

*Environmental Toxicology*

## EFFECTS OF WATER HARDNESS, ALKALINITY, AND DISSOLVED ORGANIC CARBON ON THE TOXICITY OF COPPER TO THE LATERAL LINE OF DEVELOPING FISH

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**Abstract**—Conventional water chemistry parameters such as hardness, alkalinity, and organic carbon are known to affect the acute lethality of copper to fish and other aquatic organisms. In the present study, we investigate the influence of these water chemistry parameters on short-term (3 h), sublethal (0–40 µg/L) copper toxicity to the peripheral mechanosensory system of larval zebrafish (*Danio rerio*) using an in vivo fluorescent marker of lateral line sensory neuron (hair cell) integrity. We studied the influence of hardness (via CaCl<sub>2</sub>, MgSO<sub>4</sub>, or both at a 2:1 molar ratio), sodium (via NaHCO<sub>3</sub> or NaCl), and organic carbon on copper-induced neurotoxicity to zebrafish lateral line neurons over a range of environmentally relevant water chemistries. For all water parameters but organic carbon, the reductions in copper toxicity, although statistically significant, were small. Increasing organic carbon across a range of environmentally relevant concentrations (0.1–4.3 mg/L) increased the EC<sub>50</sub> for copper toxicity (the effective concentration resulting in a 50% loss of hair cells) from approximately 12 µg/L to approximately 50 µg/L. Finally, we used an ionoregulatory-based biotic ligand model to compare copper toxicity mediated by targets in the fish gill and lateral line. Relative to copper toxicity via the gill, we find that individual water chemistry parameters are less influential in terms of reducing cytotoxic impacts to the mechanosensory system.

**Keywords**—Zebrafish    Hair cells    Mechanosensory    Bioavailability    Biotic ligand model

## INTRODUCTION

Metals such as copper are toxic to the peripheral sensory systems of fish and other aquatic organisms. Dissolved copper specifically impairs the normal function of olfactory and mechanosensory neurons by reducing their physiological responsiveness to environmental cues [1] and, at higher exposure concentrations, via cell death [2]. Sensory isolation in turn interferes with behaviors mediated by these senses, including predator detection and avoidance [3–7], social interaction [8], prey detection [9], and rheotaxis (orienting toward flow [10]). Copper and other so-called information disruptors [11] can therefore have important impacts on the survival, distribution, and reproductive success of fish.

In fish and other aquatic organisms, the acute lethality of copper is known to be mediated by the gill epithelium. The relative concentrations at which copper kills fish are influenced by various water chemistry parameters such as hardness, alkalinity, pH, and dissolved organic carbon [12]. Cations (e.g., Ca<sup>2+</sup> and Na<sup>+</sup>) reduce the bioavailability of metal ions to the binding site (biotic ligand) on the gill by competition, whereas anions (e.g., HCO<sub>3</sub><sup>-</sup>, CO<sub>3</sub><sup>2-</sup>, Cl<sup>-</sup>, and SO<sub>4</sub><sup>2-</sup>) and dissolved organic carbon (DOC) bind to the free metal ions to form inorganic and organic complexes, respectively. Because complexation reduces the availability of copper to the biotic ligand, the lethal effect of copper is reduced in waters enriched in anions and DOC [12]. The influence of water chemistry is commonly estimated using the biotic ligand model (BLM).

The BLM was developed to predict the acute toxicity of copper to freshwater aquatic organisms in waters with varying chemical compositions [13]. The BLM has been used more recently to derive site-specific water quality criteria that are intended to be protective of the most sensitive freshwater taxa (e.g., *Ceriodaphnia dubia* [14]).

Despite several important assumptions and limitations [15], the BLM has served as a useful predictor of copper toxicity mediated via the fish gill in waters of different chemistries [12]. However, for biotic ligands in fish tissues other than the gill, water chemistry may have less of an influence on copper toxicity. For example, copper-induced neurotoxicity to the peripheral olfactory epithelium of juvenile coho salmon (*Oncorhynchus kisutch*) was less influenced by changes in water hardness, alkalinity, and DOC than was predicted from an ionoregulatory-based rainbow trout (*Oncorhynchus mykiss*) BLM [16,17].

In the present study, we used larval zebrafish to investigate the effects of water chemistry on copper toxicity to the mechanosensory lateral line. In teleosts, the peripheral mechanosensory system is comprised of assemblages of receptor neurons (neuromasts) along the surface of the fish. Each neuromast contains a rosette of ciliated hair cells. These receptor cells are responsive to water displacement around the body of the animal. Mechanosensory information is transduced by hair cells and then propagated to the central nervous system. Zebrafish are a convenient experimental model with a diversity of established molecular markers that allow the direct visualization of the mechanosensory system in vivo. Also, the ontogeny and anatomy of the zebrafish lateral line have been described in considerable detail [18,19],

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Table 1. Test water characteristics and toxic effect concentrations (EC50; concentration at which copper caused a 50% loss of hair cells): All physiochemical water quality values are nominal except pH and organic carbon. All water parameter characteristics of the test waters were measured (except those in italics) and were within 20% of the nominal value. Underline indicates which chemical properties of the test media were manipulated. SE = standard error of the nonlinear regression; x = values not measured

