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Copper-induced changes in locomotor behaviors and neuronal physiology of the freshwater oligochaete, *Lumbriculus variegatus*

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Abstract

The behavioral and neurotoxic effects of copper exposure were examined in the freshwater oligochaete, *Lumbriculus variegatus*. The 24 h LC_{50} for worms exposed to copper sulfate in an artificial pond water was 0.45 μM. Almost all animals that died due to copper exposure died during the first day of exposure. Immersion in water containing 0.2 or 0.4 μM copper produced time- and concentration-dependent reductions in the ability of tactile stimulation to evoke two stereotyped locomotory behaviors, body reversal and helical swimming. Helical swimming was more severely affected by copper exposure than was body reversal behavior. Upon return to clean water, both behaviors returned to normal levels within 1–2 days. Noninvasive electrophysiological testing indicated that copper exposure produced time- and concentration-dependent reductions in the conduction velocities of the medial and lateral giant nerve fibers. An 8 h exposure to 0.2 μM copper produced significant reductions in giant fiber conduction velocities that returned to normal levels within 3 days of return to clean water. It is likely that copper exposure can significantly degrade the ability of aquatic oligochaetes to avoid predators.

Keywords: Copper; Metals; Oligochaete; Sublethal; Behavior; Electrophysiology

1. Introduction

Copper is an essential trace element; however, excessive levels are toxic to essentially all forms of life (reviewed by Barceloux, 1999; Pena et al., 1999; Rotilio et al., 2000; Burkitt, 2001; Gaetke and Chow, 2003). Copper enters the aquatic environment through several pathways, including runoff from mineral deposits & mining operations, industrial activities, corrosion of copper plumbing, and leaching from wood preservatives. In addition, copper compounds are used as fertilizers, fungicides, algicides, molluscicides, and in marine paint as an antifouling agent.

Sublethal copper exposure alters a number of behaviors in invertebrates. In some cases, the altered behavior reduces the exposure of the animal to copper (McLeese, 1975; Pynnönen, 1996; Leynen et al., 1999; Sambongi et al., 1999; Dhawan et al., 2000; Curtis et al., 2000; Salminen and Haimi, 2001a). In other cases, copper exposure reduces the ability of decapod crustacea to detect and orient to food odors in a water plume (McLeese, 1975; Sherba et al., 2000). Locomotory behavior can also be adversely affected by copper exposure (Sullivan et al., 1983; Rondelaud, 1988;

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Charoy and Janssen, 1999; Dhawan et al., 2000; Praël et al., 2001). Such adverse effects on locomotory and sensory systems can also be correlated with adversely affected predator avoidance behaviors (Sullivan et al., 1983; Clements et al., 1989).

Lumbriculus variegatus is a small freshwater oligochaete worm (Order Lumbriculida, Family Lumbriculidae) that lives in the shallows of freshwater ponds, lakes, and marshes. *Lumbriculus* is widely distributed in North America and Europe and has been introduced into Africa, Australia and New Zealand (Brinkhurst and Jamieson, 1971). Aquatic oligochaetes, such as *Lumbriculus*, are important members of freshwater aquatic communities where they serve in such diverse roles as aiding in the decomposition of organic materials in the sediment and serving as food for animals at higher trophic levels. Due to these reasons, as well as other practical considerations, it has been proposed to use *Lumbriculus* as a standard organism in bioaccumulation tests (ASTM, 1995; OECD, 1995).

In its natural habitat, *Lumbriculus* normally positions itself so that its head is buried in the substrate and its tail is extended into the water column. The worm consumes the bottom sediments and obtains its nourishment from the organic material and microorganisms contained therein. The positioning of the tail into the water column renders this portion of the animal vulnerable to predation. To protect the tail, *Lumbriculus* has developed rapid withdrawal behaviors that are elicited by tactile stimulation or shadow (Zoran and Drewes, 1987; Drewes and Fourtner, 1989). The neuronal circuits mediating rapid withdrawal responses in *Lumbriculus* are similar in many ways to those in other terrestrial & aquatic oligochaetes (Drewes, 1984; Zoran and Drewes, 1987). Two distinct reflex systems respond to tactile stimulation of the anterior and posterior regions of the body respectively. Tactile stimulation of approximately the anterior one-third of the worm's body will activate the medial giant fiber (MGF), while stimulation of the posterior three-fourths of the worm's body will activate the paired lateral giant fibers (LGF). When activated, the giant fibers subsequently activate motor neurons, which in turn activate longitudinal body wall muscles. Contraction of the longitudinal body wall muscles underlies the rapid shortening response of the animal.

Lumbriculus also possesses several context-specific locomotor "escape" behaviors that are not mediated by the giant fibers. When on wet surfaces or in confined spaces, tactile stimulation of the head or tail will elicit backwards or forward crawling respectively (Ding et al., 2001). In open underwater spaces, touching the head elicits a reversal in body position, and touching the tail elicits helical swimming (Drewes, 1999). Helical swimming consists a series of helical waves that progress from the anterior end to the posterior end. Successive waves alternate between clockwise and counterclockwise helical waves. The animals literally appear to corkscrew through the water. All of these locomotor behaviors (rapid withdrawal, crawling, body reversal, helical swimming) are stereotyped behaviors that are amendable to quantification; and thus, can be used for sublethal toxicology testing (Rogge and Drewes, 1993; Ding et al., 2001).

In this study, we examine the effects of copper exposure on the ability of tactile stimulation to evoke body reversal, helical swimming and giant fiber spiking activity. We also quantify the effects of copper on the electrophysiological properties of the giant fibers. In addition, we also quantify the time course of recovery from copper-induced behavioral and electrophysiological effects.

