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Effects of Continuous Copper Exposure and Calcium on the Olfactory Response of Fathead Minnows

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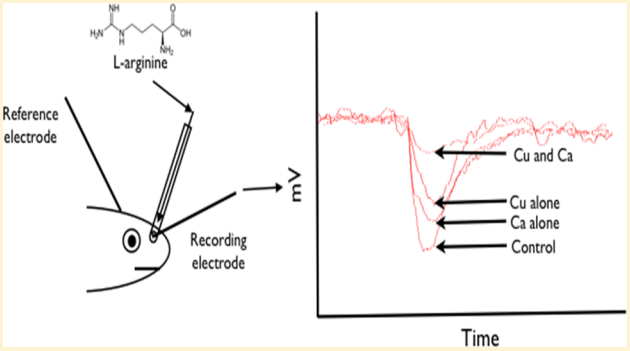
Supporting Information

ABSTRACT: The current gill-based Biotic Ligand Model (gbBLM) is an acute-toxicity model used to predict site-specific safe copper (Cu) concentrations. Recent effort to develop a chronic BLM has focused on the olfactory epithelium. To further this effort, the current study looked at the effect of varying Cu concentration and exposure duration on Cu-induced olfactory dysfunction, and whether calcium (Ca) protected against Cu-induced impairment as it does at the gill. Fathead minnows (*Pimephales promelas*) were treated with five Cu concentrations for varying exposure durations in hard and soft water. A neurophysiological technique, electro-olfactography (EOG), was employed to determine the level of olfactory dysfunction. At the low, ecologically relevant Cu concentrations tested there was significant inhibition of EOG function; however, over time there was at least a partial recovery of olfactory function, despite the continuous Cu exposure. Calcium did not appear to protect against Cu-induced olfactory dysfunction; and even alone, Ca appeared to interfere with the olfactory response to the amino acid L-arginine. Safe copper concentrations as predicted by the gbBLM, chemosensory-based BLMs, the USEPA BLM, and hardness-adjustment equations based on the exposure waters were not entirely protective against olfactory dysfunction.



INTRODUCTION

The Biotic Ligand Model (BLM) is a powerful predictive toxicological model that is used to predict site-specific acute metal toxicity to freshwater fishes and invertebrates.[1−3] The BLM is based on the complex relationship among metal binding to a physiologically sensitive binding site (e.g., fish gills), metal speciation as it relates to bioavailability, and acute toxicity.[4,5] Questions about the ecological relevance of the acute, gill-based BLM (gbBLM) have emerged given model assumptions that metals are only taken up via a waterborne source and that metal concentrations typically found in contaminated waters are rarely sufficient to induce acute toxicity.[6] Consequently, research attention has shifted focus to the development of chronic BLMs in order to improve the ecological relevance of BLM predictions.[6]

To address some of these concerns, Pyle and Wood[7] and Meyer et al.[8] proposed the olfactory epithelium as an alternative biotic ligand to the gill in support of a chemosensory-based BLM (cbBLM). Meyer and Adams[9] proposed an early version of such a model for coho salmon (*Oncorhynchus kisutch*) exposed to copper (Cu). The cbBLM considers metal binding at the olfactory epithelium instead of the gill and its relationship to olfactory dysfunction instead of mortality.[7] Such a model is not confounded by dietary metal exposure. The predicted effects have been shown to occur at environmentally relevant metal concentrations, and are ecologically relevant because olfaction mediates a range of antipredator, feeding, and reproductive behaviors. However, Meyer and Adams[9] changed only the Cu-sensitivity parameter [i.e., they replaced the medial lethal accumulation (LA50) in the gbBLM with a lower median inhibitory accumulation (IA50) of Cu that optimized the fit to olfactory impairment data in McIntyre et al.[10] without changing the binding constants for Cu, Ca, Mg, and Na to the biotic ligand]. Therefore, that early cbBLM ignored potential differences in binding affinities for cations between branchial and olfactory epithelia.

Green et al.[11] published the first empirical olfactory tissue Cu-binding constant for fathead minnows (*Pimephales promelas*). They showed that olfactory dysfunction occurred at approximately 160 nM (10 $\mu g L^{-1}$), as demonstrated by both behavioral and neurophysiological assays. Calcium (Ca), which protects the gill epithelium against Cu binding and subsequent toxicity, did not appear to be protective against olfactory intoxication following low-concentration Cu exposure. The data reported in Green et al.[11] showed neurophysiological impairment with exposure to 160 nM Cu at 1 h (their Figure 2), but they compared that result to Cu and Ca coexposures for

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3 h. Therefore, increasing exposure time from 1 to 3 h could have shifted the concentration−response curve to the left (Figure 1); that is, a lower Cu concentration might have been