Test water	Cations (mM)				Anions (mM)				Carbon (mg/L)	Hard- ness (mg/L)	Alka- linity (mg/L)	pH	EC50 ± SE <sup>a</sup>	
	Ca <sup>2+</sup>	Mg <sup>2+</sup>	Na <sup>+</sup>	K <sup>+</sup>	HCO <sub>3</sub> <sup>-</sup>	CO <sub>3</sub> <sup>2-</sup>	SO <sub>4</sub> <sup>2-</sup>	Cl <sup>-</sup>					(μg/L)	r <sup>2</sup>
Embryo medium	0.3	0.15	0.3	0.13	0.15	0	0.15	1.0	0.1 ± 0.01	45	9.2	7.46	11.5 ± 0.5	0.992
CaCl <sub>2</sub> -1	<u>0.75</u>	0.15	0.3	0.13	0.15	0	0.15	<u>1.9</u>	x	<u>90</u>	9.2	7.43	11.9 ± 0.5	0.998
CaCl <sub>2</sub> -2	<u>1.65</u>	0.15	0.3	0.13	0.15	0	0.15	<u>3.7</u>	x	<u>180</u>	9.2	7.37	13.6 ± 0.6	0.998
CaCl <sub>2</sub> -3	<u>3.05</u>	0.15	0.3	0.13	0.15	0	0.15	<u>6.5</u>	x	<u>320</u>	9.2	7.32	17.4 ± 2.0	0.984
MgSO <sub>4</sub> -1	0.3	<u>0.6</u>	0.3	0.13	0.15	0	<u>0.6</u>	1.0	x	<u>90</u>	9.2	7.37	13.2 ± 0.3	0.999
MgSO <sub>4</sub> -2	0.3	<u>1.5</u>	0.3	0.13	0.15	0	<u>1.5</u>	1.0	x	<u>180</u>	9.2	7.37	16.6 ± 1.2	0.993
MgSO <sub>4</sub> -3	0.3	<u>2.9</u>	0.3	0.13	0.15	0	<u>2.9</u>	1.0	x	<u>320</u>	9.2	7.34	19.5 ± 2.4	0.975
CaCl <sub>2</sub> :MgSO <sub>4</sub> -1	<u>0.6</u>	<u>0.3</u>	0.3	0.13	0.15	0	<u>0.3</u>	<u>1.6</u>	x	<u>90</u>	9.2	7.45	11.9 ± 0.1	0.998
CaCl <sub>2</sub> :MgSO <sub>4</sub> -2	<u>1.2</u>	<u>0.6</u>	0.3	0.13	0.15	0	<u>0.6</u>	<u>2.8</u>	x	<u>180</u>	9.2	7.43	14.3 ± 0.6	0.996
CaCl <sub>2</sub> :MgSO <sub>4</sub> -3	<u>2.1</u>	<u>1.1</u>	0.3	0.13	0.15	0	<u>1.1</u>	<u>4.6</u>	x	<u>320</u>	9.2	7.42	17.1 ± 1.6	0.985
NaHCO <sub>3</sub> -1	0.3	0.15	<u>1.6</u>	0.13	<u>1.5</u>	0	0.15	1.0	x	45	<u>75</u>	<u>8.23</u>	12.1 ± 0.4	0.999
NaHCO <sub>3</sub> -2	0.3	0.15	<u>3.1</u>	0.13	<u>3.0</u>	0	0.15	1.0	x	45	<u>150</u>	<u>8.40</u>	12.7 ± 0.2	1.000
NaHCO <sub>3</sub> -3	0.3	0.15	<u>6.1</u>	0.13	<u>6.0</u>	0	0.15	1.0	x	45	<u>300</u>	<u>8.47</u>	17.7 ± 3.0	0.966
NaCl-1	0.3	0.15	<u>1.6</u>	0.13	0.15	0	0.15	<u>2.3</u>	x	45	9.2	<u>7.59</u>	12.5 ± 0.3	0.999
NaCl-2	0.3	0.15	<u>3.1</u>	0.13	0.15	0	0.15	<u>3.8</u>	x	45	9.2	<u>7.62</u>	15.3 ± 0.5	0.999
NaCl-3	0.3	0.15	<u>6.1</u>	0.13	0.15	0	0.15	<u>6.8</u>	x	45	9.2	<u>7.59</u>	17.7 ± 0.9	0.996
NOM-1 <sup>b</sup>	<i>0.3</i>	<i>0.15</i>	<i>0.3</i>	<i>0.13</i>	<i>0.15</i>	<i>0</i>	<i>0.15</i>	<i>1.0</i>	<u>1.1 ± 0.05</u>	45	9.2	<u>7.29</u>	21.7 ± 0.8	0.996
NOM-2 <sup>b</sup>	<i>0.3</i>	<i>0.15</i>	<i>0.3</i>	<i>0.13</i>	<i>0.15</i>	<i>0</i>	<i>0.15</i>	<i>1.0</i>	<u>2.4 ± 0.23</u>	45	9.2	<u>7.20</u>	38.8 ± 2.4	0.998
NOM-3 <sup>b</sup>	<i>0.3</i>	<i>0.15</i>	<i>0.3</i>	<i>0.13</i>	<i>0.15</i>	<i>0</i>	<i>0.15</i>	<i>1.0</i>	<u>4.3 ± 0.04</u>	45	9.2	<u>6.97</u>	50.3 ± 8.8	0.941
System water <sup>c</sup>	<i>0.27</i>	<i>1.5</i>	<i>12.9</i>	<i>0.29</i>	<i>0.09</i>	<i>0</i>	<i>0.8</i>	<i>14.9</i>	x	177	4.5	<u>7.44</u>	28.3 ± 1.8	0.985

<sup>a</sup> Calculated from nonlinear regression of the dose–response relationship with dissolved copper (degrees of freedom = 3).