2. Materials and methods

2.1. Animal culture

Lumbriculus variegatus were laboratory-reared in aquaria containing aerated artificial pond water and pieces of brown paper towels to act as a substrate. Worms were fed commercial fish food 2–3 times per week. The asexually reproducing cultures were derived from animals originally purchased from Aquatic Foods (Fresno, CA). The previous exposure of the worms to copper while in the care of the vendor is unknown. The initial LC_{50} determination and behavioral experiments (Figs. 1–3) were conducted within three months of the worm's arrival in the lab. Electrophysiology experiments (Figs. 4–7) were conducted approximately 1 year after establishment of the cultures. Although not reported here, subsequent LC_{50} determinations conducted for other purposes produced similar values as reported here. The soft artificial pond water

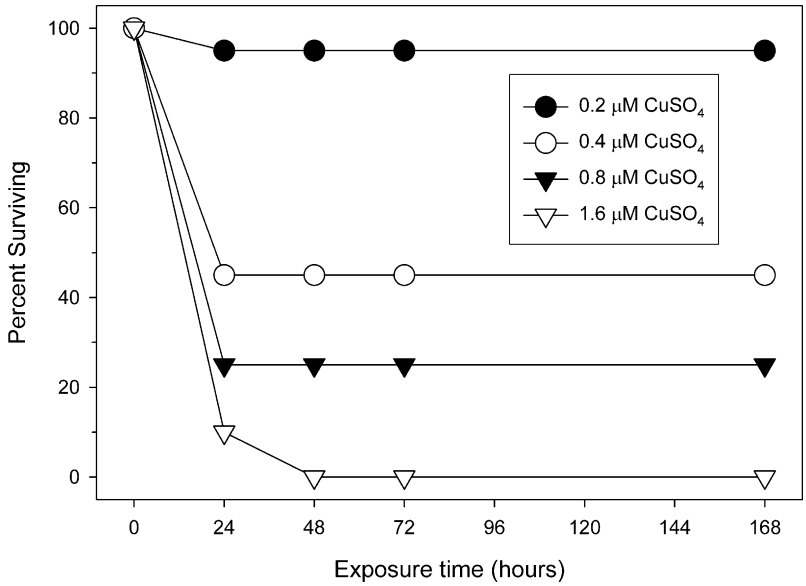


Fig. 1. Time course of mortality following copper exposure. Worms were placed in one of four copper concentrations (0.2, 0.4, 0.8, and 1.6 μM; $n = 30$ per concentration) and mortality was checked daily. Almost all worms that succumbed to copper exposure died during the first 24 h of exposure.

had the following composition: 1 mM NaCl; 13 μM KCl, 4 μM $Ca(NO_3)\cdot 4H_2O$; 17 μM $Mg(SO_4)\cdot 7\ H_2O$; 71 μM HEPES buffer. During the LC_{50} determination, the initial pH of the artificial pond water was 6.45±0.02 while worms used in electrophysiology experiments were raised in water with a pH of 7.0±0.05. No attempt was made to monitor or adjust the pH once the worms were placed in the water. Water used for the artificial pond water and all other solutions was produced by a Barnstead NANOpure system, using house RO water as the source water.

Worms used in these experiments were randomly chosen and were 2–5 cm in length as well as lacking any obvious morphological defects.

2.2. Determination of LC_{50} for copper exposure

We used copper sulfate (as opposed to other copper salts) for the experiments reported here because it is the most widely investigated copper salt and it is used frequently for agricultural and pest control purposes. Worms were exposed to one of four concentrations of $CuSO_4\cdot 5H_2O$ (nominal concentrations of 0.2, 0.4, 0.8, and 1.6 μM [12.5, 25.5, 51 and 102 μg/L Cu]) for 24 h with 30 worms per concentration (120 animals total). Each worm was placed into a separate 100 mm × 15 mm Petri dish containing 40 ml of copper-containing water. Determination of lethality was made after 24 h for all worms, and at 48, 72 and 168 h (7 days). Death was indicated by decomposition of the worm. The mean lethal concentration (LC_{50}) and 95% confidence interval were calculated by the trimmed Spearman–Karber method (Hamilton et al., 1977) using a computer program developed at Montana State University and modified by the United States Environmental Protection Agency.

2.3. Behavioral testing

We assessed the effect of copper exposure on the ability of touch to evoke stereotyped helical swimming and body reversal behaviors. These behaviors were studied in a manner similar to previous studies (Drewes, 1999; Ding et al., 2001). In normal worms, touching the head evokes body reversal while touching the tail evokes helical swimming. Individual worms were placed into a 100 mm × 15 mm Petri dish filled with 45 ml of artificial pond water or artificial pond water containing copper sulfate (0.4 or 0.2 μM). Test-

