calibrated with pH 4, 7, and 10 standards.

Alarm cue preparation and delivery

We used rainbow trout to produce the conspecific alarm cue, and these fish were from the same group of fish we used to conduct this bioassay, which we removed from the holding tank and euthanized with a blow to the head. Each fish was 7.5 to 8.0 cm long and weighed approximately 6.1 g. After euthanizing, we scored the skin of each fish several times with a scalpel in a cross-hatch pattern to simulate damage that would be caused if the fish had been attacked by a predator, and then we rinsed each fish with 50 mL of deionized water. The scoring process did not cause bleeding, and blood was not introduced into the rinsate. This rinsate was the alarm cue, and we produced all necessary alarm cues for the 120-h bioassay at one time and then froze it in 75-mL aliquots (i.e., enough cue for each time point when we administered the cue). We conducted preliminary tests using alarm cues produced using this method, including freezing and thawing the cue, prior to use during this bioassay to ensure that fish responded to the cue in a predictable manner.

To facilitate cue delivery without disturbing the fish, we added 3 mL of cue to the exposure water delivery tube for each tank directly below the splitter box on the proportional diluter, which was shielded from the exposure tanks by a plastic tarp. The exposure water continued to flow during cue addition so that the cue bolus was pushed down the tube and delivered to each tank within 1 min of addition. We added alarm cues to each tank on test hours 3, 24, 48, 96, and 120.

Behavior recording and quantification

We recorded fish behavior using digital video cameras (Logitech) mounted above each aquarium for approximately 20 min before and after cue addition. The top-down view of 3 aquaria was simultaneously recorded by one camera (Figure 1). Therefore, we used a total of 8 cameras to record all 24 aquaria. We cropped these videos using Wondershare video conversion software (Ver 8.8.1) so that only one tank was included in each video to facilitate separate analysis. In addition, we clipped these cropped videos into five 5-min segments including 1 pre cue addition segment and 4 sequential post cue segments using Wondershare. We quantified the total area covered by fish in each tank over each 5-min pre- and post cue segment for each cue addition time point using the Fiji distribution of ImageJ (Ver 1.51h; Schindelin et al. 2012; Schneider et al. 2012) to generate videograms similar to the method described for analysis of

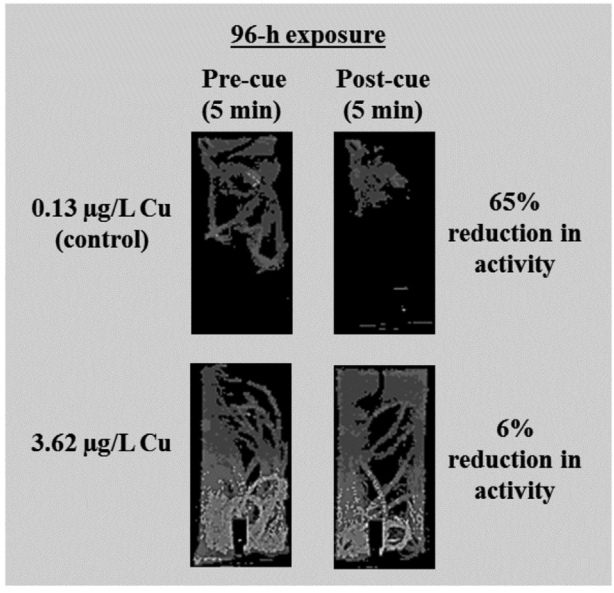

FIGURE 2: Example videogram of pre- and post cue activity in 2 exposure tanks. The reduction in activity in the 3.62–µg/L Cu treatment (6%) is within ±20% of pre cue activity and would, therefore, be classified as no change in activity for the purposes of conducting our binary logistic regression (see Figure 3). In contrast, the 65% reduction in activity in the control tank would be classified as a positive response (i.e., expected behavior) to the alarm cue.

TABLE 2: Copper exposure concentrations during rainbow trout 120-h olfactory bioassay in laboratory water[b] Filtered through a 0.45-μm filter.

Copper concentrations (μg/L)[a]		
Nominal exposures	Measured (dissolved[b])	Measured (total)
Control	0.13	0.13
0.5	0.57	0.57
1	0.98	1.00
2	1.93	1.85
4	3.62	3.71
8	7.12	7.14

[a] Copper detection limit = 0.02 μg/L.
[b] Filtered through a 0.45-μm filter.

zebrafish behavior by Wyeth et al. (2011). This videogram technique allowed us to compare pre- versus post cue activity as a function of total area occupied by the fish over each of the 4 post cue segments. See Figure 2 for an example of pre- and post cue videograms for 2 exposure tanks recorded at 96 h.

According to our preliminary testing, we expected non-Cu-exposed fish activity to decrease following cue addition because this is the normal behavioral response when a fish detects the scent of an injured fish (usually conspecific). Therefore, to determine if Cu exposure affected the fish's olfactory system we compared pre- and post cue activity levels to determine if activity decreased following cue addition. We categorized each tank's response to the cue by assigning a binomial value of 1 to tanks that exhibited a post cue decrease in activity (expected response to the cue) of at least 20% or a value of 0 to tanks that did not exhibit at least a 20% reduction in activity (i.e., did not respond to the cue). We chose a 20% reduction in activity to align with common toxicological metrics where a 20% effect is on the low end of positively attributing the observed effect to the contaminant exposure (i.e., 20% lethal concentration [LC20] or 20% effect concentration [EC20]). Binomial scores were calculated for each tank at each of the 4 post cue 5-min observation segments for all 5 cue addition time points over the course of the test. If a tank received a score of 1 for at least one of the 4 post cue segments, it was assigned this value for that cue addition time point; if not, it received a 0 for that cue addition time point.