<sup>b</sup> Natural organic matter.

<sup>c</sup> Based on previous data [2]. System water was distilled water amended with Instant Ocean Salt. Values of water constituents based on the Typical Ion Composition of Instant Ocean Salt, which was provided by United Pet Group (personal communication).

and the neurotoxic effect of copper on zebrafish mechanosensory receptors has been previously described [2,20,21]. In the present study, using conventional *in vivo* methods for measuring hair cell death [2], we determined how environmentally relevant variations in water hardness, alkalinity, sodium, and DOC influence copper-induced neurotoxicity in the lateral line of larval zebrafish. These empirical results were then combined with empirical data from the olfactory system [16,17] and modeled data from the fathead minnow (*Pimephales promelas*) BLM to compare the relative influence of different water chemistry parameters on acute toxicity to the fish lateral line system, olfactory system, and gill.

## MATERIALS AND METHODS

### Animals

Zebrafish were obtained from our breeding colony maintained at the Northwest Fisheries Science Center (Seattle, WA, USA). Adult wild-type (AB strain) zebrafish were allowed to spawn, and the embryos were collected and sorted into 100-mm plastic petri dishes (Falcon®, VWR) containing embryo medium or modified embryo medium (artificially constituted test waters with different chemical properties). Embryo medium consisted of distilled water amended with CaCl<sub>2</sub>·2H<sub>2</sub>O, MgSO<sub>4</sub>·7H<sub>2</sub>O, NaCl, NaHCO<sub>3</sub>, and KCl at the concentrations in Table 1 (all chemicals minimum 99.0% purity; Sigma). Embryo medium or test water was renewed every 24 h. Embryos and larvae were incubated at 28.5°C in the appropriate test water (except for DOC manipulations, wherein all fish were in embryo medium) and raised until 4 d postfertilization (dpf).

### Test waters

To determine the effects of water chemistry parameters on copper-induced hair cell death, different salts or organic matter

were added to the embryo medium to alter hardness, alkalinity, and DOC across a range of environmentally relevant surface water concentrations as determined from the U.S. Geological Survey's National Water Information System Web Site (NWIS-Web; <http://nwis.waterdata.usgs.gov/nwis>). The six different water chemistry formulations (Table 1) varied hardness (via CaCl<sub>2</sub> alone, MgSO<sub>4</sub> alone, or both at a 2:1 molar ratio), alkalinity (via NaHCO<sub>3</sub>), sodium (via NaCl), and DOC (via natural organic matter [NOM]). Test waters were prepared by adding stock solutions made from CaCl<sub>2</sub>·2H<sub>2</sub>O, MgSO<sub>4</sub>·7H<sub>2</sub>O, NaCl, NaHCO<sub>3</sub> (Sigma), and NOM (52.5% carbon, Suwannee River, International Humic Substances Society; <http://www.ihss.gatech.edu/>) to embryo medium. The test media were stored at 28.5°C.

For the DOC test, a NOM stock solution was prepared 24 h before the copper exposure and allowed to dissolve at room temperature. The day of the copper exposure, the NOM test waters were then diluted to the final concentrations (0, 2.5, 5, and 10 mg/L NOM) to which the animals were exposed. Each test water was then spiked with the copper stock solution to achieve the appropriate metal concentration (see below). In a separate experiment to test whether toxicity is reduced by copper complexation to NOM (a process which requires time [22]), a NOM stock solution was prepared 48 h before exposure. Copper was then added to the diluted NOM test water (~1.1 mg carbon/L) 24 h prior to exposure and allowed to equilibrate overnight at room temperature.

### Copper exposures

For each test water formulation, larvae were exposed to five copper concentrations (0, 5, 10, 20, and 40 μg/L) diluted from a copper stock solution using CuCl<sub>2</sub>·2H<sub>2</sub>O (Sigma; min-

imum 99.0% purity). Copper stock solutions were prepared 24 h before the exposure and stored at 4°C.

Larvae at 4 dpf were separated into plastic six-well plates (Costar®, Fisher Scientific). Fifteen larvae were added to each well containing 6 ml of the water treatment. Triplicates (3 wells of 15 larvae each) were performed for each water treatment and copper concentration. For experiments involving the different water formulations, the 6 ml of water treatment was renewed for each well before the copper exposure. Copper (from stock) was then added to each well to achieve the appropriate concentration. In the equilibrated NOM and copper experiment, the embryo medium was exchanged with 6 ml of the appropriate premixed solution. The six-well plates containing zebrafish larvae in test water amended with dissolved copper were incubated at 28.5°C for 3 h. Hair cell death within the zebrafish neuromast has previously been shown to be complete by 3 h [2].