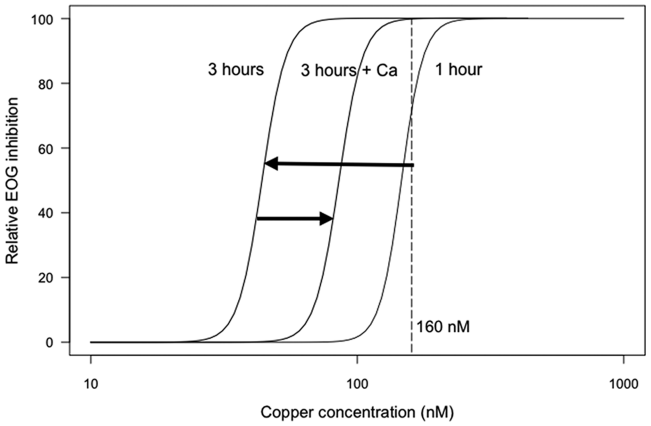

Figure 1. Concentration−response curves depicting the predicted theoretical reduction in the EOG response of fathead minnows by increasing time with the same exposure concentration of Cu, and predicted recovery, or "shift to the right" of the 3-h concentration−response curve with the addition of Ca. Note that in this theoretical model, at 160 nM no difference would be seen between a Cu alone and a Cu with Ca treatment at 3 h because the concentration−response curve is still at its upper asymptote of 100% inhibition in both exposures.

required to produce the same degree of olfactory impairment after the longer exposure duration, as would be expected for acute lethality.[12] Then, if Ca were protective against Cu toxicity, the left-shifted curve could be shifted back to the right at the higher Ca concentration. Because Green et al.[11] held Cu concentration constant at 160 nM while varying Ca concentration, this Cu concentration might have been too high to demonstrate a protective effect of Ca against impairment of neurophysiological function by Cu (Figure 1).

To further inform the development of a cbBLM, the purpose of the present study was to determine (i) the interaction between Cu concentration and exposure duration on neurophysiological function in fathead minnows, (ii) if Ca protects against Cu-induced olfactory dysfunction in fathead minnows, and (iii) if current gbBLMs, reparameterized cbBLMs, the USEPA BLM, or hardness-adjustment equations used for setting site-specific water quality criteria would have been protective against the Cu-induced olfactory dysfunction. We exposed fathead minnows to very low, but environmentally relevant, Cu concentrations and tested their olfactory acuity using electro-olfactography (EOG), a neurophysiological assay, at time intervals bracketing those used in Green et al.,[11] and tested whether Ca could protect against Cu-induced olfactory dysfunction. We also parameterized three new cbBLMs based on the current understanding of the olfactory epithelium, and compared their predictions with standard gbBLM predictions and experimental data to determine if the current framework of the gill-based BLM can be adapted to the olfactory epithelium. For comparison, the acute (criterion maximum concentration, or CMC) and chronic criteria (criterion continuous concentration, or CCC) were also calculated using the USEPA BLM and hardness-adjustment equations.[13] Data generated by this study will be useful for the development and refinement of a cbBLM for fathead minnows.

EXPERIMENTAL PROCEDURES

Fish. Adult fathead minnows (1−4 g) were held in the Lakehead University Biology Aquatic Facility on a 16 h light:8 h dark photocycle in dechlorinated Thunder Bay, ON municipal water (Table S1). All fish were held in a flow-through system at a density not exceeding one fish per liter. Fish were fed *ad libitum* once daily, with *Artemia* spp. and commercial fish flakes (Tetra, Blacksburg, VA, USA) on alternate days. All fish were acclimated to laboratory conditions for a minimum of two weeks before being used in experiments. All experimental treatments were conducted in 4 L of aerated holding water, with a 50% water change every 24 h. All water quality measurements were performed by the Lakehead University Centre for Analytical Services (LUCAS), which is accredited through the Canadian Association for Laboratory Accreditation (CALA). All QA/QC procedures followed internal standard operating procedures of the LUCAS lab, including analysis of NIST traceable reference material standards.

Electro-Olfactography Procedure. Electro-olfactography (EOG) experiments were performed as previously described.[11] Fish were anaesthetized in 0.42−0.46 mM (110−120 mg L^{-1}) pH 7.4 buffered MS-222. Water used to irrigate the olfactory epithelium had the same Cu and Ca content as the experimental treatment water detailed below, and was made just prior to the EOG measurements.

The stimulus used to induce an olfactory response was 10^{-4} M L-arginine, and was made fresh daily from powdered L-arginine (Sigma, Oakville, ON, Canada) in dechlorinated Thunder Bay municipal water (Table S1). For animals treated with Ca, the stimulus was prepared in dechlorinated Thunder Bay ON municipal water containing the same concentration of Ca as the experimental treatment waters. Calcium stock solutions were prepared by dissolving $Ca(NO_3)_2$ (Sigma, Oakville, ON, Canada) in dechlorinated water, and were diluted to the appropriate concentration for each experiment. A minimum of three 3-s pulses of the stimulus were delivered to the olfactory epithelium, with a minimum of 2 min between each of the stimulus deliveries to minimize potential chemosensory attenuation to the stimulus.