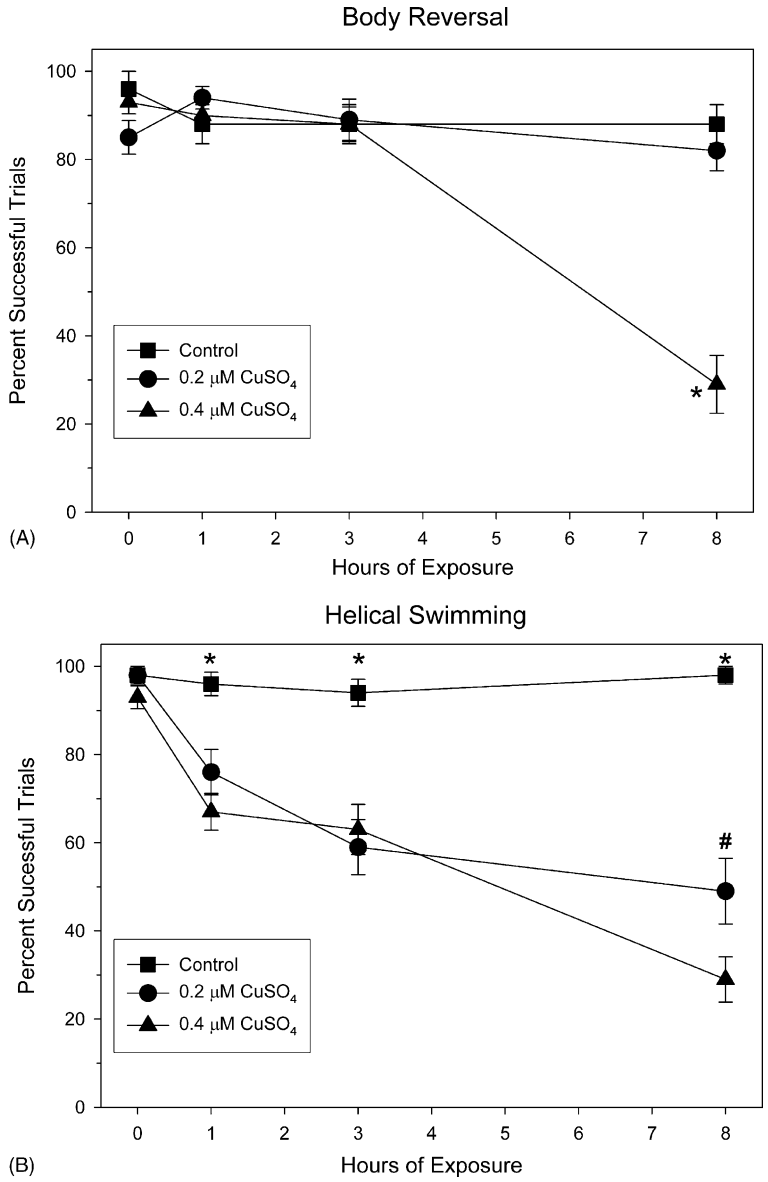


Fig. 2. Effect of copper exposure upon body reversal and helical swimming behaviors. Worms were exposed to one of three copper concentrations (0, 0.2, or 0.4 μM; $n = 10$ per concentration) and tested for the ability of tactile stimulation to elicit body reversal (A) or helical swimming (B). (A) Exposure to 0.4 μM copper reduced the ability of tactile stimulation to elicit body reversal at 8 h exposure time. A two way repeated measures ANOVA indicated a significant effect due to copper concentration ($P < 0.001$; $F = 8.624$; d.f. = 2, 47), duration of exposure ($P < 0.001$; $F = 24.302$; d.f. = 3, 141), and the interaction ($P < 0.001$; $F = 18.435$; d.f. = 6, 141). Subsequent Tukey tests indicated that animals exposed to 0.4 μM copper first exhibited a significant decrease in reversal behavior at 8 h (∗) ($P < 0.05$), and at 8 h this group was significantly different from control or 0.2 μM copper groups. (B) Exposure to either copper concentration produced a significant decrease in evoked helical swimming within 1 h of exposure. A two way repeated measures ANOVA indicated a significant effect due to copper concentration ($P < 0.001$; $F = 25.516$; d.f. = 2, 47), duration of exposure ($P < 0.001$; $F = 29.016$; d.f. = 3, 141), and the interaction ($P < 0.001$; $F = 7.2343$; d.f. = 6, 141). Subsequent Tukey tests indicated that the control group was significantly different ($P < 0.05$) from both copper exposed groups at 1, 3 and 8 h (∗). The two copper-exposed groups did not significantly differ from one another at 1 and 3 h, but there was a significant difference between the two groups at 8 h (#).

ing of swimming and reversal behaviors occurred in this same dish. In one experiment, the worms were exposed to copper for 8 h and tested for swimming and reversal behaviors at 0 (prior to exposure), 1, 3, and 8 h of exposure. In a separate experiment, the worms were exposed to copper for 8 h and then transferred into clean artificial pond water. These animals were tested for swimming and reversal behaviors at 0 and 8 h of copper exposure, and then every 24 h after transfer to clean water for 4 days.

At each testing time, the worm was touched 10 times alternatively at its anterior or posterior ends (5 touches per end). The interval between successive touches was 2–6 s. The tactile stimulus was delivered with a rubber probe (made from a rubber band) mounted at the end of a wooden applicator stick. The dimensions of the rubber probe were 2 mm × 1 mm × 7 mm long. Each trial was videotaped using a Panasonic AG-188 Proline VHS video camera. The videos were played back frame-by-frame on a Panasonic GX4 Multifunctional AG-1950 Proline VCR. A response was scored as successful only when the worm showed stereotypical helical swimming or body reversal movements (Drewes, 1999). Data is reported as the percentage of trials in which stereotypical swimming or reversal movements were successfully evoked.

2.4. Electrophysiological testing of giant nerve fiber function

Techniques for noninvasive electrophysiological testing of giant nerve fiber function in oligochaetes have been previously described (Drewes, 1984; Zoran and Drewes, 1987; Rogge and Drewes, 1993). A worm was placed perpendicular to a grid of recording electrodes on a printed circuit board. The worm was held next to a smooth strip of Plexiglass (4.8 cm × 0.8 cm) by the surface tension caused by a drop of water placed on the circuit board. Tactile stimulation, applied to the anterior or posterior end using a human hair (attached to a wooden applicator stick), was used to evoke medial or lateral giant fiber (MGF and LGF) activity, respectively. Evoked spikes were detected by two pairs of recording electrodes located in the midbody region of the worm. Signals from the electrodes were filtered and amplified by AC-coupled amplifiers (Warner Instruments DP-301, Hamden, CT), and then displayed as two channels on a computer-based data acquisition system (WinDaq, Dataq Instruments, Dayton, OH) at a sampling rate of 20,000 samples/s/channel. Giant fiber conduction velocity was determined over a 10-mm conduction distance. To obtain conduction velocity, conduction distance (10-mm) was divided by conduction time, as measured by the peak-to-peak interval between the two recording channels as the spike propagated between the two recording sites. During a testing session, the mean conduction velocity of each giant fiber was calculated from 3 to 6 measurements.

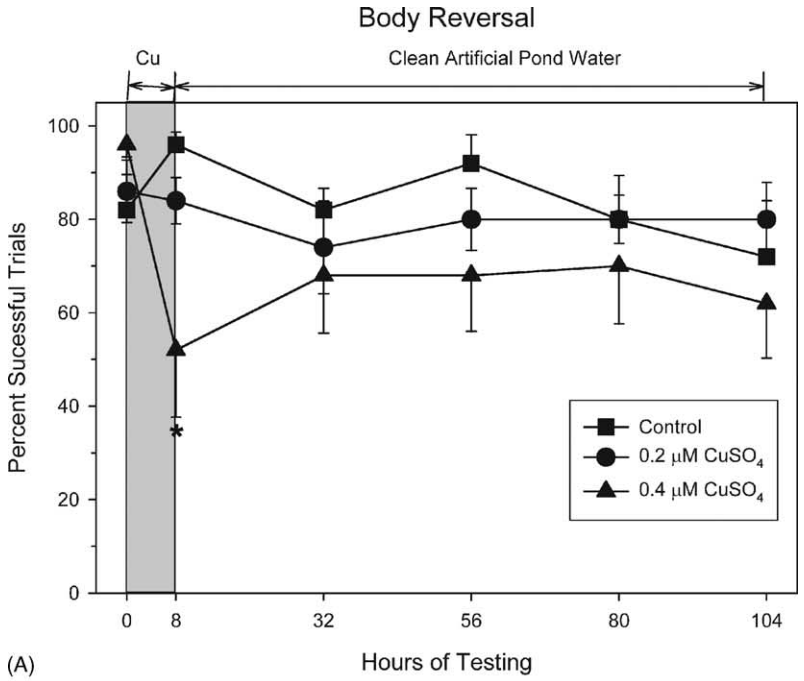
2.5. Data analysis

Except for the determination of the LC_{50} to copper exposure (described above), all statistics were performed using SigmaStat 2.03 (SPSS Inc., Chicago, IL). Data are usually reported as means ± the standard error of the mean (S.E.M.). When data was not normally distributed, it is reported as median ± median absolute deviation (MAD).