Statistical analysis

We conducted binary logistic regressions for each cue addition time point, regressing each tank's binomial response score against its Cu exposure concentration using the statistical software package Minitab (2010; Ver 16.2.2). The output from this analysis also included the probability of responding to the alarm cue at each Cu exposure concentration, the standard error for this estimate, and the *p* value for each regression.

RESULTS

Exposure water chemistry and measured total and dissolved Cu concentrations for this 120-h bioassay are reported in Tables 1 and 2, respectively. Although we did not design this bioassay to determine lethal exposure concentrations of Cu, we did use an exposure concentration range (0.13–7.12 μg Cu/L; Table 2) that spanned into the 96-h LC20 range we determined from our preceding bioassay (LC20 was 7.83 μg/L; 95% CI 4.89–10.8; Morris et al. 2019). Therefore, we did expect and observe some elevated mortality in our higher exposure concentrations toward the end of the test (Table 3).

The probability of responding to the alarm cue at each cue exposure time point relative to the pre cue 5-min segment is presented in Figure 3. Raw data for all observations (fish activity quantified as pixels) are included in Supplemental Data, Table S1. After 3 h of Cu exposure, there was no dose–response relationship for the probability of responding to the cue versus Cu exposure, although there was higher variability in the probability of responding in the highest Cu exposure concentration (Figure 3A). All Cu exposure durations >3 h exhibited a clear, negatively correlated dose response in the probability of responding to the cue versus Cu exposure concentration (Figure 3B–E). Only the regression at 24 h of Cu exposure was statistically significant ($p = 0.023$; Figure 3B). One control tank (tank 14) was not included in this 24-h time point analysis because the 2 fish in the tank were very inactive during the pre cue segment but then became very active and aggressive after the cue addition. This suggests that the fish did sense the cue but that their aggressive behavior was much different from the expected response our bioassay was designed to quantify.

The probability of responding after 48 and 96 h of Cu exposure was generally similar to the probability after 24 h of exposure (Figure 3B–D). After 96 h of Cu exposure, only one of the 4 replicate aquaria in our highest exposure concentration (7.12 μg Cu/L) had any fish remaining (2 fish); therefore, there was only one replicate tank for this treatment. One control tank (tank 8) had only one fish remaining, which was slowly drifting backward around the tank and occasionally repositioning itself but appeared to be nearly

TABLE 3: Average mortality during rainbow trout 120-h olfactory bioassay in laboratory water: Four replicate tanks per exposure concentration and 2 fish in each tank at the beginning of the experiment

	% Mortality (SD)				
Dissolved copper exposure (μg/L)	3-h	24-h	48-h	96-h	120-h
0.13	0 (0)	0 (0)	12.5 (25)	12.5 (25)	12.5 (25)
0.57	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
0.98	0 (0)	0 (0)	0 (0)	12.5 (25)	12.5 (25)
1.93	0 (0)	0 (0)	12.5 (25)	25 (28.9)	25 (28.9)
3.62	0 (0)	0 (0)	12.5 (25)	37.5 (47.9)	50 (40.8)
7.12	0 (0)	0 (0)	12.5 (25)	87.5 (25)	87.5 (28.9)

moribund at the 96- and 120-h time points; therefore, we did not include this tank in our analysis for either of these time points. Based on our binomial regression analysis, we observed a 20% reduction in the probability of responding to an alarm cue (i.e., olfactory inhibition) relative to the control response after 24 and 96 h of exposure at 2.7 and 2.4 μg Cu/L, respectively (Figure 4).

After 96 h of Cu exposure, we turned off the Cu supply input to the proportional diluter system and continued running the system with laboratory water without Cu for an additional 24 h. Following this 24-h recovery period (120 total h) the probability of responding to the alarm cue was very similar to the 96-h estimates (Figure 3D and E).