Interaction Between Cu Concentration and Exposure Duration. To determine the single and combined influences of Cu concentration and exposure duration, fathead minnows were randomly assigned to one of 5 concentrations of Cu, each at 4 different exposure durations (1, 3, 24, and 96 h). A copper stock solution was prepared by dissolving $CuCl_2$ (Sigma, Oakville, ON, Canada) in dechlorinated water, and was used to make dilutions at the appropriate concentration for each experiment. The concentrations of Cu selected bracketed the Ontario water quality guidelines for Cu (78.7 nM or 5 $\mu g L^{-1}$) and represent concentrations that are found in clean and Cu-contaminated lakes.[14,15] The two lowest concentrations (0 and 31.5 nM) are reported as nominal concentrations because all measurements of Cu concentration in the 0 nM exposure waters were below the detection limit (31.5 nM), two measurements of the 31.5 nM (2.0 $\mu g L^{-1}$) exposures were at the detection limit, and two were below (n = 4). All the other Cu concentrations (mean ± SEM, 72.7 ± 6.6 (4.6 μg L^{-1}), 106.2 ± 3.9 (6.7 μg L^{-1}), and 204.6 ± 17.0 nM (13.6 μg L^{-1}); n = 4−5) were measured by LUCAS using inductively coupled plasma atomic emission spectroscopy (ICP-AES). All exposure waters used to treat fish were made up immediately before

exposures began, and, if needed, immediately before any water changes.

Effect of Ca on Cu Intoxication. To determine the effect of Ca on Cu-induced olfactory dysfunction, fish were randomly assigned to each of four experimental treatments (control (nothing added), Cu added, Ca added, and Cu and Ca added) for two exposure durations (3 and 96 h; Table S2). The concentration of Cu in the Cu-added treatment approximately matched the second highest concentration used in the first experiment, and the concentration of Ca in the Ca-added treatment approximately matched the highest concentration of total Ca used in Green et al.[11] The olfactory acuity of each animal was measured using the EOG technique outlined above. All measurements of Cu and Ca were performed via ICP-AES. All exposure waters used to treat fish were made up immediately before exposures began, and, if needed, immediately before any water changes.

To establish if the effect of Cu and Ca coexposures on EOG response to the stimulus represents an additive interaction between Cu and Ca, we selected an effects addition model as Ca and Cu were expected to act dissimilarly. To this end the additivity equation (eq 2) from Norwood et al.[16] was adapted by substituting mean relative EOG amplitudes for survival proportion. Consequently, the expected EOG amplitude of a mixture of Cu and Ca can be calculated as follows:

$$EOG_{Mixture} = EOG_{Cu} \times EOG_{Ca} \quad (1)$$

where EOG_{Cu} is the mean relative EOG amplitude (i.e., the proportional amplitude relative to the mean no-Cu control response) of fish when exposed to Cu alone, and EOG_{Ca} is the mean relative EOG amplitude of fish when exposed to Ca alone. The product ($EOG_{Mixture}$) is the predicted value for the mean relative EOG amplitude of fish given a Cu and Ca coexposure, if the interaction between Cu and Ca is response-additive. The predicted $EOG_{Mixture}$ was then compared with the measured mean relative EOG response of fish given a Cu and Ca coexposure (EOG_{Cu+Ca}). If the value for EOG_{Cu+Ca} is similar to $EOG_{Mixture}$, there is a response-additive interaction between Cu and Ca. If EOG_{Cu+Ca} is substantially lower than $EOG_{Mixture}$, the interaction is classified as more than response-additive. If EOG_{Cu+Ca} is substantially higher than $EOG_{Mixture}$, the interaction is antagonistic (relative to response-additivity). Equation 1 was used to determine the expected EOG values for a mixture of Cu and Ca based on the EOG values for the individual metal exposures at the 3 and 96 h time points.

Data Handling and Statistical Analysis. The raw EOG amplitude in response to 10^{-4} M L-arginine for each animal was determined by measuring the difference between the baseline and the maximum response recorded following each stimulus delivery. The average response from a minimum of three measurements per fish was determined. Each averaged EOG amplitude was then corrected by subtracting any response measured to the appropriate blank. For the first set of experiments, the blank was dechlorinated tap water, for the second set of experiments the blank contained the same concentration of Ca as the water to which each fish was exposed. Results are presented as relative EOG responses, expressed as a proportion of the response measured for the 0 nM Cu exposures. The values at each time point/Cu concentration or time point/Cu and Ca concentration combination were then averaged. As such, all reported results are blank-corrected mean relative EOG amplitudes (with associated standard error of the mean).