#### Determination of hair cell neurotoxicity

To visualize the individual mechanosensory neurons *in vivo*, larvae were immersed in 0.05% 4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide (DASPEI; Sigma) for 8 min, rinsed in embryo medium, and anesthetized with tricaine methane sulfonate (ethyl 3-aminobenzoate methanesulfonate [MS-222]; 250 µg/ml; Sigma) for 2 min. The DASPEI staining was the basis for quantifying the number of hair cells per neuromast. Larvae were then mounted on depression slides in embryo medium, and hair cells were counted using a Nikon Eclipse E600 compound microscope (Meridian Instruments) fitted with a mercury lamp and a fluorescence filter (excitation 460–500 nm). Because a previous study [2] found that the toxicity of dissolved copper is similar across different zebrafish neuromasts, the number of hair cells in a single representative neuromast (O2, located on the otic vesicle [19]) was quantified. Hair cell counts were obtained from one of the bilaterally symmetrical O2 neuromasts for each of 10 randomly selected larvae per well to give a total of three replicates of 10 larvae per water treatment and copper concentration.

#### Water chemistry analysis

Dissolved copper concentrations were measured for all stock solutions for each water parameter test. Also, for one exposure concentration (40 µg/L), water samples were collected both before and after the exposure (a composite from 18 wells containing 15 larvae per well after 3 h at 28.5°C). Copper solutions were analyzed by inductively coupled plasma mass spectrometry by a U.S. Environmental Protection Agency (EPA)-certified laboratory (Frontier GeoSciences). The pH values of embryo media and test waters were measured using a pH meter (Accumet® basic AB15; Fisher Scientific). The pH glass electrode was calibrated daily with pH 4, pH 7, and pH 10 buffers (VWR). Analysis of ion concentrations, hardness, and alkalinity was performed for each artificially formulated test water with the exception of embryo medium containing DOC. Samples were analyzed by a U.S. EPA-certified analytical laboratory (AmTest Laboratories) using standard methods. Total organic carbon was measured in triplicate for each of the DOC test waters using a Shimadzu TOC Vcsh analyzer (University of Washington Oceanography Technical Services).

#### Data analyses

For each combination of water test medium and copper concentration, the means of the three replicates were used for anal-

ysis. With these means, the dose–response relationship between copper and DASPEI hair cell staining was calculated by the same nonlinear regression as previously described [2]:

$$y = m/[1 + (x/k)^n]$$

where  $y$  is the number of hair cells per neuromast of a copper-exposed fish,  $m$  is the mean number of hair cells per neuromast of an unexposed fish,  $x$  is the copper concentration,  $k$  is the EC50 (effective concentration resulting in 50% loss of hair cells), and  $n$  is the slope. The dose–response relationships were calculated using Kaleidagraph (Synergy Software). For comparison of the EC50s of dose–response relationships, an  $F$  test was performed using Prism (GraphPad Software).

The relationship between each water chemistry parameter (e.g., hardness as CaCl<sub>2</sub>) and copper toxicity was analyzed by linear regression for the lateral line EC50s and the BLM-predicted LC50s (concentration resulting in 50% mortality of the test animals; see below) using Prism. Prism was used to run  $F$  tests to compare the slopes and intercepts of the linear regressions.

#### Biotic ligand modeling

The copper BLM (version 2.2.3, HydroQual; <http://www.hydroqual.com/wr.blm.html>) was used to compare the relative influence of water chemistry on copper toxicity predicted by the BLM (LC50s) and the observed toxicity to the zebrafish lateral line sensory neuron loss (EC50). The water quality parameters used in the present study (Table 1) and 10% humic acid and 0.01 µM sulfide served as default input values. The fathead minnow BLM was selected for estimating LC50s because it is a warm-water-tolerant, freshwater species for which the BLM has been extensively refined and validated in previous studies (e.g., [12,23–25]).

## RESULTS

The measured compositions of the different artificial freshwaters were within 20% of the nominal or target values (Table 1). Similarly, all measured copper concentrations were within 20% of the nominal values (data not shown). The measured copper concentration of the 40 µg/L copper solution in embryo medium was reduced 44% after the 3-h exposure (from 33.2 to 14.7 µg/L). This loss during the exposure interval was likely due to uptake by the zebrafish larvae, adherence to the plastic wells, or both. Thus, the calculated copper toxicities (EC50s), which in the present study were based on initial nominal copper concentrations, may underestimate actual copper toxicity. Because hair cell death happens within 30 min of exposure [2] and the exposure time frame in the present study was 3 h, cytotoxicity likely occurred at a copper concentration between nominal and the 44% copper loss. The measured values for DOC equaled approximately 40% of the nominal concentrations for NOM. This reflects the fact that NOM is not entirely composed of DOC.

#### Influence of major ions on copper neurotoxicity

The effect of hardness on copper toxicity to the zebrafish peripheral mechanosensory system (EC50) was determined by altering the concentrations of CaCl<sub>2</sub>, MgSO<sub>4</sub>, and both salts at a ratio of 2:1 (molar). When hardness was increased from soft water (45 mg/L as CaCO<sub>3</sub>) to hard water (320 mg/L as CaCO<sub>3</sub>), EC50s increased approximately 50% across the hardness range tested, regardless of which cation was varied (Table 1). The relationship between water hardness (expressed as total Ca<sup>2+</sup> and Mg<sup>2+</sup> in

millimolar in Fig. 1) and copper toxicity (Table 2) was not significantly different among cations or ratios ( $F_{2,6} = 2.92$ ,  $p = 0.13$ ; pooled slope = 2.48, pooled intercept = 10.1).

