

DISSOLVED COPPER TRIGGERS CELL DEATH IN THE PERIPHERAL MECHANOSENSORY SYSTEM OF LARVAL FISH

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Abstract—Dissolved copper is an increasingly common non-point source contaminant in urban and urbanizing watersheds. In the present study, we investigated the sublethal effects of dissolved copper on the peripheral mechanosensory system, or lateral line, of larval zebrafish (*Danio rerio*). Zebrafish larvae were exposed to copper (0–65 µg/L), and the cytotoxic responses of individual lateral line receptor neurons were examined using a combination of in vivo fluorescence imaging, confocal microscopy, scanning electron microscopy, and conventional histology. Dissolved copper triggered a dose-dependent loss of neurons in identified lateral line neuromasts at concentrations ≥ 20 µg/L. The onset of cell death in the larval mechanosensory system was rapid (<1 h). When copper-exposed zebrafish were transferred to clean water, the lateral line regenerated over the course of 2 d. In contrast, the lateral line of larvae exposed continuously to dissolved copper (50 µg/L) for 3 d did not recover. Collectively, these results show that peripheral mechanosensory neurons are vulnerable to the neurotoxic effects of copper. Consequently, dissolved copper in non-point source storm-water runoff has the potential to interfere with rheotaxis, schooling, predator avoidance, and other mechanosensory-mediated behaviors that are important for the migration and survival of fish.

Keywords—Lateral line Behavior Storm water Neurotoxicity Non-point source

INTRODUCTION

Non-point source pollution is a growing threat to freshwater and marine species, particularly in coastal areas where human population growth and development are rapidly increasing [1]; (<http://www.pewtrusts.org/pubs/>), [2]; (<http://www.oceancommission.gov/documents/>). For example, dissolved copper is a widely distributed non-point source contaminant in lakes, rivers, and coastal marine environments. This reflects the use of copper in vehicle brake pads, building materials, pesticide formulations, and other applications. As a consequence of vehicle emissions [3], dissolved copper is particularly common in storm-water runoff from roads and other impervious surfaces. In urban and urbanizing watersheds, the loading of copper to aquatic habitats is highly variable and depends, in part, on precipitation patterns [4], traffic density [3], and the site-specific hydrological characteristics of different watersheds [5]. In northern California, for example, storm-water runoff has recently been shown to contain dissolved copper at levels that vary from 3.4 to 64.5 µg/L, with a mean of 15.8 µg/L [5].

The acute and chronic toxicity of copper to fish are well known [6]. Acute lethality (96-h median lethal concentration) can often be predicted using the biotic ligand model [7,8], which describes the uptake of copper across the surface of the gill epithelium as a function of water hardness and other physiochemical parameters. The impacts of short-term pulses (<12 h) of dissolved copper at sublethal concentrations that are representative of non-point source storm-water runoff are not as well understood. This is particularly true for pathways of copper toxicity that may be independent of the gill epithelium.

The neurotoxic effect of copper on the olfactory system of

salmonids [9,10] and other fish species [11] is a well-documented example of copper acting directly on the peripheral nervous system, presumably via a mechanism that is independent of ligand binding in the gill. Olfactory receptor neurons bind natural odorant molecules that are dissolved in surface waters. Accordingly, these peripheral sensory neurons are in direct contact with the surrounding environment, including pollutants such as copper. Salmonids will actively avoid dissolved copper in aquatic environments [10]. However, behavioral avoidance may not be possible in the absence of a defined spatial gradient. This might include, for example, surface water contamination via non-point source runoff.

Short-term exposures to copper (4 h; ≥ 25 µg/L) have been shown to trigger cell death in the olfactory epithelium of juvenile chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) [12]. At lower concentrations (≤ 20 µg/L), copper can also diminish the neurophysiological responsiveness of sensory neurons to natural odorants [9,13,14]. Although the olfactory system has received considerable attention, much less is known about the impacts of dissolved copper on other elements of the fish peripheral nervous system.

One area of potential concern is the fish mechanosensory system [6]. In teleosts, vibrational cues and other forms of water displacement are transduced by the lateral line system. The lateral line is comprised of a series of neuromasts positioned in a stereotypical location over the surface of the fish. Individual neuromasts consist of a rosette that contains both support cells and ciliated hair cells. The hair cells, or mechanosensory neurons, are the elements of the peripheral nervous system that respond to the physical displacement of water along the body axis of the animal. Similar to olfactory receptor neurons, lateral line neurons are in direct contact with the surrounding environment [15]. Water movement displaces the

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cilia on the apical surface of receptor cells, leading to signal transduction and the propagation of mechanosensory information to the fish central nervous system [15]. This information about the environment underlies several important fish behaviors, including shoaling [16], prey capture [17,18], rheotaxis (orientation to flow) [19], and predator and obstacle avoidance [20]. Therefore, a loss of lateral line function could have a range of behavioral consequences that ultimately affect the survival of contaminant-exposed fish.