All statistical analyses were performed using R (www.r-project.org), version 2.13.0,[17] with graphics made using the sciplot package.[18] Mean differences in relative EOG responses were considered statistically significant when $p \leq 0.05$. A two-way analysis of variance (ANOVA) was used to examine the single or combined influences of Cu concentration and exposure duration. A 2-way ANOVA was also used to examine the single or combined influences of treatment and exposure duration for the Cu, Ca, and Cu/Ca coexposures. A Tukey–Kramer test was performed to determine differences between individual groups for the Cu, Ca, and Cu/Ca coexposures at $p \leq 0.05$.

BLM Modeling. Predicted median lethal concentrations (LC50s) and median inhibitory concentrations (IC50s) for olfactory impairment from a total of six BLMs (two gbBLMs, four cbBLMs) were compared to empirical results to determine if the current BLMs could be used or adapted to determine safe Cu concentrations. All BLMs were based on the HydroQual Cu BLM version 2.2.3 (www.hydroqual.com/wr_blm.html).[19] The six BLMs were the rainbow trout gbBLM (RBT-gbBLM), the coho salmon cbBLM (CS-cbBLM) as parameterized by Meyer and Adams,[9] the fathead minnow gbBLM (FHM-gbBLM), and three fathead minnow cbBLMs (FHM-cbBLM 1, FHM-cbBLM 2, and FHM-cbBLM 3) parameterized based on current understanding of the olfactory epithelium.

The three FHM-cbBLMs were made by adjusting the parameter file as follows. For the first model, FHM-cbBLM 1, the parameter file was changed by deleting sections dealing with Ca binding to the ligand (but retained the effects of Ca binding to DOC), as recent evidence shows that Ca offers little to no protection against Cu-induced olfactory dysfunction (10, 11, 20, and this study). For FHM-cbBLM 2 the binding constant for Cu was adjusted to 6.7, as this is the empirical binding constant of Cu for the olfactory epithelium of fathead minnows.[11] Both modifications for FHM-cbBLM 1 and FHM-cbBLM 2 were used in FHM-cbBLM 3.

The water quality parameters in Table S1 were used as inputs to produce the BLM predictions. Running the BLMs in "toxicity mode" produced "LC50" values, however, as EOG inhibition is not a measure of lethality the LC50 estimates from cbBLMs were considered to be IC50 values for EOG inhibition. As the humic acid (HA) content of the DOC in the dechlorinated tap water was not known, two different concentrations, 10% HA and 50% HA, were used for comparison. HydroQual[19] recommends 10% HA when the exact HA content is not known. The 50% HA content was used as it is representative of the humic acid content in DOC from Ontario lakes.[21] For the experimentally derived values, concentration response curves of EOG inhibition and IC50 values for the 24 and 96 h Cu exposures were determined using the drc package in R.[22] As the lowest concentration tested was below the detection limit, $^1/_2$ the detection limit (15.75 nM) was used with the 0 inhibition level.

According to Meyer and Adams,[9] the HydroQual BLM uses the same algorithms as the USEPA BLM, therefore the HydroQual BLM was set to water quality criteria (WQC) mode and the CMC and CCC values were determined for the exposure waters (based on Table S1). The acute USEPA Criterion Maximum Concentration (CMC) specifies the highest average concentration of a toxicant in ambient water to which an aquatic community can be exposed briefly without resulting in an unacceptable adverse effect. For regulatory compliance, the CMC should not be exceeded in a 1-h

averaging period more than once every three years. The chronic USEPA Criterion Continuous Concentration (CCC) specifies the highest average concentration of a toxicant in ambient water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable adverse effect. For regulatory compliance, the CCC should not be exceeded in a 4-d averaging period more than once every 3 years. Values for CMC and CCC were also calculated based on hardness-adjustment Cu-criteria equations.[13] As these concentrations represent predicted safe Cu concentrations, the appropriate comparisons to experimental results would be the inhibitory concentration shown to cause no impairment to the animals. Currently there is no empirical evidence in the literature showing what the safe inhibitory concentration is; however, the IC20 has been proposed as a surrogate because variability in EOG responses is typically approximately 20%.[9] The IC20 value for the 96 h exposures was calculated for comparison to the CCC value using the drc package in R.[22] Any IC20 prediction based on the 1-h exposures would be highly suspect as the lowest degree of inhibition seen in the data was approximately 60%. For this reason all that can be said is the CMC is comparable to a value less than the lowest concentration that showed an effect after a 1-h exposure.