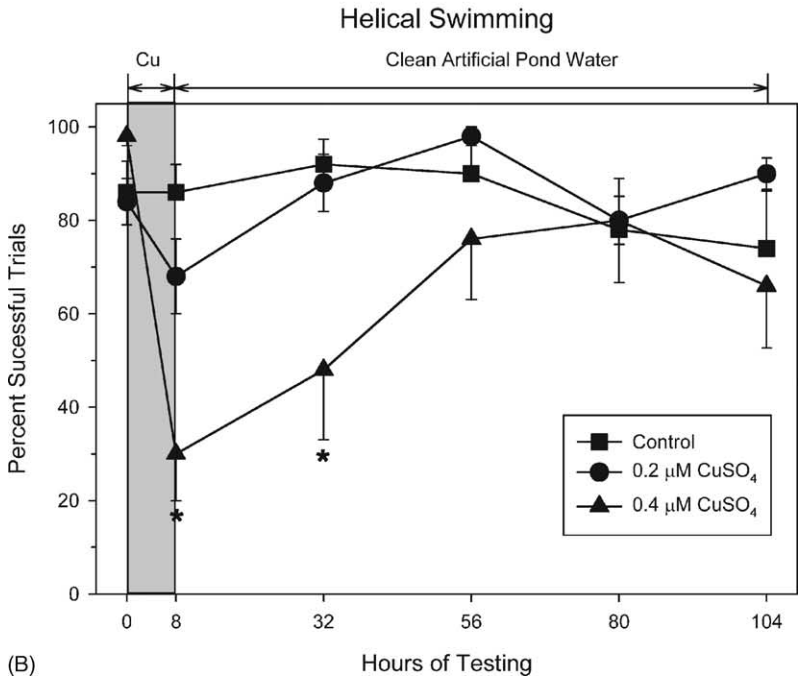
3. Results

3.1. Lethal effects

Because copper toxicity for *Lumbriculus* (and other aquatic animals) depends on various water quality parameters (Schubauer-Berigan et al., 1993; Meyer et al., 2002), we felt it desirable to use a defined water composition for these experiments. The artificial water used in this study has been previously used in this and other laboratories to maintain the medicinal leech *Hirudo medicinalis* for periods of over one year (O'Gara et al., 1991). Some of our *Lumbriculus* cultures have been successfully maintained in this water for over two years. However, this artificial pond water has not been previously used in *Lumbriculus* toxicology studies; therefore, we determined the LC_{50} for copper in this water. The LC_{50} for 24 h exposure to $CuSO_4$ was 0.45 μM (95% confidence interval: 0.36–0.54 μM). We found that almost all worms that succumbed to copper exposure died during the first 24 h of exposure (Fig. 1). We have replicated this finding in a number of other LC_{50} determinations for experiments not reported in this study.


Body Reversal
Cu
Clean Artificial Pond Water
Percent Sucessful Trials
100
80
60
40
20
0
0
8
32
56
80
104
Hours of Testing
Control
0.2 μM CuSO4
0.4 μM CuSO4
*

(A)


Helical Swimming
Cu
Clean Artificial Pond Water
Percent Sucessful Trials
100
80
60
40
20
0
0
8
32
56
80
104
Hours of Testing
Control
0.2 μM CuSO4
0.4 μM CuSO4
*

(B)

3.2. General sublethal effects

During our experiments on the sublethal effects of copper exposure, we observed the individual worms more closely than during the LC_{50} determination. Worms often became hyperactive when first placed in copper-containing water. Writhing and coiling behaviors were commonly noted, especially in higher concentration copper solutions. However, copper-exposed animals eventually became lethargic and hyporesponsive to tactile stimulation. In general, the rate of onset of the lethargy & hyporesponsiveness was related to copper concentration. Although not investigated formally, we noted that animals exhibiting lethargy and hyporesponsiveness also exhibited slowed giant fiber conduction velocity. Copper-exposed animals that exhibited near normal motility and responsiveness also had normal or near normal giant fiber conduction velocities. Lethargic and hyporesponsive animals usually exhibited slower than normal giant fiber conduction velocities. At all copper concentrations examined, the appearance of the worms changed during the test duration. The posterior regions of the worm changed in color, becoming pale from their normal reddish coloration. For worms exposed to 0.4 μM copper, this change in color appeared within 30 min of exposure, while at 0.2 μM copper, the color change occurred within 12 h. We also noted that these posterior regions were more likely to engage in autotomy following copper exposure. Another change noted was that when exposed to copper concentrations of 0.4 μM or higher, the body became shorter and thinner within 3 h of exposure.

3.3. Effects of copper exposure on nongiant fiber-mediated behaviors

Copper exposure produced both time- and concentration-dependent reductions in the ability of tactile stimulation to evoke body reversal and helical swimming behaviors. In animals exposed to copper for 8 h and tested at 0, 1, 3 and 8 h of exposure, helical swimming was more severely affected than was body reversal (Fig. 2). The percentage of trials where body reversal was successfully initiated was not altered by exposure to 0.2 μM copper, but was dramatically reduced by 8 h exposure to 0.4 μM copper (Fig. 2A). In contrast, the percentage of successful trials of touch-evoked helical swimming was reduced at 1 h of exposure time to either 0.2 or 0.4 μM copper (Fig. 2B). At the 1 and 3 h durations of copper exposure, there was no statistical difference between the 0.2 and 0.4 μM copper exposure groups; but by 8 h of exposure, the higher copper concentration produced a statistically greater decrement in swimming behavior.

3.4. Recovery of nongiant-mediated behaviors from copper exposure

We next sought to examine the time course of recovery from an 8 h copper exposure. In a separate experiment from that described in Fig. 2, we exposed worms to copper-containing or clean water for 8 h and then transferred all worms to clean water for the next 4 days. As we saw during the 8 h exposure experiment, helical swimming was more severely affected by copper exposure than was body reversal (Fig. 3).