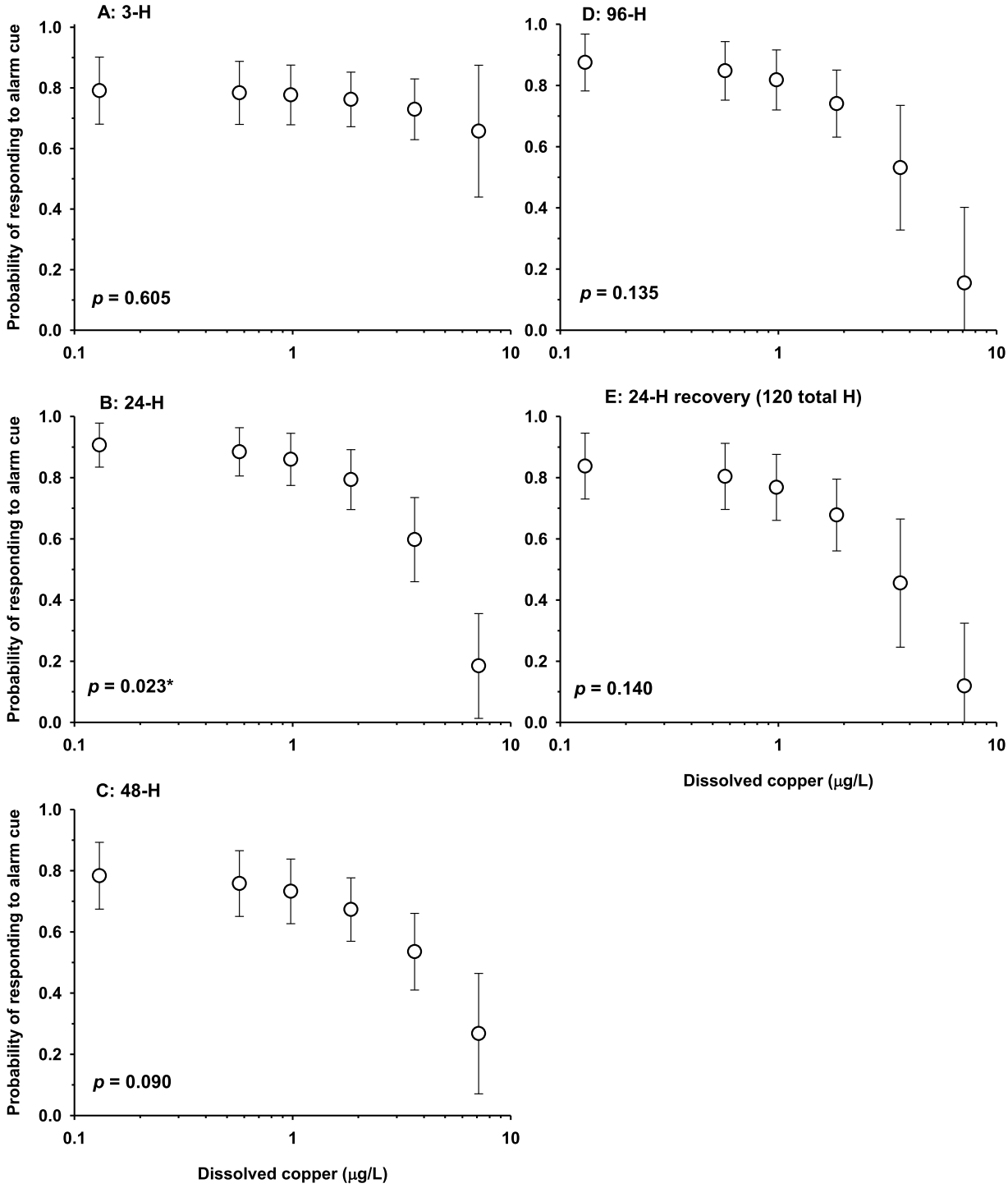

FIGURE 3: Probability of fish responding to alarm cue at each time point tested based on estimates from binary logistic regressions. A higher probability of responding indicates that the fish sensed the alarm cue and reduced their activity (expected behavior). Error bars are ± standard error of the mean. *Although there is a negatively correlated dose response between copper exposure and the probability of fish responding to the alarm cue for all of the exposure durations >3 h, only the 24-h relationship was statistically significant.

Our lowest Cu exposure concentration (0.57 μg/L), other than the control (0.13 μg/L), was between the CMC (0.63 μg/L; i.e., 24-h average concentration threshold) and the criterion continuous concentration (CCC = 0.39 μg/L; i.e., 96-h average concentration threshold) generated by the BLM for our test water. At this exposure concentration (0.57 μg/L) we observed no mortality after 24 or 96 h of exposure and only a small decrease in the probability of response compared to the control. However, the probability of trout exposed to increasing Cu concentrations responding to the alarm cue decreased in a dose-dependent manner (Figure 4).

DISCUSSION

Many experiments have tested the effects of Cu exposure on fish olfactory performance either directly using neurophysiological responses such as electroencephalogram (EEG) or electro-olfactogram (EOG) techniques or indirectly through behavioral assays using alarm cues, similar to the present study (e.g., Table 4 and Meyer and Deforest 2018). This is the first experiment to test the effects of Cu exposure on the salmonid olfactory system over time using the same fish. We designed this test specifically to evaluate if any inhibition of the olfactory system observed after shorter-term durations (i.e., 3 or 24 h) of exposure changed with prolonged exposure (up to 96 h). We also included a relatively short recovery period after the 96-h Cu exposure during which we exposed the fish to clean water in all treatments for 24 h to determine if any inhibition of the olfactory system observed after 96 h of Cu exposure was reversible.