RESULTS

Interaction Between Cu Concentration and Treatment Duration. Impaired olfaction resulting from the initial Cu exposure at all concentrations was followed by a recovery, at least partly, by 96 h. There was a statistical interaction in EOG function between Cu concentration and exposure duration ($F_{(12,83)}$ = 5.83, p < 0.001; Figure 2). At Cu concentrations of

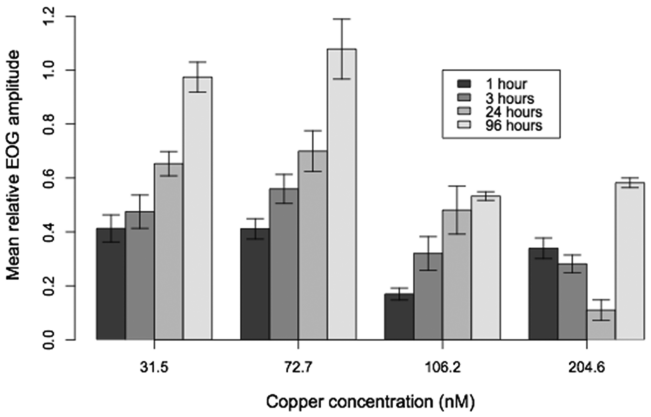

Figure 2. Interaction of exposure duration (h) and Cu concentration (nM) on mean EOG (±SEM) response of fathead minnows in response to 10^{-4} M L-arginine (n = 3–6 animals per bar). As time increases there is an increased recovery of EOG function for all concentrations, and as concentration increases there is a decrease in EOG function at each sampling point.

31.5 and 72.7 nM, there was a strong initial reduction in EOG amplitude followed by a gradual increase over time resulting in a full recovery at 96 h. At the next highest Cu concentration (106.2 nM), the same pattern of recovery was seen, except that the initial inhibition of EOG function was greater and the recovery was not complete after 96 h. Interestingly, the pattern of recovery for the highest concentration of Cu (204.6 nM) was unlike all of the other concentrations. Olfactory function, as measured by EOG, gradually decreased over 24 h, but showed significant recovery at 96 h. The amount of recovery at 96 h with the animals treated with the highest concentration of Cu was approximately the same as that with the 106.2 nM Cu exposure concentration.

Effect of Ca on Cu Intoxication. Elevated waterborne Ca offered no protection against the inhibition of olfaction caused by elevated waterborne Cu. There was a statistical interaction between treatment and exposure duration ($F_{(2,13)}$ = 25.65, p < 0.03; Figure 3). There was a reduction in EOG function across

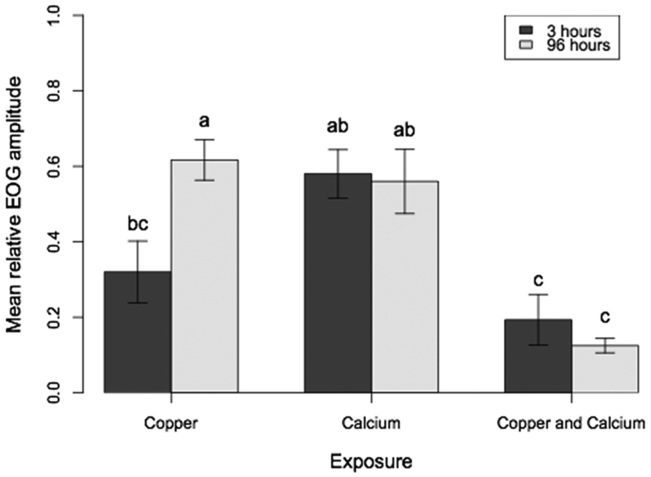

Figure 3. Individual (copper treatment has 173.1 nM Cu, calcium treatment has 1117.0 nM Ca) and combined effect of Cu and Ca (173.1 nM Cu and 1112.4 nM Ca) exposure on the mean relative EOG response (±SEM) of fathead minnows to 10^{-4} M L-arginine (n = 3 for each bar). Different letters above bars indicate a significant difference ($p \leq 0.05$).

all treatments (Cu, Ca, Cu and Ca) at both sampling points (3 and 96 h). The Cu-alone exposures showed a trend similar to that seen in the first experiment (Figure 2), in that there was an increase of EOG function between 3 and 96 h. Interestingly, when fish were treated with only Ca, EOG function decreased by almost the same percentage at the 3 and 96 h time points. Co-treatments with both Cu and Ca showed two separate patterns: at 3 h the relative EOG response was significantly lower than that with Ca alone, but was not significantly different from the Cu alone treatment, while at 96 h the relative EOG response for the combined treatment was significantly lower than both of the individual treatments.

The interaction between Cu and Ca differed depending on the exposure duration. In the short-term exposure (3 h), there was a response-additive relationship between Cu and Ca because the values for $EOG_{Mixture}$ and EOG_{Cu+Ca} were similar (Table S3). For the long-term exposure (96 h), EOG_{Cu+Ca} was much lower than $EOG_{Mixture}$, indicating a more-than-additive relationship with respect to the assumption of response additivity. Therefore, the relationship between Cu and Ca appears to shift from additive to more-than-additive inhibition over time.

BLM Predictions and Experimental IC50s. To test whether or not the current Cu BLM framework could be applied to the olfactory epithelium, IC50 predictions from two gbBLMs and four cbBLMs were compared to experimentally derived values (Table 1). The BLMs were run with HA content set to either 10% or 50% HA, and all LC50 and IC50 predictions when models were based on 50% HA were higher.