To assess the effects of increased alkalinity and  $\text{Na}^+$  ions, copper toxicity in test waters containing four different concentrations of  $\text{NaHCO}_3$  and  $\text{NaCl}$  was compared. Toxicity decreased when either sodium salt was added, with EC50s increasing approximately 55% across the range of sodium concentrations tested (Table 1). The effects of  $\text{NaHCO}_3$  and  $\text{NaCl}$  were similar, and relationships between salt concentration and copper toxicity for the two salts (Table 2) were not significantly different ( $F_{1,4} = 0.028$ ,  $p = 0.88$ ; pooled slope = 1.09, pooled intercept = 10.9). This indicates that carbonate complexation and the subsequent change in pH caused by the addition of  $\text{NaHCO}_3$  had no significant effect on copper toxicity to the lateral line.

#### *Organic carbon reduces copper toxicity to the lateral line*

Varying concentrations of NOM were used to evaluate the relationship between organic carbon and copper toxicity to the mechanosensory system. Addition of DOC to test waters significantly decreased copper toxicity to the mechanosensory system. Specifically, the EC50s for hair cell death increased by an average of 437% across the DOC range tested (Table 1). Notably, the two highest concentrations of NOM fell within the lower half of the dose–response curve for the range of copper concentrations tested in the present study. Although the values for these higher NOM concentrations were sufficient for the purposes of calculating an EC50, the smaller data sets produced standard errors that were correspondingly higher (Table 1). The reduction in copper neurotoxicity by DOC was greater than that for hardness, sodium, or alkalinity across the ranges tested (Fig. 1). Although the molecular weight of the NOM used in the present study is unknown, on a unit-equivalent basis (mM), the effect (i.e., slope) of DOC on copper toxicity would be two or more orders of magnitude greater than those for the cations (Table 2) due to the typically large molecular weights of DOC compounds [26].

#### *Premixing copper and organic matter does not reduce toxicity*

To determine the effect of allowing copper and organic matter to complex for 24 h prior to exposure, DASPEI labeling of hair cells was compared between animals exposed to copper mixed with NOM solutions for 24 h (equilibrated) and copper added to NOM solutions at the start of the exposure (spiked). Although allowing copper to equilibrate with NOM resulted in a slight shift of the dose–response curve to higher copper concentrations (Fig. 2), the resulting change in the EC50 (from  $21.7 \pm 0.8 \mu\text{g/L}$  for spiked to  $26.6 \pm 1.0 \mu\text{g/L}$  for equilibrated) was not significant ( $F_{1,4} = 5.21$ ,  $p = 0.085$ ).

#### *A gill-based BLM does not predict lateral line toxicity*

The BLM is conventionally used to predict the acute, gill-mediated lethality of copper as a function of water chemistry [12]. To assess whether these predictions also extend to the fish lateral line, we compared the slopes of the effective concentration (hair cell loss; EC50) to the BLM-predicted lethal concentration (LC50) for each water chemistry parameter. In all cases, the BLM predicted that changes in water chemistry would have greater influence at the fish gill than was experimentally determined for the lateral line system in the present study (Fig. 1). Slopes for the relationships between water quality parameter concentration and copper toxicity were signifi-