The present study design included exposing fish to Cu over 96 h using a flow-through system with aquaria contained in a water bath to maintain constant temperature. The advantages of

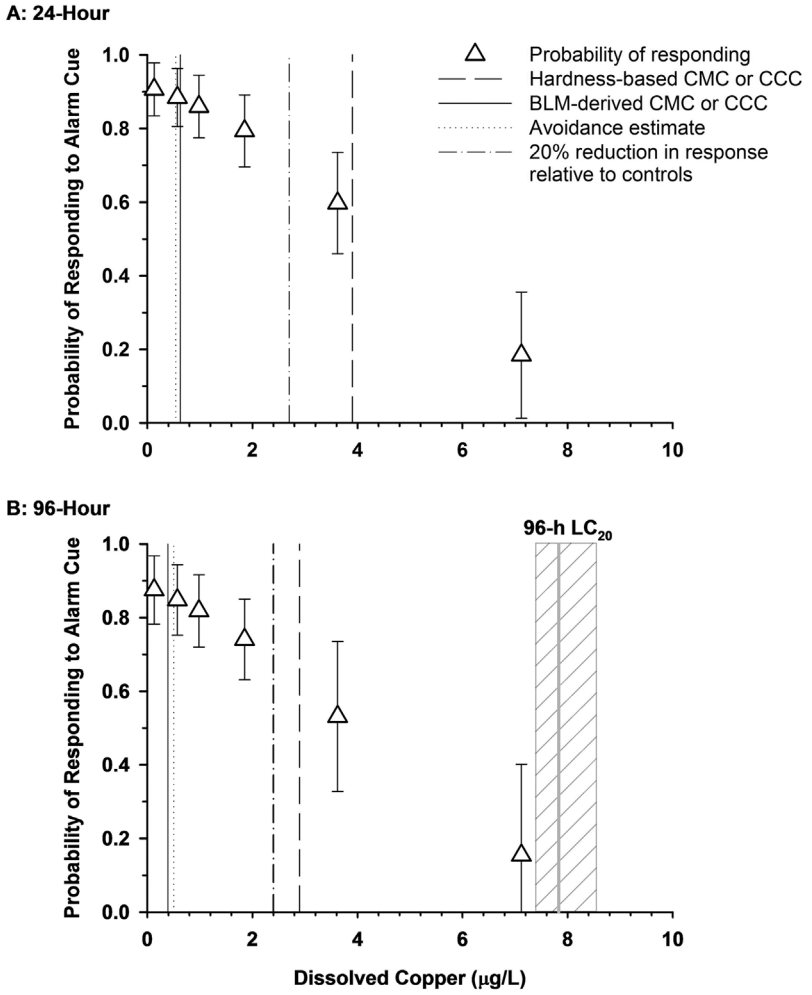

FIGURE 4: Probability of fish responding to alarm cue following (A) 24- or (B) 96-h copper (Cu) exposures. A higher probability of responding indicates that the fish sensed the alarm cue and reduced their activity (expected behavior). Biotic ligand model– and hardness-based ambient water quality criteria for 24-h exposures (criterion maximum concentration) and 96-h exposures (criterion continuous concentration) for this test water are indicated with vertical lines. The Cu concentrations representing a 20% reduction in response probability from controls and our estimated Cu avoidance concentration (see *Discussion*) based on Meyer and Adams (2010) are also indicated with vertical lines. For reference, 96-h 20% lethal concentration values (solid vertical gray line) from the bioassay preceding this test and the associated 95% confidence intervals (hatched area) are also displayed (Morris et al. 2019). Error bars are ± standard error of the mean. BLM = biotic ligand model; CCC = criterion continuous concentration; CMC = criterion maximum concentration; LC20 = 20% lethal concentration.

TABLE 4: Published effects of copper exposure on salmonid olfactory responses

Response	Species	Life stage	Exposure duration	Effect level (μg Cu/L)	Effect	Hardness (mg/L as $CaCO_3$)	DOC (mg/L)	Notes	Reference
Copper avoidance									
	Chinook salmon (*Oncorhynchus tshawytscha*)	Juvenile 6.5–16 cm	Instant	0.7 (M)[a]	Significant avoidance of Cu	25.3	NA	Countercurrent exposure chamber	Hansen et al. 1999a
	Rainbow trout (*Oncorhynchus mykiss*)	Juvenile 3.4–11 cm	Instant	1.6 (M)	Significant avoidance of Cu	25.3	NA	Countercurrent exposure chamber	Hansen et al. 1999a
	Rainbow trout	Juvenile 6–10 wk	0.25–1 h	4.8–9.2 (M)	EC50 (avoidance of Cu)	88	0.7	Y-maze exposure chamber	Van Genderen et al. 2016
	Chinook Salmon	Juvenile 12.7 cm, 23.5 g	0.5 h	2.6–7.9 (M)[a]	Significant avoidance of Cu; loss of preference for structure	100–300	0.93	Y-maze exposure chamber	Sommers et al. 2016
Neurophysiological effects									
	Coho salmon (*Oncorhynchus kisutch*)	Juvenile 4–5 mo, 4.6 cm, 0.9 g	3 h	1.9 (M)	Reduction in EOG response with all stimulants	120	NA	Stimulants: L-serine, skin extract, or bile salt	Sandahl et al. 2007
	Coho salmon	Juvenile 14 cm, 30 g	7 d	4.3–4.5 (N)	BMC20 (reduction in EOG and EEG response)	120	NA	Stimulant: L-serine	Sandahl et al. 2004
	Steelhead (*Oncorhynchus mykiss*)	Juvenile 4.9 cm, 1.2 g	3 h	5 (N)[a]	Significant reduction in EOG response	58	NA	Stimulant: L-serine	Baldwin et al. 2011
	Coho salmon	Juvenile 23 cm, 143 g	0.5–1 h	2.7 (N)	BMC25 (reduction in EOG response)	120	NA	Stimulant: L-serine; 2.7 μg Cu/L effect level is concentration above background of 3 μg Cu/L; observed incomplete recovery after 30 min	Baldwin et al. 2003
	Chum salmon (*Oncorhynchus keta*)	Juvenile 5 cm, 0.8 g	4 h	8 (M)	Significant reduction in EOG response	61	NA	Stimulant: L-serine; recovery after 1 d in clean water	Sandahl et al. 2006
	Chinook salmon	Juvenile 17–28 cm	1 h	26.2 (M)[a]	~50% reduction in EEG response	24.5	NA	Stimulant: L-serine; recovery began during 1-h recovery period	Hansen et al. 1999b
	Rainbow trout	Juvenile 20–28 cm	1 h	27.8 (M)[a]	~50% reduction in EEG response	24.5	NA	Stimulant: L-serine; recovery began during 1-h recovery period	Hansen et al. 1999b
	Coho salmon	Juvenile 14 cm, 31 g	0.5 h	48 (total) 38 (diss.) (M)[a]	Significant (62%) reduction in EOG response	85–100	0.5	Stimulant: L-serine	Sommers et al. 2016