Table 1. Comparison of LC50s and IC50s Predicted by Six BLM Models to Experimental IC50s[a]

name	fish species	end point	10% HA	50% HA
RBT-gbBLM	rainbow trout	LC50	1.89 [0.12]	2.45 [0.16]
CS-cbBLM	coho salmon	IC50	0.57 [0.04]	1.08 [0.07]
FHM-gbBLM	fathead minnow	LC50	2.28 [0.14]	2.96 [0.19]
FHM-cbBLM 1	fathead minnow	IC50	1.84 [0.12]	2.49 [0.16]
FHM-cbBLM 2	fathead minnow	IC50	4.71 [0.30]	5.58 [0.35]
FHM-cbBLM 3	fathead minnow	IC50	3.44 [0.22]	4.23 [0.27]
experimental: 24 h	fathead minnow	IC50	0.087 (0.063−0.111) [0.006]	
experimental: 96 h	fathead minnow	IC50	0.213 (0.103−0.323) [0.01]	

[a]The two gbBLMs used the default parameters specified by HydroQual;[19] all cbBLMs were parameterized as discussed in the Experimental Procedures section. The experimental values were derived from the concentration response curves in Figure S1, bracketed values represent 95% confidence intervals. All concentrations are in μM, values in square brackets represent the average concentration in mgL^{-1}.

Currently, the only chemosensory-based BLM that has been parameterized is the CS-cbBLM proposed by Meyer and Adams.[9] For a comparison the RBT-gbBLM was also used in the current study, as this source file was used to construct the CS-cbBLM.[9] The IC50s predicted by the CS-cbBLM were less than those predicted by the RBT-gbBLM, a result which was expected as the parameterization of the model assumes that the olfactory epithelium is more sensitive than the gill to Cu. Both the RBT-gbBLM LC50s and the CS-cbBLM IC50s were higher than the experimentally derived IC50s. In terms of fathead minnow models, when the FHM-gbBLM predictions were compared to the three different FHM cbBLMs, clear differences were seen. The prediction for FHM-cbBLM 1 was lower than the prediction for FHM-gbBLM; this was expected as the protective effect of Ca was removed from the model. For the second model (FHM-cbBLM 2), it was expected that as the binding constant for Cu was reduced, a higher IC50 would be predicted as compared to the FHM-gbBLMs results. The lower the binding constant of Cu, the less Cu will be binding to the biotic ligand and causing an effect. The FHM-cbBLM 2 did, in fact, predict a higher IC50 value than the FHM-gbBLM, as expected. The third model, FHM-cbBLM 3, predicted an IC50 between the other two FHM-cbBLMs. Regardless of which fathead minnow model was used, all predicted IC50s were higher than the experimental results. In fact, the closest prediction to experimental was with FHM-cbBLM 1 (at 10% HA), which predicted an IC50 over 8.5× higher than the experimental value for 96 h and over 21× higher than the experimental value for 24 h.

To determine if the USEPA BLM and hardness-adjustment equations predicted CMCs and CCCs protective of the olfactory epithelium, CMC and CCC criteria were compared to experimentally measured values (Table 2). For comparison to the CMC and CCC values, the appropriate comparison would be IC20s for 1 and 96 h. Unfortunately the data were not appropriate to predict a 1 h IC20, and all that can be stated is that the IC20 at 1 h is below the lowest concentration tested, namely <31.5 nM. The 96 h IC20 value was calculated to be 94.6 nM (Figure S1). The results show that the lowest prediction for a CMC value (the USEPA BLM set to 10% HA) is over 3× higher than the 1 h experimental value. For the CCC, the hardness-adjustment equation predicted a Cu concentration that was below the 96 h IC20, the USEPA BLM set to 10% HA predicted a Cu concentration that was lower than the 96 h IC20, and when set to 50% HA, the USEPA BLM predicted a CCC that was approximately 60% higher than the 96 h IC20.

Table 2. Comparison of USEPA BLM Predictions for CMC and CCC at Two Concentrations of Humic Acid (HA) to the Hardness-Adjusted Values and Experimentally Derived IC20 Values[a]

	CMC predictions and 1 h experimental results	CCC predictions and 96 h experimental results
gbBLM (10% HA)	96.5 (6.1)	60.0 (3.8)
gbBLM (50% HA)	246.4 (15.7)	153.1 (9.7)
hardness adjusted	105.9 (6.7)	75.2 (4.8)
experimental IC20	<31.5 (2.0)	94.6 (6.1)

[a]The values predicted for the CMC are compared with the lowest concentration used, and the values predicted for the CCC are compared to the 96 h IC20. All values are in nM, bracketed values are in μgL^{-1}.