Fig. 3. Recovery of body reversal and helical swimming behaviors from copper exposure. Worms were exposed to one of three copper concentrations (0, 0.2, or 0.4 μM; $n = 10$ per concentration) for 8 h (gray bars) and tested for the ability of tactile stimulation to elicit body reversal or helical swimming at the end of copper exposure and then once a day for 4 days. (A) The ability of tactile stimulation to evoke body reversal in worms exposed to 0.4 μM copper was significantly reduced for up to 24 h after being returned to clean water. Exposure to 0.4 μM copper reduced the ability of tactile stimulation to elicit body reversal at 8 h exposure time. A two way repeated measures ANOVA did not indicate a significant difference due to copper concentration ($P = 0.357$; $F = 1.071$; d.f. = 2, 27); however significant differences existed for both the test interval ($P < 0.001$; $F = 5.264$; d.f. = 6, 162) and the interaction ($P = 0.025$, $F = 2.028$; d.f. = 12, 162). Subsequent Tukey tests indicated that worms exposed to 0.4 μM copper were significantly different from the other two groups at 8 h. In both panels, an asterisk (∗) indicates a significant difference ($P = 0.003$) from the other groups at that test time. (B) The ability of tactile stimulation to evoke helical swimming in worms exposed to 0.4 μM copper was significantly reduced for up to 48 h after return to clean water. A two way repeated measures ANOVA did not indicate a significant difference due to copper concentration ($P = 0.102$; $F = 2.486$; d.f. = 2, 27); however, significant differences existed for both the test interval ($P < 0.001$; $F = 5.765$; d.f. = 6, 162) and the interaction ($P < 0.001$; $F = 4.342$; d.f. = 12, 162). Subsequent Tukey tests indicated that the 0.4 μM group was significantly different from the other two groups at 8 h ($P < 0.001$) and 32 h (24 h after return to clean water) ($P = 0.002$).

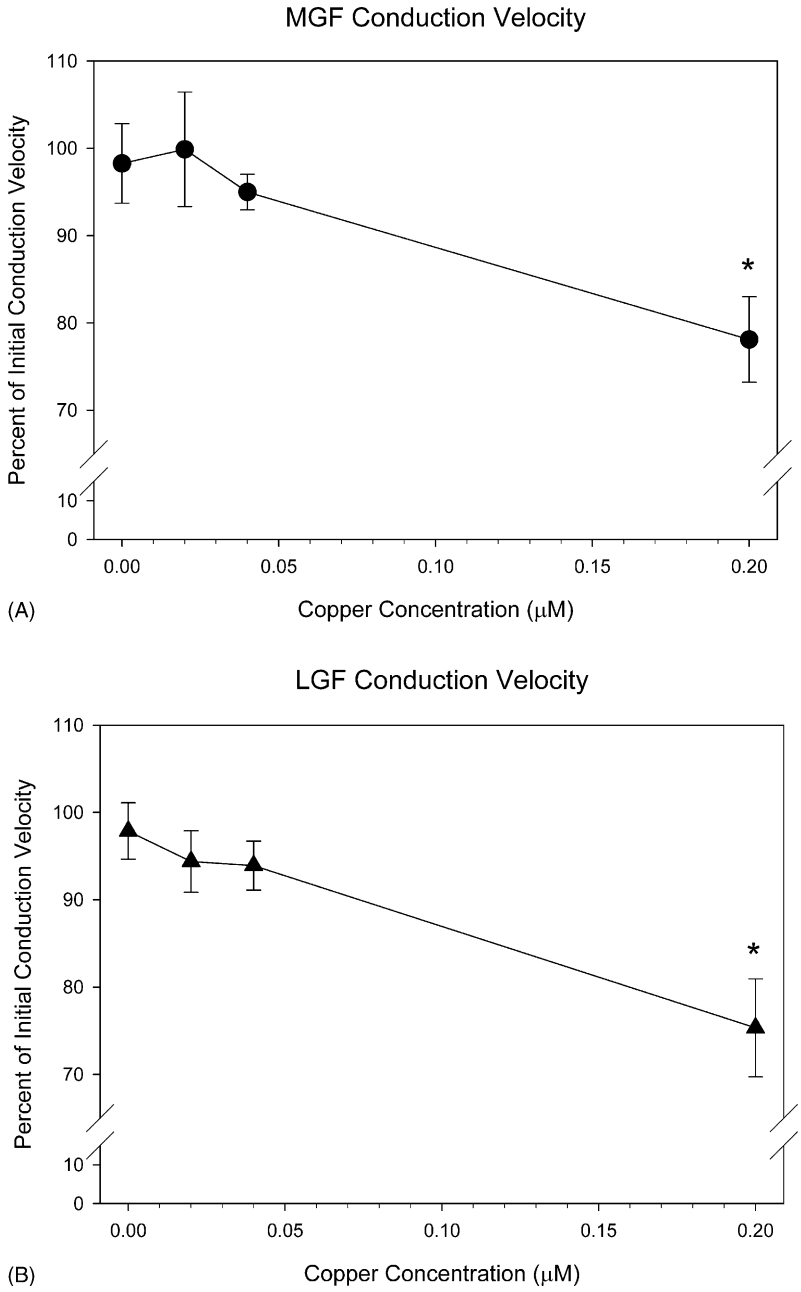


Fig. 4. Concentration–response curves for the effects of 24 h copper exposure on giant fiber conduction velocities. Exposure to 0.2 μM copper induced a significant reduction in MGF (A) and LGF (B) conduction velocities ($n = 10$ per concentration). An ANOVA on the MGF data indicated a significant effect due to copper exposure ($P = 0.01$; $F = 4.395$; d.f. = 3, 35). Likewise, an ANOVA on the LGF data indicated a significant effect due copper exposure ($P = 0.001$; $F = 6.594$; d.f. = 3, 35). Subsequent Tukey tests on both sets of data indicated only the group exposed to 0.2 μM copper (∗) was significantly different ($P < 0.02$) from animals unexposed to copper.

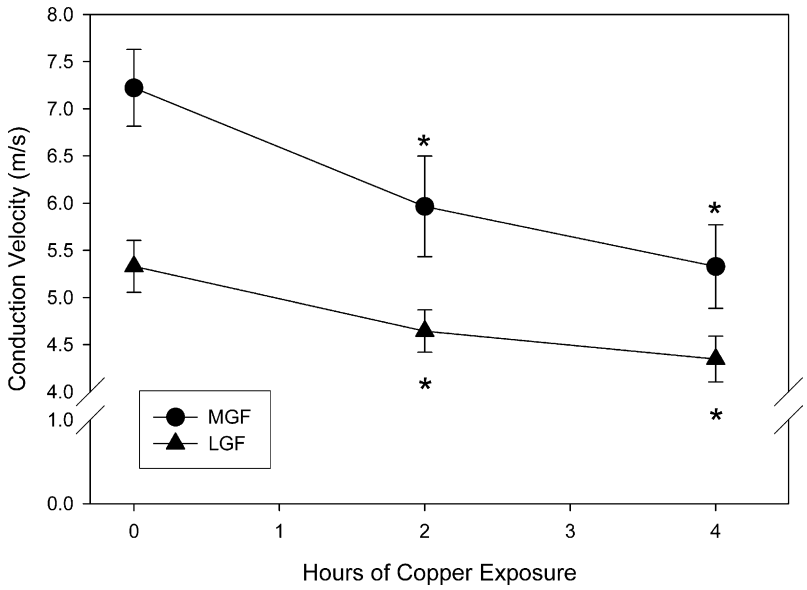


Fig. 5. Exposure to 0.8 μM copper reduced giant fiber conduction velocities within 2 h of exposure onset. Repeated measures ANOVAs on the MGF data ($P < 0.001$; $F = 12.593$; d.f. = 2, 17) and LGF data ($P < 0.001$; $F = 23.299$; d.f. = 2, 17) indicated significant effects due to exposure time ($n = 10$). Subsequent Tukey tests on both sets of data indicated that MGF & LGF conduction velocities at 2 and 4 h of exposure were significantly lower than the initial conduction velocities ($P < 0.02$).