(*Continued*)

TABLE 4: (*Continued*)

Response	Species	Life stage	Exposure duration	Effect level (μg Cu/L)	Effect	Hardness (mg/L as $CaCO_3$)	DOC (mg/L)	Notes	Reference
	Rainbow trout	Juvenile	96 h	3.6 (M)[a]	No change in EOG response at Cu concentration tested	186	1.7	Stimulant: L-alanine; reported hardness = 133 mg/L; however, reported Ca and Mg values of 44.2 and 18.5 mg/L, indicates hardness = 186	Dew et al. 2016
Olfactory Inhibition									
	Rainbow trout	Juvenile 8 cm, 6 g	96 h	2.4 (M)	20% reduction in probability of responding to alarm cue	27	0.98	Alarm cue: conspecific skin extract; no recovery after 24 h in clean water	Present study
	Rainbow trout	Juvenile 8 cm, 6 g	24 h	2.7 (M)	20% reduction in probability of responding to alarm cue	27	0.98	Alarm cue: conspecific skin extract	Present study
	Coho salmon	Juvenile 4–5 mo, 4.6 cm, 0.9 g	3 h	1.9 (M)[a]	Response to alarm cue significantly reduced	120	NA	Alarm cue: conspecific skin extract	Ellis et al. 2004
	Chinook salmon	Juvenile 9–14 g	96 h	5.8 (N)	IC50 (olfactory inhibition)	6	1	Tested avoidance of L-histidine using Y-maze; tested a range of DOC (0–20 mg/L)	Kennedy et al. 2012
	Chinook salmon	Juvenile 9–14 g	336 h (14 d)	6.2 (N)	IC50 (olfactory inhibition)	6	1	Tested avoidance of L-histidine using Y-maze; tested a range of DOC (0–20 mg/L)	Kennedy et al. 2012
	Coho salmon	Juvenile 4–6 cm	3 h	5 (N)[a]	Significantly elevated swim speed (i.e., nonresponse to alarm cue)	56	0.07[b]	Alarm cue: conspecific skin extract	McIntyre et al. 2012
	Rainbow trout	Juvenile 14 cm	13 wk	20 (N)[a]	Reduced recognition of rearing tank water	61	NA	Recognition tested using Y-maze; recovery after 2 wk in clean water	Saucier and Astic 1995
	Rainbow trout	Juvenile 3 g	12 h	21.8 (M)[a]	Response to alarm cue significantly reduced	30	NA	Alarm cue: conspecific skin extract; Cu associated with Cu nanoparticles; no recovery after 30 min	Sovova et al. 2014
	Rainbow trout	Juvenile 3 g	12 h	46.4 (M)[a]	Response to alarm cue reduced (not significant)	30	NA	Alarm cue: conspecific skin extract; Cu associated with $CuSO_4$; no recovery after 30 min	Sovova et al. 2014
Predator Avoidance									
	Coho salmon	Juvenile 4–6 cm	3 h	5 (N)[a]	66% reduction in median survival time	56	0.07 (TOC)	Predator avoidance test	McIntyre et al. 2012

(*Continued*)

TABLE 4: (*Continued*)

Response	Species	Life stage	Exposure duration	Effect level (μg Cu/L)	Effect	Hardness (mg/L as $CaCO_3$)	DOC (mg/L)	Notes	Reference
Histopathological effects									
	Rainbow trout	Juvenile 14 cm	40 wk	20 (N)[a]	Histological damage to olfactory rosette	61	NA	Recovery after 6 wk in clean water	Saucier and Astic 1995
	Rainbow trout	Juvenile 14 cm	5 wk	40 (N)[a]	Histological damage to olfactory rosette	61	NA	Recovery after 10–14 wk in clean water	Saucier and Astic 1995
	Rainbow trout	Embryo and alevin	20 wk	22 (M)[a]	Histological damage to olfactory rosette in both life stages	62–64	NA	Incomplete recovery after 10 wk in clean water	Saucier et al. 1991b
	Chinook salmon	Juvenile 17–28 cm	1 h	50 (M)	Significant reduction in number of olfactory receptor cells	24.5	NA		Hansen et al. 1999b
	Chinook salmon	Juvenile 17–28 cm	4 h	26.2 (M)[a]	Significant reduction in number of olfactory receptor cells	24.5	NA		Hansen et al. 1999b
–	Rainbow trout	Juvenile 20–28 cm	1 h	199 (M)	Significant reduction in number of olfactory receptor cells	24.5	NA		Hansen et al. 1999b
–	Rainbow trout	Juvenile 20–28 cm	4 h	27.8 (M)[a]	Significant reduction in number of olfactory receptor cells	24.5	NA		Hansen et al. 1999b