In the present study, we explore the sublethal effects of dissolved copper on the lateral line system of larval zebrafish (*Danio rerio*). Zebrafish are a model system for studying mechanosensation in teleosts in part because the ontogeny and architecture of the lateral line complex have been described in considerable detail in this species [21,22]. The lateral line of larval zebrafish consists of a bilaterally symmetrical pattern of 18 free neuromasts distributed over the head region of the animal as well as a line of nine neuromasts along the trunk [22]. Also, fluorescent markers for visualizing individual lateral line neurons in the intact animal have been developed [23]. Here we use a combination of *in vivo* fluorescent imaging, histology, and scanning electron microscopy to describe the effects of copper on the ultrastructural and pathophysiological status of individual neurons in identified neuromasts. We show that dissolved copper triggers a rapid and dose-dependent loss of mechanosensory neurons at nominal concentrations that are similar to those that have previously been shown to be cytotoxic to olfactory neurons in other fish species. Thus, dissolved copper has the potential to interfere with behaviors in fish that depend on two structurally similar but functionally distinct sensory systems.

MATERIALS AND METHODS

Animals

Adult wild-type zebrafish (AB strain) were maintained in a zebrafish breeding colony at the Northwest Fisheries Science Center (Seattle, WA, USA) according to standard protocols [24]. Male and female zebrafish were allowed to spawn, and the eggs were collected and staged [25]. Embryos were subsequently incubated at 28.5°C with 24-h renewals of reverse osmotic water amended with Instant Ocean Salts (Drs. Foster and Smith, Rhinelander, WI, USA). The amended water (system water) had a conductivity of approximately 1,500 μ S/cm, a pH of 7.0 to 7.4, a hardness (as CaCO₃) of 150 mg/L, and an alkalinity (as CaCO₃) of 5 mg/L. Fish were reared from fertilization until 4 d postfertilization (dpf).

Visualization of mechanosensory neurons in live zebrafish larvae

The fluorescent vital dye 4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide (DASPEI; Sigma, St. Louis, MO, USA) specifically labels hair cells in zebrafish [23] and was used to visualize the individual mechanosensory neurons of the lateral line. Positive DASPEI labeling served as the basis for quantifying the number of hair cells present in different neuromasts. Fish were treated with 0.05% DASPEI in system water for 10 min, rinsed in system water, and then anesthetized with tricaine methane sulfonate (MS-222; 250 μ g/ml; Sigma) for 5 min. The number of DASPEI stained hair cells in each of four identified head neuromasts (otic 2 [O2], infraorbital 4 [IO4], mandibular 2 [M2], and infraorbital 3 [IO3] [22]) were recorded for each fish. Hair cell counts were obtained using a

Nikon Eclipse E600 compound microscope (Meridian Instruments, Kent, WA, USA) fitted with a mercury lamp and the appropriate fluorescence filter (excitation 460–500 nm). A laser scanning confocal microscope (Zeiss Laser Scanning Microscope Pascal, Zeiss, Thornwood, NY, USA) was used to image the DASPEI-labeled neuromasts in zebrafish larvae at higher magnifications. Consistent settings (equivalent gain and offset) were used for both image capture and figure production.

Scanning electron microscopy

Scanning electron microscopy was used to examine the apical surfaces of individual larval neuromasts for ultrastructural evidence of copper-induced cytotoxicity. Copper-exposed and unexposed zebrafish larvae (4 dpf) were anesthetized on ice and transferred to a modified Karnovsky's fixative (3% paraformaldehyde, 0.75% glutaraldehyde in a sodium cacodylate buffer [0.1 M sodium cacodylate, 5.5% sucrose, and 0.2% calcium chloride, pH 7.4; Ted Pella, Redding, CA, USA]) for 24 h at 4°C. The larvae were then rinsed in buffer, dehydrated in a graded series of ethanol, and critical-point-dried with carbon dioxide (Pelco CPD2 critical point drier, Ted Pella). The larvae were then mounted on aluminum stubs with double-sided carbon tape, sputter-coated (Emitech K550x, EMP Direct Products, Houston, TX, USA) with gold, and imaged using a scanning electron microscope (JEOL 6360LV, JEOL USA, Peabody, MA, USA).

Histology

To evaluate the cytoarchitecture of identified neuromasts in cross section, individual zebrafish larvae were embedded in plastic. Copper-exposed and unexposed larvae were anesthetized on ice and transferred to a modified Karnovsky's fix for 24 h at 4°C. Larvae were rinsed in sodium cacodylate buffer and postfixed in 1% osmium tetroxide (Ted Pella) for 2 h. They were then rinsed in buffer and dehydrated in a graded series of ethanol, followed by electron microscopy-grade propylene oxide (Ted Pella). The samples were then infiltrated with Spurr's embedding medium (Ted Pella) overnight. Subsequently, samples were placed in fresh Spurr's and polymerized in a vacuum oven overnight. An ultramicrotome (Sorvall Porter-Blum MT2-B, Ted Pella) fitted with a glass knife was used to cut 1- to 2-micron-thick sections. Sections were stained with Richardson's solution, and images were captured with a AxioCam HRm digital camera (Zeiss).