For body reversal (Fig. 3A), exposure to 0.2 μM copper failed to produce a statistically significant inhibition of behavior at any point during the test duration. In a similar manner to that observed in the short-term test (Fig. 2A), exposure to 0.4 μM copper produced a significant reduction in reversal behavior at 8 h. However, within 24 h of transfer back into clean water (at 32 h into the experiment), the behavior of these animals was statistically indistinguishable from control animals. In other words, recovery of reversal behavior from the detrimental effects of exposure to 0.4 μM copper occurred within 24 h of return to clean water. As was true during the short-term test, helical swimming (Fig. 3B) was more severely affected than was body reversal (Fig. 3A). Worms exposed to 0.4 μM copper required 48 h of recovery time in clean water (56 h into the experiment) before their helical swimming behavior was statistically indistinguishable from control animals. It is worth noting that in both experiments (reported in Figs. 2 and 3), tactile stimulation of the individual animals could elicit reversal behavior at normal rates, while the ability to elicit helical swimming was reduced. Thus, the two behaviors (swimming and reversal) showed differential susceptibility to copper exposure, with helical swimming being the more sensitive behavior.

3.5. Effects of copper exposure on giant nerve fiber function

Copper exposure caused a concentration-dependent decrease in both MGF and LGF conduction velocities. Worms exposed to 0.2 μM copper for 24 h exhibited a statistically significant reduction in MGF and LGF conduction velocities; however, lower copper concentrations failed to produce a significant reduction in conduction velocity (Fig. 4). The MGF and LGF were equally affected by 24 h exposure to 0.2 μM copper exposure as indicated by an almost identical percentage reduction in conduction velocity (78% [MGF] versus 75% [LGF] of initial conduction velocity). We could not extend the data plotted in Fig. 4 to higher copper concentrations because 24 h exposure to higher copper concentrations always induced some death of subjects (making

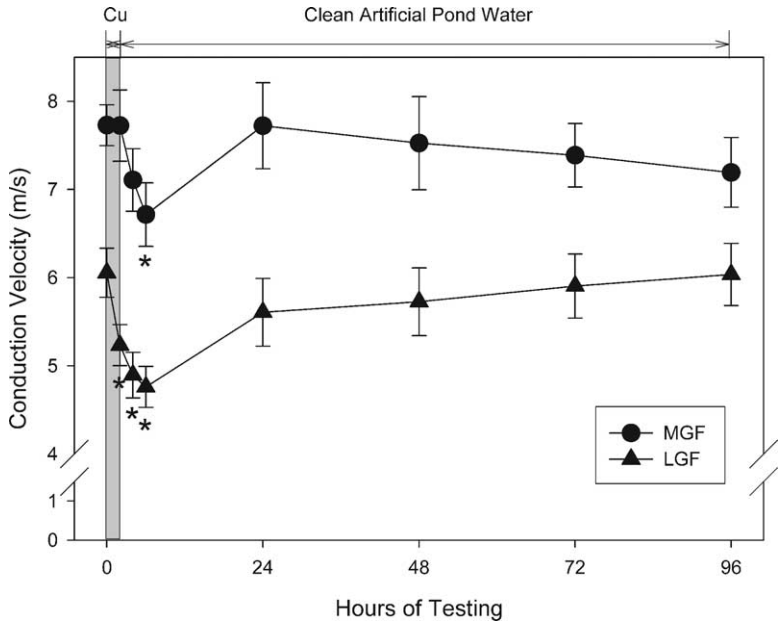


Fig. 6. Recovery from a 2 h exposure to 0.4 μM copper is complex. Worms were exposed to copper for 2 h (gray bar) and then transferred to clean water. MGF and LGF conduction velocities were determined prior to exposure, at the end of exposure, at 4 and 6 h from the beginning of exposure (2 and 4 h after return to clean water), and then at daily intervals for 4 days. Both the MGF and LGF showed a pattern where the conduction velocities continued to fall after return to clean water. The conduction velocities returned to levels statistically indistinguishable from the initial values by 24 h. The number of worms used in this experiment was 15; however not all animals were tested at each test interval. The data at each test interval was obtained from either 10 or 15 animals. Repeated measures ANOVAs indicated a significant effect due to test interval for the MGF ($P = 0.012$; $F = 2.778$; d.f. $= 7, 83$) and LGF ($P < 0.001$; $F = 11.614$; d.f. $= 7, 83$). Subsequent Tukey tests on the MGF data indicated that the conduction velocity first became significantly different ($P = 0.021$) from the initial value at 6 h into the test (4 h after return to clean water). Subsequent Tukey tests on the LGF data indicated that conduction velocity was significantly different ($P \leq 0.001$) from the initial value at 2, 4, and 6 h into the test.

statistical analysis impossible). However, we were able to examine the effects of copper concentrations higher than 0.2 μM by restricting the exposure time. Exposure to 0.8 μM copper induced significant conduction velocity reductions within 2 h, and a further reduction at 4 h of exposure (Fig. 5). In several subsequent experiments, exposure to 0.8 μM copper for durations longer than 4 h always caused the death of some individuals; thus precluding statistical analysis of longer exposure intervals. Even a brief 2 h exposure to 0.4 μM copper induced significant reductions in both MGF and LGF conduction velocities (Fig. 6). Interestingly, giant fiber conduction velocities continued to decrease after the worms were returned to clean water. In fact, for the MGF, the first significant decrease in conduction velocity occurred 4 h *after* return into clean water (6 h into the experiment).