[a]Effect level reported is the lowest copper exposure concentration tested other than controls.
[b]The dissolved organic carbon value reported refers to total organic carbon.
DOC = dissolved organic carbon; EC50 = median effect concentration; EEG = electro-encephalogram produced by measuring electrical response in the olfactory bulb (see Sandahl et al. 2004); EOG = electro-olfactogram, produced by measuring electrical response in the olfactory sensory epithelium (see Sandahl et al. 2004); IC50 = median inhibition concentration; BMC20 = benchmark concentration for 20% effect level; BMC25 = benchmark concentration for 25% effect level; M = measured concentration; N = nominal concentration; NA = not analyzed or reported; TOC = total organic carbon.

such a design included the ability to maintain constant Cu concentrations over 96 h, to quantify the responses of the same fish to our alarm cue at multiple time points over the entire testing period, and to deliver the alarm cue through the flow-through system's normal water supply route without disturbing the fish. One disadvantage to this design is that we were only able to record the fish's position in each tank from an aerial view, which only provides 2-dimensional information about the fish's position and behavior. The necessity to position our exposure tanks in a water bath obviated our ability to also record the fish position from the side view. Therefore, we were not able to quantify fish moving down out of the water column toward the bottom of the aquarium after sensing the alarm cue, which is a typical alarm cue response we observed in preliminary tests. Capturing fish activity and position in the tanks from both the aerial and side views would have allowed a more detailed quantification of response behavior and should be considered for future testing, if possible. Another potential limitation to the present study design was that our first alarm cue addition was administered 3 h after the fish were handled and placed into the exposure tanks. This was because rather than starting the Cu addition into the proportional diluter and flow-through system after adding the fish, which would have allowed a longer acclimation period to the aquaria posthandling, we wanted to

maintain constant Cu exposure concentrations during the entire experiment, so we needed to add the fish to exposure aquaria that already contained Cu. We included this 3-h alarm cue time point in the present study design to align our methods with previous research which included an olfactory assessment after only 1 to 4 h of Cu exposure (Hansen et al. 1999b; Baldwin et al. 2003, 2011; Sandahl et al. 2006, 2007; McIntyre et al. 2012). Even though this first cue addition and observation period was only 3 h after adding fish to the exposure tanks, the fish were quickly netted and transferred into the exposure tanks, to minimize handling stress and any subsequent effects on fish behavior 3 h later. Ellis et al. (2004) determined that the rate of corticosteroid (cortisone and cortisol) release from rainbow trout exposed to handling stress peaked within the first hour following handling and was not significantly different from their controls after 3 h. In addition, to induce the stress response, the authors held the fish in a net out of water for 90 s. In the present experiments, fish were quickly transferred from the holding tank to the exposure tanks by net but were not out of the water for more than 5 to 10 s.

Cu olfactory toxicity in salmonids

As with much of the toxicological literature, there is often too much variability in the study designs among similar experiments for a direct comparison of results. Literature describing the effects of Cu on salmonid olfactory systems is similarly difficult to directly compare with numerous studies conducted over the last 3 to 4 decades that include different salmonid species, life stages, exposure durations, water quality conditions, endpoint measurements, and data analysis techniques. The focus of most of the available literature is to determine at which Cu concentrations the salmonid olfactory system becomes impaired and cannot sense various chemical odors and (or) the concentrations of Cu that fish can detect and actively avoid. Avoidance tests present fish with a choice of clean or contaminated water with constant Cu concentrations and are meant to test the immediate response of fish exposed to Cu. Olfactory inhibition tests can be conducted by exposing fish to Cu for a certain duration and then directly measuring the neurological response of the sensory epithelium on the fish's olfactory rosette (i.e., EOG) or measuring the subsequent neurological response that is relayed from the nose to the olfactory bulb in the fish's brain (i.e., EEG). The chemical odor or stimulant in these types of tests is usually an amino acid or other odor known to evoke a neurological response. Another method of measuring olfactory inhibition is to monitor and analyze fish behavior in the presence of a chemical cue known to elicit a measurable and predictable behavioral response. These tests generally involve exposing fish to Cu for a short- or long-term duration and then quantifying their behavioral response once a cue is introduced. A typical response cue is the odor of a conspecific fish being attacked and injured by a predator. These cues have been successfully generated as skin extracts (Sandahl et al. 2007; McIntyre et al. 2012; Sovova et al. 2014) or the rinsate from whole fish with dermal lacerations (present study), which is less labor-intensive than generating skin extracts. Finally, researchers have also reported on certain Cu exposure concentrations and durations that cause histological damage to one or more portions of the fish's olfactory rosette (Saucier et al. 1991b; Saucier and Astic 1995; Hansen et al. 1999a).