Dissolved copper exposures

To determine the dose-dependent effects of copper on zebrafish mechanosensory neurons, larval zebrafish (4 dpf) were transferred to plastic six-well plates (Costar® or Falcon®, VWR, Brisbane, CA, USA) containing 6 ml of system water or system water containing dissolved copper (CuCl₂·2H₂O Sigma; minimum purity 99.0%). Fish were exposed to copper for 5 h and then transferred to clean system water. The number of DASPEI fluorescent-labeled hair cells present in each of the four neuromasts (O2, IO4, M2, and IO3) was recorded as described previously.

Nominal dissolved copper exposure concentrations were 0, 5, 15, 25, 30, 40, 50, or 65 μ g/L. For each identified neuromast, the mean number of labeled hair cells per replicate was averaged and normalized to the values for unexposed fish (percent controls; $n = 52$). Fish were exposed in individual wells (20–25 larvae per well), and each exposure was replicated two or three times for a total of 36 to 56 animals at each copper

concentration (a few larvae in each well were not monitored because of time constraints inherent in the fluorescent screen).

To establish the time to effect for mechanosensory neurotoxicity, larvae (4 dpf) were exposed to copper at nominal concentrations of 0, 5, 25, and 50 $\mu\text{g/L}$ in six-well plates. Individual fish were examined at six time points (0, 0.5, 1, 2, 4, and 6 h) during the continuous exposure. For each identified neuromast, the number of DASPEI fluorescent-positive receptor neurons was recorded for 15 to 25 larvae per time point and exposure concentration. Larvae were exposed in separate wells, and individual animals were examined only once. The experiment was repeated twice (for a total of 30–50 animals per time point and concentration).

To determine the time course for hair cell regeneration following dissolved copper exposure, 4-dpf zebrafish larvae were divided into three treatment groups: larvae raised for 77 h in the absence of copper, larvae exposed to 50 $\mu\text{g/L}$ copper for 5 h and then transferred to clean system water for 72 h, and larvae exposed to 50 $\mu\text{g/L}$ copper continuously for 77 h. Exposures were conducted in six-well plates, and exposure solutions were renewed at 24-h intervals. At each time point (24, 48, and 72 h after the transient 5-h exposure interval), 20 to 25 individual animals were analyzed for the presence and number of labeled sensory neurons, as indicated by DASPEI fluorescent staining. Larvae were exposed in separate wells, and individual animals were examined only once. This procedure was repeated twice to give a total of 40 to 50 animals per time point for each of the three treatment groups.

Data analysis

The dose-dependent effect of copper on the zebrafish lateral line was determined using the sigmoid function:

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

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 (control) fish, x is the copper concentration, k is the effective concentration indicating a 50% loss of hair cells (EC50), and n is the slope. Kaleidagraph (Synergy Software, Reading, PA, USA) was used to perform nonlinear regressions and to determine effective concentrations of EC20 and EC50, or a 20 and 50% loss of sensory cells, respectively. Two-factor analysis of variance followed by Tukey's honestly significantly different test for pairwise comparisons ($p < 0.05$) were calculated for the time course of cell death and regeneration.

RESULTS

The lateral line system of larval zebrafish is specifically labeled by DASPEI

The stereotypical positions of neuromasts are schematically illustrated in Figure 1A. Four neuromasts (O2, IO4, M2, and IO3; circled in Fig. 1A) were chosen for subsequent analyses of hair cell number. The labeled neuromasts in an unexposed 4-dpf zebrafish are shown in Figure 1B. The mean number of fluorescent-labeled hair cells varied for each of the four neuromasts in different locations (Table 1). Among individual sensory neurons, DASPEI staining was confined to the cytoplasm of each cell. No staining was observed in the nucleus (not shown).