3.6. Recovery of giant fiber function from copper exposure

The results presented in the previous section showed that copper exposure slowed giant fiber conduction velocity. We next sought to determine if the giant fibers could recover from copper exposure, as well as the time course of recovery. As noted above, recovery from a 2 h exposure to 0.4 μM copper was not immediate, and in fact, conduction velocities continued to decrease for several hours after return to clean water (Fig. 6). However, both MGF and LGF conduction velocities returned to levels statistically indistinguishable from their pretreatment velocities by 24 h after return to clean water. Conduction velocities remained at control values for at least the next 3 days (4 days from the start of the experiment). In other words, there was no evidence of delayed toxicity.

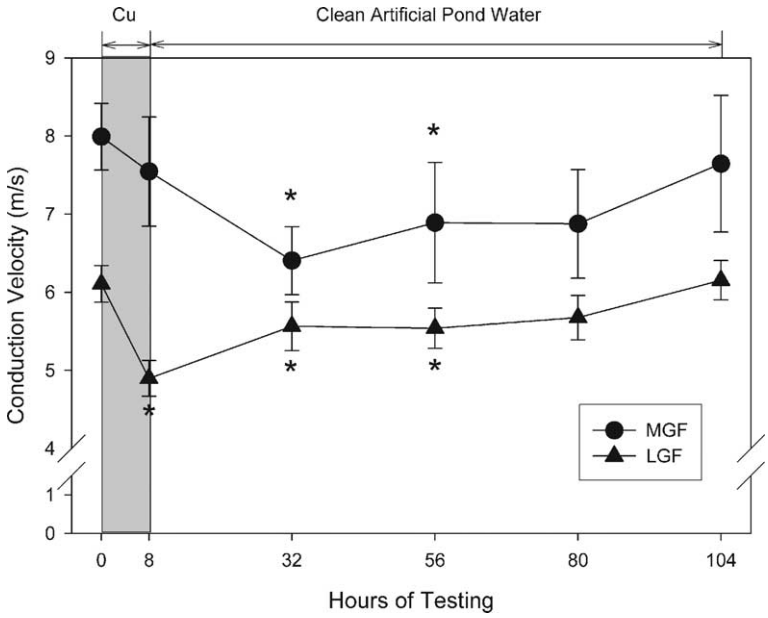


Fig. 7. Recovery of the giant fibers from an 8 h exposure to 0.2 μM copper. Worms ($n = 10$) were exposed to 0.2 μM copper for 8 h (gray bar) and then placed in clean pond water for the next 4 days. Giant fiber conduction velocity was determined prior to copper exposure, at the end of the 8 h exposure, and then once per day for the next 4 days. An asterisk by a point indicates a significant difference from the initial conduction velocity. The data for the MGF was not normally distributed and is plotted as the median ± MAD. LGF data is normally distributed and is plotted as the mean ± S.E.M. A Friedman repeated measures ANOVA on ranks indicated a significant effect of copper on MGF conduction velocity ($P = 0.034$; $\chi^2 = 12.57$; d.f. = 5). Subsequent Tukey tests indicated that the MGF conduction velocity first became significantly different ($P < 0.05$) from the initial value 24 h after being returned to clean water and returned to levels statistically indistinguishable from control values at 72 h after being returned to clean water (80 h into the experiment). For the LGF data, an ANOVA indicated a significant effect of copper on conduction velocity ($P < 0.001$; $F = 15.696$; d.f. = 5, 45). Subsequent Tukey tests indicated that LGF conduction velocity first became significantly different ($P < 0.001$) from the initial value at 8 h of exposure and returned to levels statistically indistinguishable ($P < 0.05$) from control values at 72 h after being returned to clean water.

When worms were exposed to 0.2 μM copper for 8 h, the subsequent recovery of giant fiber conduction velocities required a longer period than was required for a 2-h exposure (Fig. 7). Following an 8 h exposure to 0.2 μM copper, giant fiber conduction velocities did not return to levels statistically indistinguishable from their initial values until 72 h after being returned to clean water (80 h into the experiment).

4. Discussion

4.1. Lethal effects of copper exposure

The LC_{50} for *Lumbriculus variegatus* to copper exposure as determined in this study (0.45 μM) is at the low end of the range of LC_{50} values determined by Meyer et al. (2002) (range of 0.4–5.3 μM in a variety of water compositions) and is somewhat lower than the lowest LC_{50} determined by Schubauer-Berigan et al. (1993) (range of 2.0–7.9 μM). There are several possible reasons for the relatively high toxicity of copper found in this study compared to the studies of Meyer et al. (2002) and Schubauer-Berigan et al. (1993). First, the hardness of the water (combined Ca^{2+} and Mg^{2+}) in the current study was considerably lower than in the other two studies. Second, the concentrations of other cations differed in the waters used in each study, with the water used in this study having the lowest concentration. Third, in this study, animals were exposed to copper in individual containers, while in the studies of Meyer et al. (2002) and

Schubauer-Berigan et al. (1993) several worms were placed in each container. This may have produced higher total organic carbon levels in the assay containers in the two other studies as compared to this study. Living worms would be expected to contribute some organic carbon to the water; however, larger quantities would be released from dead worms during decomposition. The biotic-ligand model predicts that increased levels of each of these three parameters (hardness, cation concentration, total organic carbon) should reduce the bioavailability of metals and thus their toxicity (Paquin et al., 2002; De Schamphelaere and Janssen, 2002). As predicted by the biotic-ligand model, the conditions used during the LC_{50} determination in the current study would be expected to produce relatively high levels of copper toxicity compared to the conditions used in the studies of Meyer et al. (2002) and Schubauer-Berigan et al. (1993). However, it should be noted that Meyer et al. (2002) determined that regardless of any variations in the apparent copper LC_{50} due to water composition, the LA_{50} (lethal accumulation) within the worm's body is approximately constant, regardless of water composition.

An unusual aspect of the LC_{50} data in the present study is that animals that succumbed to the toxic effects of copper usually did so within 1 day of initial exposure (Fig. 1). If the worm was able to survive this first day of exposure, it continued to live for the duration of the test. This suggests that the surviving animals possessed some mechanism (or mechanisms) to deal with copper toxicity that the susceptible animals lacked. The ability of annelids to develop resistance to the toxic effects of copper varies depending on the species examined. Some earthworms apparently do not develop resistance to copper following natural exposure to contaminated soils (Bengtsson et al., 1992; Mariño and Morgan, 1999; Aziz et al., 1999). In contrast, the polychaete *Nereis diversicolor* and the asexually reproducing enchytraeid earthworm *Cognettia sphagnetorum* have apparently developed copper resistance in natural populations exposed to copper (Bryan and Hummerstone, 1971; Salminen and Haimi, 2001b). Whether such adaptation is due to physiological acclimation or genetic adaptation is unknown. However, cadmium resistance of the tubificid oligochaete *Limnodrilus hoffmeisteri* is apparently under control of a single gene (Klerks and Levinton, 1989; Martínez and Levinton, 1996).