Given the salmonid's well-known sensitivity to Cu in terms of acute-lethal toxicity (e.g., Meyer et al. 2007), it is not surprising that olfactory effects manifest at rather low concentrations over short durations. For example, Cu avoidance behavior has been reported for rainbow trout and Chinook salmon (*Oncorhynchus tshawytscha*) at concentrations ranging from 0.7 to 9.2 μg/L (Table 4; Hansen et al. 1999a; Sommers et al. 2016; Van Genderen et al. 2016). Similarly, many neurophysiological studies on juvenile salmonids reported inhibitory effects on sensory epithelium (i.e., EOG) or the olfactory bulb (i.e., EEG) at either the lowest concentration tested or in the 1.9 to 8 μg/L range over 0.5 to 4 h of exposure (Table 4; Hansen et al. 1999b; Baldwin et al. 2003, 2011; Sandahl et al. 2006, 2007; Dew et al. 2016; Sommers et al. 2016) or up to 7 d of exposure (Table 4; Sandahl et al. 2004). These ranges for neurophysiological inhibition also overlap with effect ranges for inhibition quantified through behavioral assays, which range from 1.9 to 6.2 μg/L over 3 h to 14 d of exposure (Table 4; Saucier and Astic 1995; Ellis et al. 2004; Sandahl et al. 2007; Kennedy et al. 2012; McIntyre et al. 2012; Sovova et al. 2014). This overlap in effect concentrations among neurophysiological and behavioral assays seems logical and was studied in detail by Sandahl et al. (2007), who found a significant correlation in the reduction in swim speed and EOG response using a skin extract stimulant in companion experiments with coho salmon (*Oncorhynchus kisutch*; see Table 4). The results of the present study also indicate that adverse effects on the olfactory system occur at relatively low Cu concentrations with a 20% reduction in the probability of responding to the alarm cue relative to controls occurring at 2.7 and 2.4 μg/L after 24 and 96 h of exposure, respectively (Figure 4 and Table 4).

Many of these studies also included a clean-water exposure period to determine if recovery of any adverse effects of Cu exposure occurred. Of the studies that examined this, some recovery of EOG responses was observed after 0.5 to 24 h of recovery time in clean water (Table 4; Hansen et al. 1999b; Baldwin et al. 2003; Sandahl et al. 2006). However, studies examining recovery based on rainbow trout responses to an alarm cue did not detect any recovery after 30 min and 1 d (Table 4; Sovova et al. 2014; present study). Recovery was observed by Saucier and Astic (1995) in juvenile rainbow trout using a Y-maze measuring rearing tank recognition after 2 and 29 wk following several weeks of exposure to 20 or 40 μg/L, respectively (Table 4).

In addition to disruptions in behavioral and neurological responses, exposure to Cu has been implicated in histological damage to the olfactory rosette and olfactory receptor cell densities after both short- and long-term exposures. For example, Saucier et al. (1991b) and Saucier and Astic (1995) investigated the histopathological effects of Cu exposure on the olfactory rosette in early–life stage and juvenile rainbow trout from 4 to 41 wk and determined that significant damage occurred at all concentrations tested (20, 22, and 40 μg/L). Furthermore, the authors reported that recovery from these exposures took anywhere from 6 to 14 wk once the fish were moved to clean water (Table 4). Hansen et al. (1999b) also observed deleterious impacts on the olfactory system of Chinook salmon and rainbow trout with significant reductions

in olfactory receptor cell density after only 1 to 4 h of exposure to 26 to 28 μg Cu/L (Table 4).

As with adverse effects on the gill and subsequent ion transport and balance within the fish attributable to Cu exposure, the toxicity of Cu exposure on the olfactory system may also be influenced by water chemistry. For instance, similar to how pH, hardness, and organic carbon concentrations in water can alter the toxicity of dissolved Cu as a function of bioavailability and binding at the gill, these parameters may also alter Cu binding to receptors in the olfactory rosette. However, many olfactory toxicity assays on salmonids in the literature do not measure or report organic carbon content in their exposure water, so it is not possible to conduct a meta-analysis of the effects of water quality over a broad range of such tests. Kennedy et al. (2012) determined that increased DOC concentrations decreased the inhibitory effects of Cu on juvenile Chinook salmon regarding their ability to detect and avoid L-histidine in a Y-maze assay. McIntyre et al. (2008) also interpret their work with coho salmon EOG responses as indicating differences in the ameliorative effects of hardness, pH, and DOC on olfactory toxicity compared to lethal toxicity; therefore, they concluded that the Cu binding affinity of the gill and olfactory tissue is likely different. This is something for researchers or regulators to consider when utilizing models or criteria that rely on some aspect(s) of water chemistry to determine exposure and subsequent adverse effects on the olfactory system.