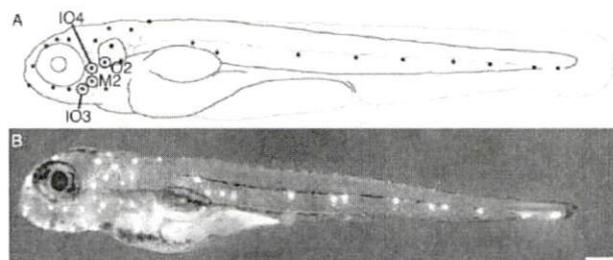


Fig. 1. Lateral line neuromasts are distributed over the surface of a zebrafish larva in a stereotypical pattern. (A) A schematic diagram illustrating the position of individual neuromasts along the left side of a zebrafish at 5 d postfertilization (dpf); adapted from T.T. Whitfield; <http://www.zfin.org>). The number of individual sensory neurons in the four identified neuromasts (circles) were monitored in copper-exposed and unexposed fish. These neuromasts included otic 2 (O2), infraorbital 4 (IO4), mandibular 2 (M2), and infraorbital 3 (IO3) [22]. (B) A photomicrograph of a larval zebrafish (4 dpf) labeled with the fluorescent vital dye, 4-(4-diethylaminostyryl)-N-methylpyridinium iodide (DASPEI). Under fluorescence, labeled neuromasts are evident in stereotypical locations on the head and along the trunk. Since zebrafish larvae are transparent, more neuromasts exist in the fluorescent micrograph than in the schematic. Scale bar = 0.5 mm.

Copper neurotoxicity is dose dependent

Laser scanning confocal microscopy was used to evaluate the effects of dissolved copper on DASPEI labeling of individual receptor neurons. A representative neuromast (located on the epidermis overlying the otic vesicle) from unexposed zebrafish larvae at 4 dpf and the same neuromast from larvae exposed to copper for 5 h at 25 and 50 $\mu\text{g/L}$ are shown in Figure 2. Individual hair cells were brightly stained with DASPEI in control fish (Fig. 2A; $n = 5$). By comparison, larvae exposed to the intermediate concentration of copper (25 $\mu\text{g/L}$) showed reductions in both the intensity of DASPEI staining and the number of labeled cells (Fig. 2B; $n = 5$). At a higher exposure concentration (50 $\mu\text{g/L}$), the DASPEI staining in the neuromasts was largely absent. In the example shown (Fig. 2C; $n = 5$), faint fluorescence is restricted to a single sensory neuron.

Copper reduced DASPEI staining in 4-dpf zebrafish in a dose-dependent manner (Fig. 2D). To evaluate the dose-re-

Table 1. Each of the four identified neuromasts (otic 2 [O2], infraorbital 4 [IO4], mandibular 2 [M2], and infraorbital 3 [IO3]; circled in Fig. 1A) of zebrafish larvae had a distinct complement of individual sensory neurons as indicated by 4-(4-diethylaminostyryl)-N-methylpyridinium iodide (DASPEI; Sigma, St. Louis, MO, USA) fluorescence. Although the neuromasts had different numbers of receptor cells, the slopes of the dose-response curves for copper-induced neurotoxicity were similar (Fig. 2D). No apparent difference was observed in the EC50 or the EC20, which describe the effective concentrations at which copper causes a 50% and 20% loss of hair cells, respectively. Values are the means of two to three replicates ($n = 13-25$ per replicate)

NE ^a	Mean ^b HC/NE ^c	Slope \pm SE ^d	EC50 \pm SE ^d ($\mu\text{g/L}$)	EC20 \pm SE ^d ($\mu\text{g/L}$)
O2	11.0 \pm 0.6	4.0 \pm 0.5	27.4 \pm 0.8	19.4 \pm 1.1
IO4	7.2 \pm 0.9	4.5 \pm 0.6	27.7 \pm 0.8	20.4 \pm 1.2
M2	6.0 \pm 1.1	4.4 \pm 0.8	29.8 \pm 1.2	21.8 \pm 1.5
IO3	6.2 \pm 0.7	4.4 \pm 0.6	29.7 \pm 1.0	21.7 \pm 1.3

^a Neuromast.

^b Value \pm 95% confidence interval.

^c Hair cells per neuromast.

^d Standard error.