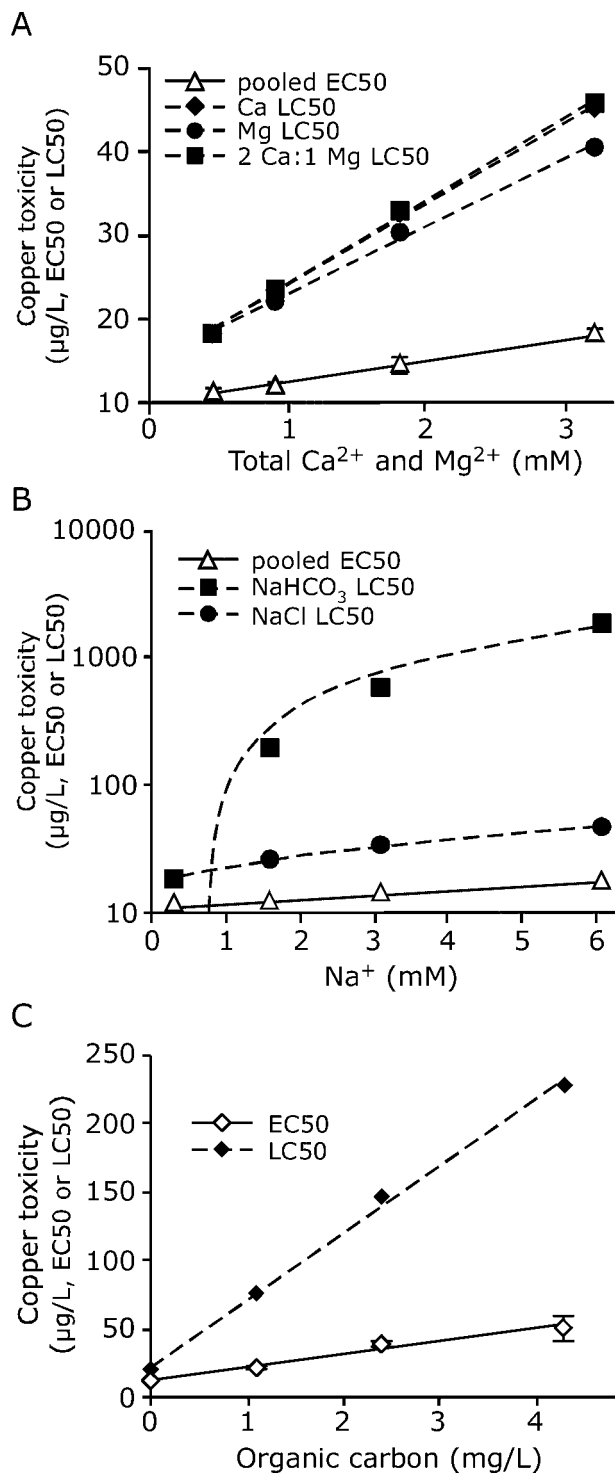


Fig. 1. Water quality parameters have less influence on copper toxicity to mechanosensory receptors (EC50, effective concentration that causes 50% loss of hair cells; open symbols and solid lines) than on the biotic ligand model (BLM)-predicted lethality (LC50, lethal concentration of 50% of test animals; solid symbols and dashed lines) for the water quality conditions of (A) hardness (as combined  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ), (B) sodium, and (C) dissolved organic carbon in Table 1. For hardness and for sodium, EC50s are pooled because there is no difference in the slope of their relationships (see Results section). Open symbols are estimates of EC50s  $\pm$  standard error based on nonlinear regressions. Note: the y axis of panel B is on a logarithmic scale.

Table 2. Linear regression relationships between concentrations of various water parameters and measures of copper toxicity for mechanosensation (EC50 = copper concentration resulting in a 50% loss of hair cells of the zebrafish lateral line), olfaction (IC50 = copper concentration resulting in a 50% inhibition of electrophysiological olfactory response in the coho salmon [16,17]), and mortality (LC50 = copper concentration resulting in 50% mortality of fathead minnow using the biotic ligand model [BLM])

Water parameter (units)	Lateral line EC50 ( $\mu\text{g Cu/L}$ )			Olfactory IC50 ( $\mu\text{g Cu/L}$ )	BLM LC50 ( $\mu\text{g Cu/L}$ )
	Slope (95% CI) <sup>a</sup>	Intercept	$r^2$	Slope (95% CI) <sup>a</sup>	Slope (95% CI) <sup>a</sup>
CaCl <sub>2</sub> (mM)	2.31 (1.41–3.21)	9.72	0.984	5.52 (2.74–8.31)	9.7 (8.4–11.0)
MgSO <sub>4</sub> (mM)	3.12 (0.990–5.26)	10.0	0.952		8.1 (6.7–9.5)
CaCl <sub>2</sub> :MgSO <sub>4</sub> (mM)	2.02 (1.07–2.96)	10.6	0.977		9.9 (8.6–11.3)
NaHCO <sub>3</sub> (mM)	1.06 (–0.039–2.17)	10.6	0.896	2.54 (1.53–3.55)	318 (123–515)
NaCl (mM)	1.11 (0.54–1.68)	11.1	0.972		4.8 (3.7–6.0)
DOC <sup>c</sup> (mg/L)	9.12 (4.71–13.5)	12.9	0.975	9.91 (6.48–13.35)	48.5 (38.9–58.1)

<sup>a</sup> 95% confidence interval of the slope.

<sup>b</sup> All slopes were significantly different from zero ( $p < 0.05$ ), except for NaHCO<sub>3</sub> where  $p = 0.053$ .

<sup>c</sup> Dissolved organic carbon.

cantly greater for LC50s than those for EC50s (above the 95% confidence interval for EC50s). Specifically, they were greater by more than a factor of three for CaCl<sub>2</sub>, MgSO<sub>4</sub>, and NaCl, more than 300 times for NaHCO<sub>3</sub>, and five times higher for DOC (Table 2).

The relative differences between the predicted and observed slopes demonstrate that the influences of water quality parameters on copper toxicity via the gill and the lateral line were dissimilar. The most notable difference between the BLM-predicted toxicity to the fish gill and the observed toxicity to the fish mechanosensory system was for the effect of the two sodium salts. In addition to the change in pH, NaHCO<sub>3</sub> was predicted to reduce copper toxicity more than 10-fold over the effect of NaCl at the fish gill. By contrast, NaHCO<sub>3</sub> and NaCl had the same influence on the toxicity of copper to the mechanosensory system (Fig. 1B), irrespective of the increase in pH (Table 1).