Cu water quality standards and the Bristol Bay watershed

The olfactory system is very important to many aspects of a fish's life history. Impacts on the olfactory system or changes to normal behavior attributable to Cu exposure may result in numerous adverse effects including disruptions in prey capture and predator avoidance, chemical imprinting during downstream migration and smolting, and upstream migration to natal spawning grounds. We observed a downward trend in the probability of fish responding to an alarm cue as Cu exposure concentrations at the 24, 48, and 96 h of exposure, likely attributable to an inhibition of the fish's olfactory system (Figure 3). There was no apparent recovery following 24 h of exposure to clean water (hours 96–120; Figure 3E). The concentrations causing a 20% reduction in the probability of responding to the cue (i.e., olfactory inhibition) compared to the controls at 24 and 96 h of exposure were 2.7 and 2.4 μg Cu/L, respectively. This 24-h inhibitory concentration was between the BLM-derived CMC (0.63 μg Cu/L) and the hardness-based CMC (3.9 μg Cu/L; Figure 4A). Similarly, our 96-h IC20 was between the BLM-derived CCC (0.39 μg Cu/L) and the hardness-based CCC (2.9 μg Cu/L; Figure 4B). Therefore, the hardness-based criteria, which is the current method used to estimate the Cu aquatic life criteria by the Alaska Department of Environmental Conservation, does not appear to protect against olfactory inhibition given our test water chemistry. The BLM criteria do appear to be protective of olfactory inhibition given our test water chemistry. This is relevant to the Alaska Department of Environmental Conservation's current 2018 to 2020 Triennial Review of existing state water quality standards because they have indicated they will consider developing guidance pertaining to use of the BLM on a site-specific basis as part of this review process.

Meyer and Adams (2010) conducted an analysis of Cu EOG/EEG sensory inhibition data for rainbow trout and Chinook salmon from Hansen et al. (1999b) and Cu avoidance behavior data for rainbow trout and Chinook salmon from Hansen et al. (1999a), in which they estimated IC20 values for sensory inhibition and avoidance. In addition, Meyer and Adams (2010) compared these values to BLM estimates and calculated ratios of avoidance and olfactory response IC20 values to BLM CMC and CCC criteria. The USEPA applied these ratios to BLM CMC values estimated for average water quality conditions in the Bristol Bay watershed to derive site-specific avoidance and sensory inhibition IC20 values for 3 drainages that could be impacted by development of the Pebble Mine (see Table 8-14 in the USEPA's Bristol Bay Assessment; US Environmental Protection Agency 2014). If we take the same approach and multiply our BLM-calculated CMC (0.63 μg Cu/L) by the IC20:BLM CMC ratios for rainbow trout avoidance and olfactory inhibition (2.2 and 11.1, respectively) from Table 1 in Meyer and Adams (2010), that results in IC20 estimates of 1.39 and 6.99 for avoidance and olfactory inhibition, respectively. However, we observed a 20% reduction in the probability of responding to an alarm cue (i.e., olfactory inhibition) in our test at 2.7 μg Cu/L after 24 h. Similarly, if we make the same comparison using our BLM-calculated CCC (0.39 μg Cu/L) and the IC20:BLM CCC ratios for rainbow trout avoidance and olfactory inhibition (3.7 and 17.6, respectively) from Table 1 in Meyer and Adams (2010), that results in IC20 estimates of 1.44 and 6.86 μg Cu/L for avoidance and olfactory inhibition, respectively. However, we observed a 20% reduction in the probability of responding to an alarm cue in our test at 2.4 μg Cu/L after 96 h. This suggests that the approach described by Meyer and Adams (2010) underestimates Cu toxicity attributable to olfactory inhibition given the chemistry of our exposure water.

The IC20 estimates for avoidance using Meyer and Adams (2010) ratios based on BLM CCC and CMC values (1.39 and 1.44 μg Cu/L, respectively) are lower than our 24- and 96-h estimates of the 20% reduction in the probability of responding to the alarm cue (i.e., olfactory inhibition), which were 2.7 and 2.4 μg Cu/L, respectively. This is expected because, by definition, avoidance should be an endpoint that manifests at a lower concentration than olfactory inhibition because the fish must be able to sense the Cu to avoid it. Furthermore, the proportional difference between the avoidance and olfactory inhibition ratios, and subsequent IC20 values, reported by Meyer and Adams (2010), based on the BLM CMC and CCC values, suggests that avoidance manifests at Cu concentrations 4.8 and 5.0 times lower than olfactory inhibition, respectively. If we reduce the olfactory inhibition values from our tests accordingly, our predicted avoidance concentrations for the 24- and 96-h exposures are 0.54 and 0.50 μg Cu/L, respectively. This predicted avoidance concentration for our 24-h exposure is slightly lower than the BLM-derived CMC of 0.63 μg Cu/L, and our predicted avoidance concentration for our 96-h exposure was slightly higher than the BLM-derived CCC of 0.39 μg Cu/L. Our predicted avoidance concentration is within ±0.11 μg Cu/L for either the CMC or CCC, suggesting that the BLM-derived criteria are not protective in

regard to Cu avoidance behavior. However, this should be verified through site-specific laboratory and (or) field assays.

In summary, the hardness-based ambient water quality criteria for Cu do not appear to be protective of olfactory inhibition or avoidance behavior in low-hardness waters, such as those in the Bristol Bay watershed. In addition, BLM-derived ambient water quality criteria appear to be protective against olfactory inhibition but potentially not protective against avoidance behavior in our low-hardness (27 mg/L as $CaCO_3$), low-DOC (0.98 mg/L) test water. Therefore, protective criteria should be further developed on a site- or reach-specific basis for streams in the Bristol Bay watershed and other similar low-hardness watersheds with regionally important salmonid species such as sockeye salmon (*Oncorhynchus nerka*).

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4295.

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Data Accessibility—Data, associated metadata, and calculation tools are available from the corresponding author (Jeff_Morris@abtassoc.com).

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