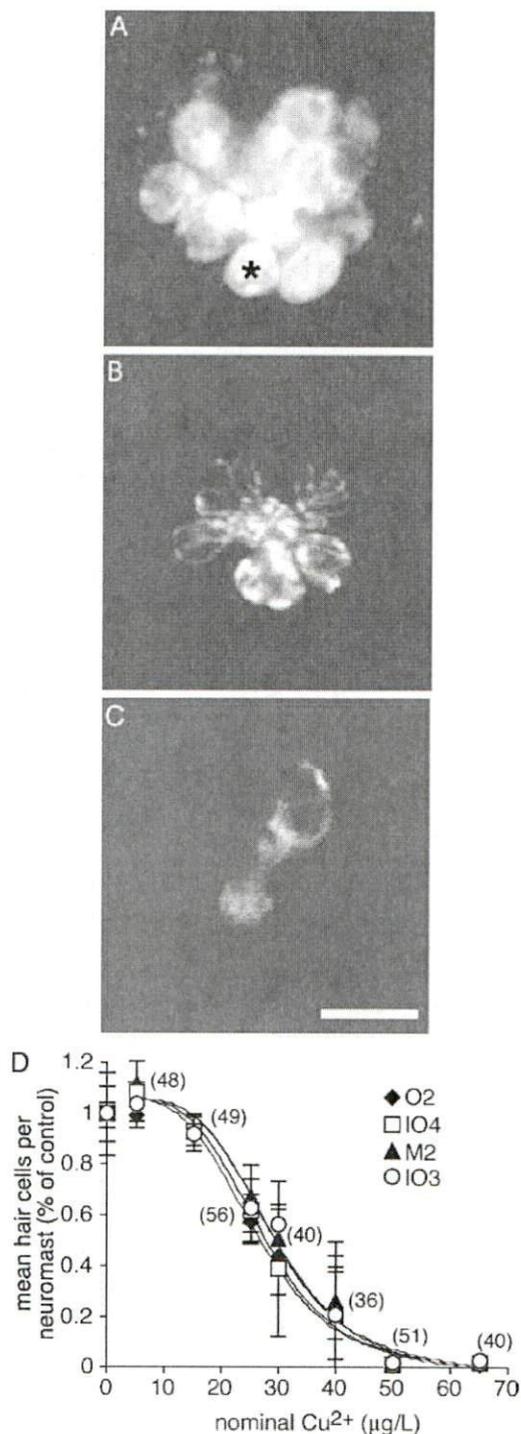


Fig. 2. Dissolved copper triggers a dose-dependent loss of 4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide (DASPEI) staining among individual mechanoreceptor neurons. (A) A confocal image of a cluster of hair cells was brightly stained with DASPEI in an unexposed zebrafish larva. The asterisk indicates a single hair cell. (B) Fewer hair cells were fluorescently labeled in larvae exposed to 25 µg/L copper for 5 h. (C) At 50 µg/L, a single neuron was evident as faint DASPEI fluorescence. $n = 5$ for each treatment. Scale bar = 10 µm. (D) Dose-dependent loss of DASPEI-labeled sensory neurons from four neuromasts—otic 2 (O2), infraorbital 4 (IO4), mandibular 2 (M2), and infraorbital 3 (IO3)—identified in Figure 1. Larval hair cell counts at each exposure concentration were normalized to the mean values for unexposed controls. Error bars are mean \pm standard deviation. Numbers of individual animals per exposure are in parentheses.

sponse relationship, larval neuromasts were labeled with DASPEI after a 5-h exposure to copper at eight nominal concentrations ranging from 0 to 65 µg/L. The number of DASPEI-labeled mechanoreceptor neurons in each of the four identified neuromasts (O2, IO4, M2, and IO3) was determined for the different exposure concentrations. Sigmoid functions were used to estimate the dose–response relationship for sensory neuron loss in each of the four neuromasts (Fig. 2D). In each case, the functions were a close fit ($r^2 = 0.981–0.989$).

The cytotoxic effects of copper on each of the four identified neuromasts were similar, as indicated by the EC₅₀ (Table 1). Nominal copper concentrations ranging from 19 to 22 µg/L were sufficient to trigger a 20% loss of hair cells in each of the four neuromasts. Since the numbers of labeled neurons at these concentrations are near the lower end of the 95% confidence interval for control animals (the mean number of hair cells per neuromast), the EC₂₀s serve as approximate indicators of the threshold for copper's toxicity to the lateral line system.

Loss of stereocilia and kinocilia from the apical surface of mechanosensory neurons

Neuromasts from 4-dpf larvae were examined using scanning electron microscopy to determine the ultrastructural effects of copper on ciliated sensory neurons. A representative neuromast of an unexposed animal is shown in Figure 3A ($n = 5$). The neuromast characteristically protruded from the surrounding epidermis. Long kinocilia, each from a single sensory neuron, projected from the center of the neuromast. Stereocilia, which are smaller and more numerous, were evident at the base of each kinocilia. Although the length and number of kinocilia varied, the example in Figure 3A is representative of the general structure of all neuromasts at 4 dpf regardless of position over the surface of the fish. When zebrafish larvae were exposed to 50 µg/L of copper for 5 h, both kinocilia and stereocilia were absent (Fig. 3B; $n = 5$). These structures, which are required for mechanical signal transduction, were missing from all the neuromasts examined at all locations along the body.

Time course for cell death and regeneration

Examination of histological sections revealed a time-dependent pattern of cell death in the neuromasts of zebrafish exposed to 50 µg/L copper. Long kinocilia projecting from the apical surface of hair cells were evident in neuromasts from unexposed larvae (Fig. 4A; $n = 5$). Elongated hair cells were darkly stained, and smaller support cells were present below the hair cells in the basal portion of the neuromast. After 1 h of exposure to copper, sensory neurons were largely absent, and dark round necrotic bodies were present in locations where hair cells would normally be located (Fig. 4B; $n = 5$). This process of cell death appeared to be largely complete following 3 h of exposure (Fig. 4C; $n = 5$). Collectively, these results suggest that the onset of copper-induced neurotoxicity in the zebrafish lateral line is relatively rapid (less than 1 h).