DISCUSSION

This study demonstrates that Cu exposure at environmentally relevant concentrations (in this case below the analytical detection limit of 31.5 nM) can inhibit a fish's olfactory function. Although short-term Cu exposure effectively inhibited the EOG response at all concentrations tested, continuous exposure to Cu for up to 96 h resulted in at least partial EOG recovery. This pattern of recovery illustrates that olfactory sensory neurons (OSNs) have a capacity to recover from Cu-induced inhibition while still being exposed to Cu. Similar exposures using a behavioral end point have also shown recovery over time during continuous copper exposure.[23,24] The degree of recovery seen in the current study is dependent on exposure concentration, because the fish exposed to the two lowest concentrations were able to fully recover their EOG function by 96 h. Fish exposed to the two highest Cu concentrations, however, did not fully recover, indicating that the ability of the OSNs to recover within 96 h was exceeded. At the longest exposure durations the effect appeared to be concentration-dependent in agreement with a previous study.[25] Previous studies have shown that removal of contaminants such as Cu[20,26] or pesticides[27] results in recovery of OSN response to stimuli; however, no studies to date have shown recovery during continuous exposure to a contaminant. A study comparing yellow perch (*Perca flavescens*) from metal-contaminated and clean lakes showed higher EOG response in fish from metal-contaminated lakes than those from clean lakes.[28] The same study showed that even though EOG response was intact, behavioral response to an antipredator cue was impaired.[28] The current study suggests that fish continuously exposed to Cu-contaminated water may recover from short-term neurophysiological (EOG) deficits via some unknown mechanism; whether or not they recover from

corresponding behavioral deficits remains unknown. Work that may elucidate this mechanism was done by Tilton et al.[29] In their study zebrafish (*Danio rerio*) were exposed to Cu at concentrations similar to ours for 24 h, and microarrays were performed using their entire olfactory system (olfactory epithelium, nerve, and bulb). In the present study, there was some recovery at 24 h, so the gene expression patterns discussed in their paper may hold clues into this mechanism of recovery. An alternative explanation of these results is that the differences in EOG response between time points is not due to recovery, but due to the length of time it takes DOC and Cu to come to equilibrium.[30] This could mean that the longer a fish is left in the Cu exposure waters, the less Cu it is being exposed to as more is being bound by the DOC, resulting in less effect on the olfactory epithelium. We do not believe that this is occurring in this study, as the irrigation water used for the EOG experiments was made up just prior to performing the experiments (from a common stock), and therefore animals across all time points were exposed to water of the same age and concentration during the EOG measurements. Earlier studies have shown that when fish have been exposed to Cu in the irrigation water, impairment can occur within 10 min of exposure.[11] In addition, investigations on the accumulation of Cu at the gill showed that the age of DOC-Cu mixtures has no effect on Cu accumulation.[31] This result conflicts with the work by Ma et al.,[30] who showed a difference. However, the latter study was done to test an acute end point on an invertebrate, while the work in Hollis et al.[31] measured accumulation of Cu in a fish tissue. As the current study is with fish and measures a subacute end point, it is likely that the age of DOC-Cu mixtures does not explain why there is a greater EOG recovery with longer Cu exposures. Further research is required, especially to elucidate the potential effects of varying water chemistry parameters on the effect concentrations.

As was seen in previous studies,[11,20] the addition of Ca offered no protection from Cu-induced olfactory dysfunction. In our experiments we held the Cu and Ca concentrations constant and varied the exposure duration, meaning we do not know the full spectrum of responses across mixture scenarios. However, at the concentrations used, the combined effect of Cu and Ca was different depending on the exposure duration. A 3 h coexposure to Ca and Cu yielded an additive effect on EOG response, while a 96 h coexposure to Ca and Cu showed a more than additive effect. The additive interaction between Cu and Ca at 3 h indicates that both Ca and Cu are exerting their effect on olfaction through independent mechanisms. The more than additive interaction between Ca and Cu after a 96 h exposure is most likely due to Ca interfering with the ability of the OSNs to recover from the Cu treatment. This means that not only does Ca fail to protect against Cu-induced olfactory dysfunction, but it also prevents recovery from Cu intoxication. These results seem to conflict with a study published by Bjerselius et al.,[32] which showed that the effect of short-term exposures (4 min) with high concentrations of Cu (10 μM) on EOG response was reduced by the addition of Ca (up to 4 mM). However, those authors also showed that adding enough magnesium (Mg) to the water to match the ionic strength found in the 4 mM Ca exposure resulted in the same protective effect. Therefore, this apparent protective effect is most likely due to a general protective mechanism involving the ionic strength of the exposure water, and not a specific protective effect of Ca. In fact, the conclusion of the authors was that the reduced effect of Cu was most likely explained due to a lower Cu^{2+} activity in these solutions due to an increased ionic strength of the solution.