## DISCUSSION

Our findings indicate that water chemistry parameters can influence copper toxicity to the peripheral mechanosensory system of larval fish. Increasing hardness ions, sodium ions, and DOC all resulted in some reduction of copper neurotoxicity to individual sensory neurons in zebrafish lateral line

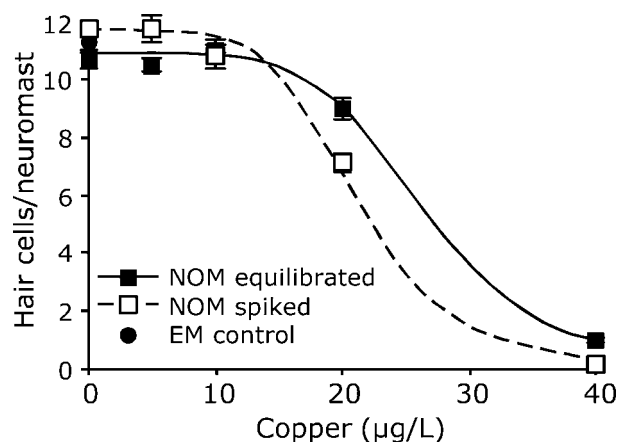


Fig. 2. Copper equilibrated with natural organic matter (NOM;  $\sim 1.1$  mg carbon/L) for 24 h before exposure to zebrafish (equilibrated, solid squares with solid line) is not less toxic than copper added simultaneously to the organic matter and zebrafish (spiked, open squares with dashed line). The number of hair cells per neuromast in embryo medium (EM; solid circle) is present as a control. Symbols are mean of  $n = 3$  replicates of 10 embryos  $\pm$  standard error of the mean.

neuromasts. Changes in the divalent cations Ca<sup>2+</sup> and Mg<sup>2+</sup> over an environmentally relevant hardness range slightly decreased copper toxicity. For example, an increase in hardness of 1 mM or 100 mg/L (as CaCO<sub>3</sub>) decreased toxicity (as indicated by an increase in the EC50) by approximately 2.5  $\mu\text{g/L}$  (Fig. 1A). The contributions of each ion were similar for the different Ca<sup>2+</sup>/Mg<sup>2+</sup> ratios examined. Alkaline waters also slightly reduced copper neurotoxicity. For example, an increase in NaHCO<sub>3</sub> of 5 mM (alkalinity change of  $\sim 250$  mg/L as CaCO<sub>3</sub>) decreased toxicity by approximately 5.5  $\mu\text{g/L}$ . However, because the influences of NaCl and NaHCO<sub>3</sub> were approximately equal, the effect of alkalinity can be attributed to the sodium content of the artificial freshwaters and not to an increase in bicarbonate ions or to a shift in pH. An example of how Na<sup>+</sup> influences copper toxicity in the zebrafish lateral line is evident when comparing the present results with a previous study [2]. Specifically, the higher EC50 for copper toxicity to lateral line neurons that was reported earlier [2] is attributable to the relatively high concentration of Na<sup>+</sup> in the water used in that study (Table 1; see also the formula used in Table 2). Overall, our present findings indicate that the most effective parameter for reducing copper toxicity to lateral line neurons was DOC. This effect of DOC presumably reflects complexation and reduced copper bioavailability.

Although most of the water chemistry parameters had a limited effect on the copper-induced degeneration of mechanosensory neurons, hair cells have the ability to regenerate when zebrafish larvae are restored to clean water [2]. Zebrafish sensory neurons regenerate over the course of approximately 48 h. The extent to which different water chemistries influence the time course and extent of hair cell regeneration remain to be investigated.

The findings in the present study for the peripheral mechanosensory system are similar to the results of previous studies focused on the peripheral olfactory system of fish. Investigations involving Atlantic salmon (*Salmo salar*) and coho salmon (*O. kisutch*) have found only a limited influence of hardness [16,17,27,28] or alkalinity [16,17,29] on copper-induced neurotoxicity to olfactory receptor neurons. In studies involving the salmon olfactory system, copper neurotoxicity to sensory neurons has generally been measured as a reduction in physiological responsiveness to olfactory stimuli (odorants). The influence of water chemistry on copper toxicity by changes in calcium hardness, bicarbonate alkalinity, and DOC was similar for both the coho olfactory system [16,17] and the zebrafish lateral line system (the present study). This is indicated by overlapping confidence

intervals (95%) for the slope coefficients (Table 2). Therefore, in terms of water chemistry and copper toxicity, the two fish sensory systems appear to be similar to each other and distinct from the gill as a separate target organ.

Given the similar form (ciliated sensory neurons), function (signal transduction), and locations of mechanosensory and olfactory receptors (in direct contact with copper dissolved in water), it is perhaps not surprising that the different chemical properties of water produced similar outcomes for the two sensory systems. The sensory receptors of lateral line neuro-masts and the olfactory epithelium are comprised of ciliated neurons embedded in a mucous membrane directly exposed to ambient water. These receptors transduce mechanical and chemical stimuli, respectively, into electrical information relayed to sensory processing centers in the fish brain. Similarities in receptor form and function could also explain why both sensory systems appear to diverge substantially from toxicity predictions based on gill epithelial cells that are very different from sensory receptors in terms of both architecture and physiology [30,31].