To monitor the onset of copper-induced cell death in the lateral line in more detail, zebrafish larvae were exposed to four concentrations (0, 5, 25, and 50 µg/L) and observed at six time intervals (0, 0.5, 1, 2, 4, and 6 h) during the exposure. The time course for cell loss in neuromast M2 (Fig. 4D) was representative of all four neuromasts examined. At the highest exposure concentration (50 µg/L), the number of DASPEI-labeled sensory neurons in M2 significantly declined within

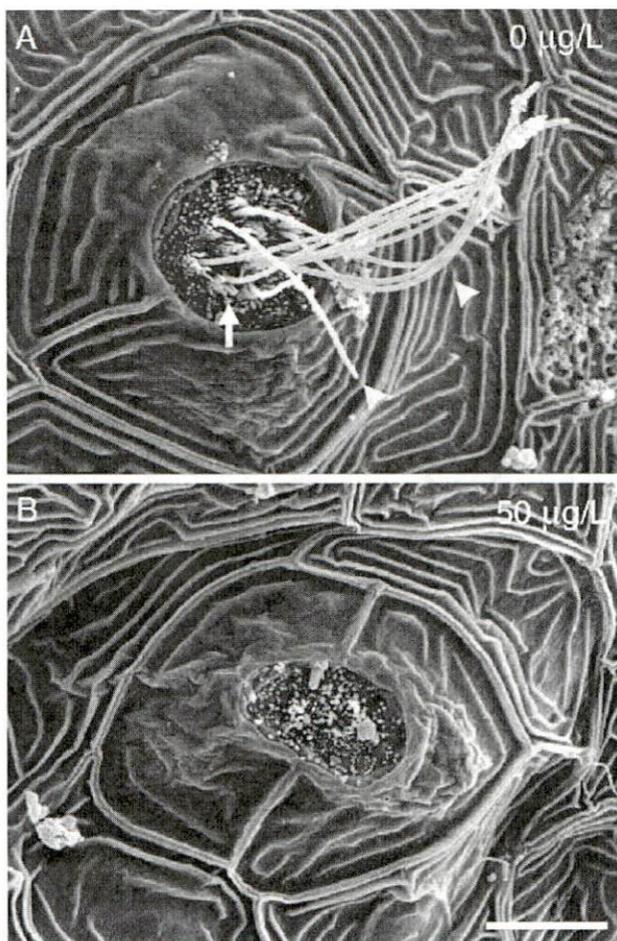


Fig. 3. Loss of apical cilia in lateral line neurons exposed to copper. (A) A scanning electron micrograph of an untreated zebrafish larva (4 d postfertilization) showing the lateral line as a dome-shaped structure protruding from the epidermal surface. Long kinocilia (arrowheads) projected from the apical surface of individual sensory neurons in the neuromast. Numerous stereocilia (white arrow) were clustered around the base of each kinocilia. (B) Kinocilia and stereocilia were absent from the neuromasts of the larvae exposed to 50 $\mu\text{g/L}$ of copper for 5 h. $n = 5$ for each treatment. Scale bar = 5 μm .

the first half hour of copper exposure ($p < 0.05$). After approximately 2 h of copper exposure, the mean number of hair cells per neuromast at the higher concentrations (25 and 50 $\mu\text{g/L}$) did not change ($p > 0.05$). By comparison, the number of fluorescently labeled cells remained constant in control animals and fish in the 5- $\mu\text{g/L}$ exposure group during the same 6-h developmental window ($p > 0.05$).

Mechanosensory neurons have previously been shown to regenerate following acute chemical injury [26]. In zebrafish larvae, mechanosensory neurons regenerated (defined as a recovery in DASPEI labeling) when copper-exposed animals were returned to clean water (Fig. 4E). To determine the time course for recovery, hair cell regeneration in the four identified neuromasts was monitored at 24, 48, and 72 h after a 5-h exposure to 50 $\mu\text{g/L}$ copper. As a representative example, data for neuromast M2 are shown in Figure 4E. Note that the mechanosensory system is still developing at 4 dpf, as indicated by an increase in the number of hair cells per neuromast in control fish during the first 24 h of this exposure interval ($p < 0.05$). In larvae exposed to copper, partial hair cell regeneration was evident within 24 h of transfer to uncontaminated water ($p <$

0.05). After approximately 48 h in clean water, the mean number of hair cells per neuromast in the transferred larvae was not significantly different from the unexposed larvae ($p > 0.05$), indicating that recovery was complete. In contrast, zebrafish larvae that were continuously exposed to copper showed no mechanoreceptor regeneration.