That exposure to Ca alone causes olfactory dysfunction in fish has received little attention in the literature. McIntyre et al.[10] observed a small but significant negative relationship between EOG amplitude and Ca concentration (from $CaCl_2$) in juvenile coho salmon (*Oncorhynchus kisutch*). This Ca-induced reduction in EOG response could be occurring via a variety of mechanisms. As calcium is essential in numerous steps of olfactory signal transduction,[33] adding Ca may directly interfere with the olfactory response to the cue. In addition, there may be cross-adaptation between Ca and the amino acid stimulus used. Cross-adaptation occurs when one odorant activates OSNs that overlap with those activated by a second odorant, causing a decreased response to the second odorant.[34−36] Calcium has been shown to be an odorant in a variety of fish species including the gilthead seabream (*Sparus aurata*) and goldfish (*Carassius auratus*),[37,38] and may interfere with the response to L-arginine due to an overlap in receptors that recognize both odorants. The fact that Ca causes a reduction in response by itself and that it offers no protection from Cu-induced olfactory dysfunction suggests that any future cbBLM should consider Ca, not as a competing cation, but as a metal capable of inducing its own independent effects on fish olfaction.

It may be argued that the counterion of the Ca salt may explain some of the observed Ca-induced effects, including the apparent lack of protection against Cu intoxication of olfactory epithelium, the exacerbation of the Cu effect, or the induction of a unique olfactory response. Although the current study used $Ca(NO_3)_2$ as the Ca salt [i.e., the same salt used by Green et al.[11]], McIntyre et al.[10] found that $CaCl_2$ produced effects similar to those reported here. Bodznik (1978) reached a similar conclusion regarding the counteranions of sodium salts.[39]

Predictions from six different BLMs (two gbBLMs and four cbBLMs) were compared to experimentally derived results to answer two questions: first, are LC50s predicted by the current gbBLMs lower than experimentally derived IC50s (i.e., are they protective against Cu-induced olfactory dysfunction)?; and second, can a simple reparameterization of current gbBLMs make cbBLMs which predict IC50s lower than experimentally derived values? To answer the first question, the RBT-gbBLM and FHM-gbBLM were used with the water quality measures of the exposure waters, assuming either 10% or 50% HA content. When compared to the experimentally derived results, it is clear that the LC50s predicted by the current gbBLMs are higher than experimentally derived values. To answer the second question, four cbBLMs were made based on different reparameterizations of the gbBLMs. All cbBLMs predicted IC50 values in excess of the experimentally derived IC50s. This result indicates that a simple reparameterization of currently existing gbBLMs is not sufficient to produce a model that predicts IC50s below experimentally derived values for the olfactory epithelium. Taken together, there is currently no BLM, gill or chemosensory based, that is capable of predicting IC50s for Cu intoxication at or below experimentally derived values for the olfactory epithelium. This is not surprising as the olfactory epithelium and gills have very different functions, and as this study has demonstrated, the interactions between ions (Cu, Ca, etc.) at the olfactory epithelium and gills appear to be quite different. Much more work must be undertaken to

understand the interactions at the olfactory epithelium before a useful cbBLM can be constructed.

The USEPA BLM and hardness-adjustment equations were used to determine Cu concentrations which are considered "safe" from a regulatory perspective (i.e., the CMC and CCC). For the acute criterion (CMC), all concentrations predicted were higher than the experimental value, suggesting that the USEPA BLM and hardness-adjustment equations are not protective for acute Cu-induced olfactory dysfunction. For the chronic criterion (CCC), the hardness-adjustment equation predicted a value below the experimental 96 h IC20, and the USEPA BLM predictions bracketed this result. This means that the CCC as predicted by the hardness-adjustment equation was protective; however, the USEPA BLM may or may not be protective, depending on the %HA used in the calculations. Taken together, these results demonstrate that the current USEPA BLM and hardness-adjustment equations are not always protective against Cu-induced olfactory dysfunction.

In contrast to what was predicted in Figure 1, a comparison of the concentration response curves in Figure S1 clearly demonstrates that increasing the length of Cu exposure results in a decreased effect of Cu. A comparison of the IC50 value for 24 h (87 nM) with that for 96 h (213 nM) clearly shows this trend. At the concentrations used in this study, pulses of Cu into waterways may be more detrimental to olfaction in fish than increased background Cu levels.

In conclusion, this study shows that interaction of Cu and Ca at the olfactory epithelium is much more complex than expected. This work demonstrates that (i) fathead minnows are able to at least partially recover olfactory function during continuous exposure to waterborne Cu, (ii) Ca is not protective against Cu-induced olfactory dysfunction and has its own effect, (iii) current gbBLMs and simple reparameterizations to make cbBLMs do not predict realistic IC50 values for Cu-induced olfactory dysfunction, and (iv) current models do not produce CMC values which are protective against Cu effects at the olfactory epithelium. To construct a cbBLM that will be protective of Cu-induced olfactory dysfunction, the ability of OSNs to recover and the role of Ca (and other ions) at the olfactory epithelium must be further investigated.

■ ASSOCIATED CONTENT

Supporting Information

Water quality data (Table S1), measured Cu and Ca concentrations (Table S2) for the data found in Figure 3, measured and predicted combined effect on EOG response of Cu and Ca (Table S3), and the concentration response curves (Figure S1) used to derive the IC50 and IC20 values found in Tables 1 and 2. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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