4.2. General sublethal effects

Copper-exposed *Lumbriculus* often exhibited altered body morphology, with posterior body regions often appearing thinner and paler in coloration. A similar phenomenon was also noted following copper exposure in the tubificid oligochaete *Tubifex tubifex* (Khangarot, 1991). We also noted that following copper exposure, *Lumbriculus* was more likely to engage in autotomy or fragmentation. Such autotomy could occur "spontaneously" (i.e., no experimental manipulation) or following touch (during the swimming and reversal tests). A similar phenomenon also occurs following copper or other metal exposure in both aquatic and terrestrial oligochaetes (Brković-Popović and Popović, 1977; Khangarot, 1991; Guérin et al., 1994; Sjogren et al., 1995; Meller et al., 1998; Lucan-Bouché et al., 1999). Correlated with this propensity for posterior fragmentation is the finding that a number of oligochaete species preferentially sequester heavy metals in their posterior regions (Ireland, 1975; Morgan and Morgan, 1990, 1998; Lucan-Bouché et al., 1999). At least in *Tubifex*, the worm can autotomize these metal-containing segments in a decontamination process that rids metals from the surviving anterior portion of the worm (Lucan-Bouché et al., 1999).

4.3. Effects of copper on locomotory and escape behaviors

Given the nature of the experiments reported here, it is impossible to definitively determine the site (or sites) of action where copper acts to produce the behavioral and neural abnormalities noted here. Although the behavioral and neural effects noted suggest the copper acts upon the nervous or muscular system, these derangements may be secondary to copper-mediated effects on other physiological systems. A nonexhaustive list of general physiological impairments induced by copper exposure include: disturbances in osmotic balance (Bjerregaard and Vislie, 1986; Boitel and Truchot, 1990; Grosell et al., 2002), metabolic abnormalities (Babu and Rao, 1985; Stürzenbaum et al., 2001; Viant et al., 2002; Khangarot and Rathore, 2003), as well as production of reactive oxygen species and lipid peroxidation (Pourahmad and O'Brien, 2000; Burkitt, 2001; Geracitano et al.,

2002; Gaetke and Chow, 2003). Copper may also alter the physiology of specific organ systems. For example, copper exposure induces a slowing of heart rate in mussels (Scott and Major, 1972; Grace and Gainey, 1987; Gainey and Kenyon, 1990; Curtis et al., 2001). In unpublished work in our laboratory, we have also noted that copper exposure slows the heart rate of *Lumbriculus*. Obviously, inadequate perfusion of the tissues could produce physiological abnormalities in the nervous system or other organ systems. The behavioral and neural abnormalities we note in this study could be secondarily induced effects that are a consequence of copper-induced effects on other organ systems.

Copper exposure adversely affected the ability of worms to produce body reversal or helical swimming behaviors following tactile stimulation (Figs. 2 and 3). Of the two behaviors, helical swimming was more severely affected by copper exposure than was body reversal behavior. The difference in the susceptibility of the two behaviors to copper exposure probably reflects the greater complexity of movements underlying swimming as compared to those of body reversal. The antiparasitic drug ivermectin also more severely affects helical swimming than body reversal behavior (Ding et al., 2001). However, the physiological mechanisms underlying copper-induced and ivermectin-induced deficits in these two behaviors are likely to be different. Ivermectin activates ligand-gated chloride channels (Martin et al., 1998), while copper inhibits at least one of these channels, the $GABA_A$ channel (Ma and Narahashi, 1993; Narahashi et al., 1994; Horning and Trombley, 2001).

Copper exposure also caused a slowing of giant fiber conduction velocity (Figs. 4–7). Although the degree of slowing of giant fiber conduction velocity was both time and concentration dependent, there appeared to be a limit to the amount of slowing that copper could produce. This limit appeared to be an approximately 25% slowing of the conduction velocity compared to the conduction velocity in the same worm prior to copper exposure. Death occurred if the copper concentration or exposure time was increased over those conditions producing an approximately 25% reduction in conduction velocity. However, this 25% reduction in conduction velocity does not represent a physiological limit for action potential conduction in *Lumbriculus*. Rogge and Drewes (1993) documented several neurotoxicants that could reduce giant fiber conduction velocity to about half of untreated animals.

How might copper interact with the nervous system to produce the reduction in conduction velocities noted in this study? Copper is known to directly block a number of voltage-gated ion channels (Århem, 1980; Wojtczak et al., 1996; Horning and Trombley, 2001; Morera et al., 2003). Among the channels affected by copper are the voltage-gated sodium and potassium channels that underlie the production of action potentials. The inhibition of such channels could underlie the reduction in giant fiber conduction velocity induced by copper in the present study. Interestingly, other heavy metals such as cadmium often interact with the same ion channels; however, cadmium does not alter *Lumbriculus* giant fiber conduction velocity (Rogge and Drewes, 1993).

Copper can disrupt synaptic transmission by inhibiting several ligand-gated channels (Lovinger, 1991; Ma and Narahashi, 1993; Narahashi et al., 1994; Trombley et al., 1998; Horning and Trombley, 2001) or by altering neurotransmitter levels (Nemcsók et al., 1984; Reddy and Chari, 1985; Salánki and Hiripi, 1990; Salánki et al., 1993; Kufcsák et al., 1994). In addition, copper may disrupt neurotransmitter release by inhibition of voltage-gated calcium channels (Horning and Trombley, 2001; Morera et al., 2003). Disruption of synaptic transmission could underlie the copper-induced hyposensitivity noted in this study. An even more profound hyposensitivity to tactile stimulation than was noted in the current study is produced in *Lumbriculus* following exposure to the well-known calcium channel blocker cadmium (Rogge and Drewes, 1993).

5. Conclusion

This study documents the adverse effects of copper exposure on the locomotory and escape behaviors of the aquatic oligochaete *Lumbriculus variegatus*. The adverse effects of copper exposure on the neural function noted in this study would be expected to reduce the ability of *Lumbriculus* to escape from predators, an effect noted following copper exposure in other invertebrates (Sullivan et al., 1983; Clements et al., 1989). Such copper-induced disruptions of predator avoidance behaviors may increase predation compared to

unexposed worms; and thus, facilitate the transfer of copper from benthic oligochaetes to animals occupying higher trophic levels.

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