DISCUSSION

The sensory systems of fish convey critical information about habitat conditions, predators, prey, competitors, mates, and other features of the aquatic environment. By rendering animals unresponsive to social and ecological cues, chemical contaminants have the potential to adversely affect behaviors that ultimately determine the survival and reproductive success of exposed fish [27]. As an example, dissolved copper has long been known to be toxic to the peripheral olfactory system of fish [9,12–14]. Here we extend these earlier observations to the lateral line system of zebrafish. Specifically, we show that dissolved copper triggers a rapid loss of ciliated mechanoreceptor neurons from identified neuromasts as indicated by DASPEI staining. Copper therefore has the potential to disrupt behaviors in fish that depend on olfaction, mechanosensation, or a combination of these two distinct sensory modalities.

The zebrafish is a useful experimental model for studying both the ontogeny and the regeneration of the lateral line in contaminant-exposed fish. Inexpensive fluorescent markers such as DASPEI allow for rapid *in vivo* screens of receptor neuron cytotoxicity [23]. Since the basic architecture and function of the lateral line is highly conserved across teleosts [28], it is possible that copper is also cytotoxic to the lateral line systems of other fish, including cold-water species such as salmonids.

Storm events tend to mobilize non-point source contaminants such as copper over relatively short time intervals [4]. Thus, the onset of copper neurotoxicity is an important consideration for fish exposed to dissolved copper in transient intermittent pulses. In the present study, fluorescent imaging and histopathology indicated a relatively rapid (<1 h) onset of copper-induced neurotoxicity in the zebrafish lateral line. This interval is well within the duration of typical storm events that transport pulses of pollutants to fish habitats via non-point source storm-water runoff [4].

We did not evaluate the influence of water chemistry on the bioavailability or toxicity of copper to the zebrafish mechanosensory system. The biotic ligand model, which has been widely used to predict the acute lethal toxicity of copper to fish, is based on the binding and accumulation of copper in gill tissues [29]. While dissolved organic matter in ambient waters might reduce the bioavailability of copper to the fish peripheral nervous system via complexation, it is presently unknown whether hardness or other aspects of water chemistry affect sublethal sensory neurotoxicity via competition at biotic ligands. Interestingly, the impacts of copper on the neurophysiological responsiveness of olfactory sensory neurons in coho salmon do not appear to be altered by varying water hardness (as extracellular CaCO_3 [14]). Sensory neurons are very different from gill epithelial cells in terms of their architecture, extracellular proteins, ion channels, transporters, intracellular signaling machinery, and overall function [30,31]. Consequently, our current understanding of water chemistry, as developed from studies on the gill (reviewed by Niyogi et al. [8]), may not extend to sensory neurons, particularly if the ligands for copper in the different tissues are not the same.