Although an ionoregulatory BLM may be useful for predicting the influence of site-specific water quality conditions on the acutely lethal effects of copper to fish, the accuracies of these predictions are diminished when this form of the BLM is applied to the fish peripheral mechanosensory system. The cations  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Na}^+$ , in conjunction with the anions  $\text{Cl}^-$  or  $\text{SO}_4^{2-}$ , do not appear to compete with copper as effectively for the as-yet-unidentified biotic ligand(s) in the lateral line compared to the ligands of the fish gill. Also, the BLM assumption that inorganic or organic constituents of surface waters render dissolved copper unavailable to the biotic ligand did not hold for the mechanosensory system. Results from the present study also show that the inorganic ligand  $\text{HCO}_3^-$ , along with an increase in pH, did not reduce the bioavailability of copper to the lateral line beyond that of its associated cation ( $\text{Na}^+$ ), in contrast to the predicted effect of  $\text{HCO}_3^-$  and pH at the gill. This suggests that copper may be available and toxic to zebrafish lateral line neurons in inorganic complexes (e.g., Cu-carbonate) that are biologically unavailable to the gill. Also, although DOC did reduce the toxicity of copper to mechanoreceptors, this was less of a reduction than that predicted by an ionoregulatory BLM. Interestingly, the BLM assumes that copper has reached equilibrium in the exposure water. Although toxicity via the fish gill is reduced when copper has reached equilibrium with DOC [22], this was not the case for the lateral line. Copper toxicity to the zebrafish peripheral mechanosensory system was not significantly decreased when copper was allowed to equilibrate with DOC for 24 h. Hence, the lateral line may be vulnerable to some Cu-DOC complexes, as has been shown in some studies for the fish gill [32,33]. Alternatively, the binding affinity of the biotic ligand for copper in the mechanosensory system may be greater than that of the biotic ligand for copper at the fish gill. For example, the biotic ligand(s) associated with mechanosensory receptor neurons may be binding dissolved copper that remains bound to carbonates and DOC at the gill.

There is an important distinction between a BLM that is predictive of sublethal, sensory-dependent behavioral effects in fish and BLM-derived water quality criteria for copper that may be protective for neurobehavioral toxicity. As we have shown here, the gill-based BLM does not accurately predict copper toxicity to fish sensory systems, particularly under conditions of varying alkalinity. It may be possible to improve

predictive accuracy with a modified BLM specifically parameterized for sensory toxicity (see below). This will require an improved biological understanding of copper binding affinities in sensory tissues, among other parameters. It should be noted, however, that the intended use of the BLM as a regulatory tool [14] may result in site-specific criteria that generally protect against toxicity to the olfactory and lateral line systems of fish. This is because the criteria mode of the BLM considers toxicity data for the most sensitive aquatic species (e.g., *C. dubia*). Relative to fish (e.g., fathead minnows), the parameter for critical copper accumulation (LA50) is much lower for *C. dubia*. The net effect is that the BLM-derived criteria are below our calculated EC50s for copper toxicity to the lateral line in waters with different chemical compositions. A corresponding criterion maximum concentration (CMC) can be calculated for each of the different water chemistries shown in Table 1 (data not shown). In all cases, the resulting CMC is below the respective EC50 for copper-induced toxicity to the zebrafish lateral line. For example, the exposure water with a DOC of 2.4 mg/L (NOM-2 in Table 1) has a BLM-derived CMC of 6.05  $\mu\text{g/L}$  copper, which is below the EC50 of 38.8  $\mu\text{g/L}$  for hair cell death. This comparison is simplistic because an EC50 for sensory neuron cell death is likely well above the threshold for adverse impacts on fish behavior. Nevertheless, the example illustrates how the inclusion of sensitive taxa in the BLM-derived CMC produces criteria that are below the EC50s calculated in the present study.

The basic structure and function of the lateral line are highly conserved across teleosts [34]. Therefore, the zebrafish can serve as a useful model to investigate the effects of physiochemical water properties on dissolved copper toxicity to the lateral line of native fish species. Additionally, by providing important sensory input regarding environmental cues, such as the presence of predators, the mechanosensory system underlies behavioral responses, such as predator avoidance, that are critical to the survival of fish [35]. Not surprisingly, studies have found that impairing the mechanosensory system of fish can lead to reductions in important behaviors, such as startle and orienting responses [6,9,21]. Therefore, the mechanosensory system is an important endpoint to consider when assessing the potential impacts of copper on fishes. Moreover, the simple fluorescent imaging procedures used in the present study should be useful in terms of screening a wide range of dissolved toxicants for lateral line neurotoxicity in fish. Extending our current findings to the mechanosensory-mediated behaviors of wild fish species remains an important area of future research.

In summary, we have shown that the fish mechanosensory system is sensitive to dissolved copper at low exposure concentrations (e.g., EC50s of 11–20  $\mu\text{g/L}$ ) that last for only a short duration (3 h). Copper toxicity to the fish lateral line is a particularly important consideration in urban and urbanizing areas, where storm events (<12 h) transport pulses of dissolved copper into aquatic habitats at concentrations that span or exceed the copper levels used in the present study (e.g., 3.4–64.5  $\mu\text{g/L}$  in northern California, USA [36]). Overall, our results indicate that environmentally relevant levels of dissolved copper from stormwater discharges and other inputs to fish habitats have the potential to impair critical behaviors that depend on a properly functioning lateral line. Although copper toxicity to the lateral line may be ameliorated by DOC (and to a much lesser extent hardness and alkalinity), this reduction will be less than that predicted from an ionoregulatory BLM parameterized for ligands in the fish gill. In the future, it should

be possible to use the empirical data generated in the present study, as well as recent data for the salmon olfactory system [16,17], as a basis to develop a set of BLM parameters that are more representative of sublethal sensory endpoints in fish. This holds promise for a model with greater predictive accuracy than the current gill-based BLM.

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