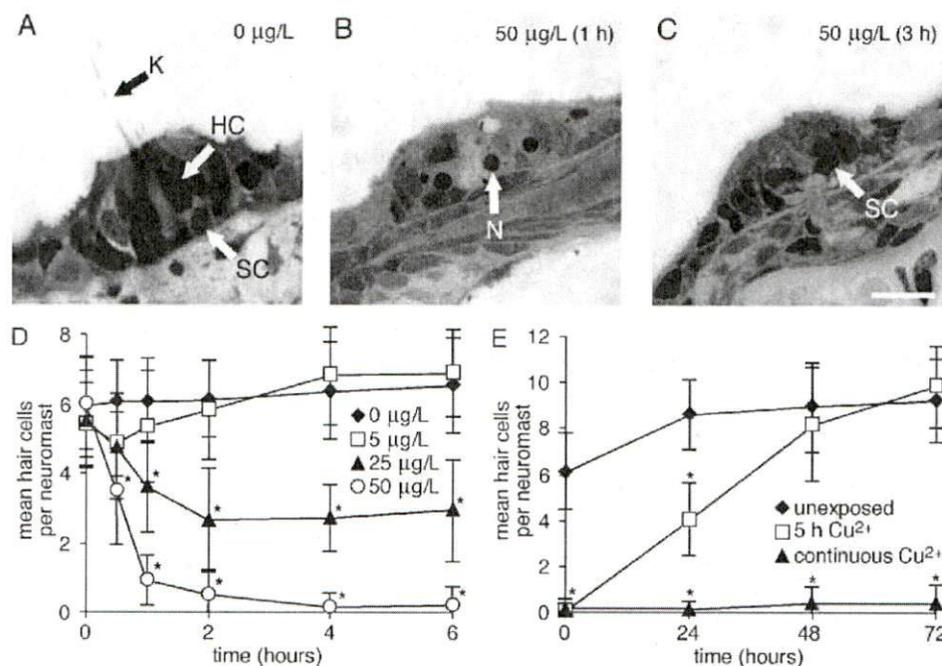


Fig. 4. Time-dependent effects of copper on the zebrafish lateral line system. (A) A cross section of an unexposed zebrafish (4 d postfertilization [dpf]) neuromast shows darkly stained hair cells (HC) and kinocilia (K) projecting from the apical surfaces of the receptor. Smaller support cells (SC) were located below the hair cells. (B) Within 1 h of copper exposure (50 µg/L), the hair cells and kinocilia were absent. In place of the sensory neurons were dark round necrotic bodies (N). (C) The process of cell death appeared to be complete by 3 h of exposure. Although kinocilia and hair cells were absent, fewer necrotic bodies were present. In contrast to the hair cells, the support cells in the basal portion of the zebrafish neuromast do not appear to be affected by dissolved copper (arrow in C). $n = 5$ for each treatment. Scale bar = 10 µm. (D) The effect of dose and time on the onset of copper-induced cytotoxicity in a representative neuromast (M2). Asterisks indicate a significant difference from unexposed larvae ($p < 0.05$; two-way analysis of variance, Tukey's honestly significant different test). Error bars are mean \pm standard deviation (SD) ($n = 30$ –50 fish). (E) Time course for lateral line regeneration in the M2 neuromast following a 5-h exposure to copper at 50 µg/L. Hair cells are added to the M2 neuromast of unexposed larvae as the developing lateral line matures between 4 and 6 dpf (0–48 h on the x-axis). A 5-h copper exposure eliminated 4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide (DASPEI) labeling (open squares and filled triangles at $t = 0$). Zebrafish larvae restored to clean water (open squares) gradually regenerated hair cells in the M2 neuromast over 48 h. The neuromasts of larvae exposed continuously to copper (filled triangles) showed no recovery. Asterisks denote a significant difference from unexposed larvae ($p < 0.05$). Error bars are mean \pm SD ($n = 40$ –50 fish).

This has not been investigated, however, and the influence of water chemistry on the bioavailability and toxicity of copper to fish sensory systems remains an important area for future research.

Sensory neuron regeneration is a well-known feature of both the fish olfactory system [32] as well as the lateral line [26]. Toxicant-induced cell death in the zebrafish lateral line has previously been shown to stimulate proliferation among supporting cells in a neuromast [26]. Over time, this leads to the genesis of new ciliated receptor cells [26]. In the present study, when copper-exposed (50 µg/L for 5 h) zebrafish larvae were transferred to clean water, the process of sensory cell regeneration occurred over approximately 2 d. However, the zebrafish lateral line showed no signs of recovery when animals were exposed to 50 µg/L copper continuously for 3 d. Therefore, at least within this exposure interval, no compensatory mechanism that might allow the lateral line to regenerate during continuous copper exposures appears to exist.

In juvenile chinook salmon (*O. tshawytscha*), cell death was observed in the olfactory sensory epithelium following exposures to copper concentrations at 25 µg/L or higher (4-h exposures [12]). In the present study, the death of mechanosensory neurons (20% loss) was observed at nominal copper concentrations at or above 20 µg/L. In the olfactory system, dissolved copper impairs the sensory physiology of fish at concentrations that are well below the levels that cause cell

death in the peripheral receptor neurons. For example, neurophysiological recordings from the olfactory epithelium of coho salmon (*Oncorhynchus kisutch*) have recently shown that copper reduces olfactory sensitivity at concentrations as low as 5 µg/L or less [13,14]. It is therefore possible that copper inhibits lateral line function at concentrations lower than those that trigger cell death in this study.

The contributions of the lateral line are important to a range of fish behaviors [16–20]. In instances where copper causes a loss of mechanoreceptors (at concentrations greater than 20 µg/L in the present study), considerable potential exists for loss of mechanosensory function and thus behavioral impairment. Although the impacts of copper on lateral line-mediated behaviors are not presently known, other chemicals have been shown to affect mechanosensory behaviors. Cadmium has been shown to interfere with rheotaxis in the banded kokopu whitebait (*Galaxias fasciatus*), presumably via sublethal effects on the mechanosensory system [33]. Moreover, gentamicin sulfate has been shown to cause mechanosensory cell death and disrupt rheotaxis behavior in the mottled sculpin (*Cottus bairdi*) [34]. The effects of dissolved copper on behaviors relying on mechanosensory function are not known, however, and remain an important area for future investigation.

In summary, it is now well established that storm events periodically transport copper and other non-point source pollutants to freshwater and coastal marine environments [1,2].

Moreover, within watersheds, the loading of copper to fish habitats typically increases with human population growth and urban development. The sublethal neurotoxicity of copper is therefore likely to be an expanding concern for many fish species throughout the United States. Primary receptor neurons in the fish lateral line system appear to be similar to olfactory receptor neurons in terms of their vulnerability to the cytotoxic effects of dissolved copper. Our results highlight the importance of understanding the interrelationships between short-term copper exposures, sensory neurotoxicity, behavior, and the ecological interactions that ultimately influence fish survival and reproduction.

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