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The effect of $CuSO_4$ on visual development in larval *Danio rerio*

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Abstract

This study sought to learn the effect of copper sulfate ($CuSO_4$) exposure on the visual development of larval *Danio rerio*, also known as zebrafish. Zebrafish were acquired at 1 day post fertilization (dpf) and exposed to either 0, 0.25, 0.75, or 1.25 mg/L $CuSO_4$ solution for 72 hours, and then moved out of solution to continue developing until 9 dpf. On 9 dpf, zebrafish were photographed under a microscope for later length measurement using ImageJ. Zebrafish were also run through an optokinetic response (OKR) behavior assay to determine if the fish are visually processing information. The OKR was run for 30 seconds clockwise and 30 seconds counterclockwise, and the summed number of saccades, or eye movements, was determined. The difference in mean summed saccades between the 0 mg/L, 0.75 mg/L, and 1.25 mg/L treatments was significant, with a decrease in the number of saccades matching the increase in $CuSO_4$ concentration. The difference in average length between treatments was not significant, with no strong visual trend in the results. These results imply that visual development in larval *Danio rerio* is negatively impacted by $CuSO_4$ concentrations. As the vertebrate eye is highly conserved, the zebrafish can serve as a model for human visual development, and negative impacts of $CuSO_4$ exposure on human visual development might be of concern.

Introduction

There is a known problem of chemical pollution in waters all over the globe. Coming from a variety of sources and causing a plethora of health concerns, people are worried about how water pollution could be affecting them (Wang et al. 2018). There is concern about the sources of pollutants and what short- and long-term health effects could result from exposure to different chemicals (Wang et al. 2018).

Copper is a toxic pollutant found in waterways across the planet and it has a variety of sources. One source of copper is antifouling paint. Boats and other water vessels' hulls are coated in antifouling paint to reduce the presence of marine life buildup on the surface. These paints are most frequently comprised of copper due to the effect of the chemical's toxic properties on marine life (Turner et al. 2007). The antifouling paint is designed for the copper to slowly dissolve into the water to kill off the fouling organisms on the boat (Turner et al. 2007). In a 2021 study by Paz-Villarraga, Castro, and Fillman, where data was reviewed from six countries and over one thousand paint products, cuprous oxide (Cu_2O) was found in 76.1% of antifouling compounds, followed copper pyrithione ($C_{10}H_{10}CuN_2O_2S_2$) found in by 28.8% of the compounds. Multiple sources of copper are released into any body of water occupied by a painted boat, as copper leaches into the water from the antifouling paint. Paint chips also flake off the boat during routine cleaning processes, which is thought to cause heavy pollution near marinas and other boat storage locations (Krahforst et al. 2002; Turner et al. 2007).

Another source of copper pollution in waterways is copper piping. Copper pipes were used as a lead pipe replacement in areas with soft water because lead pipes were more likely to corrode due to the relative softness of the metal (Campbell 2021). While copper pipes are no longer common practice, many areas throughout the world still have them as a part of their water systems (Campbell 2021). Corrosion of pipes over time can lead to excess amounts of copper in drinking water (Alam & Sadiq 1989; Insanullah et al. 2016). A study by Alam and Sadiq (1989) found levels of copper over 2 μg/mL in drinking water that moves through copper pipes, which is over the safety limit recommended by the World Health Organization (WHO), the U.S. Environmental Protection Agency (EPA), and the Center for Disease Control (CDC). The copper limit in drinking water set by the EPA is 1.3 μg/L, which has been supported by other regulatory agencies as a safe limit to avoid gastrointestinal harm that has been reported from concentrations as low as 2 μg/L (Taylor et al. 2020). Alam and Sadiq (1989) found that an increase in copper pipe length travelled led to an increase in copper concentrations in the water, especially when travelling through branch distribution pipes.

Copper is even intentionally put into bodies of water as a treatment. Copper sulfate has been used for over a century to treat lakes for harmful algal blooms due to its algicidal properties, its relatively low impact on other aquatic organisms compared to alternative treatment methods, and its lower cost compared to other effective methods such as ozonation, biochar, and electrochemical oxidation (Ahmed et al. 2021; Goudey R.F. 1936; Haughey et al. 2000; Watson et al. 2024). The copper sulfate treatment method is still commonly used, even though multiple studies have been published that note abnormally high levels of copper in the water (Haughey et al. 2000; Watson et al. 2024).

Copper bonds easily with particulate matter, which causes it to build up in the sediments of waterways (Haughey et al. 2000). Copper can also bind with suspended sediment in the water column of surface water (Whitehead 1988). Additionally, copper ends up in waterways when it is released from sediments, especially in deeper bodies of water. Copper is released slowly from the sediment, taking months to desorb out into the water (Haughey et al. 2000).

Despite the low level of threat perceived by groups that use $CuSO_4$ to treat waterways, copper has many possible dangers to organisms that ingest or otherwise accumulate too much of the chemical (Haughey et al. 2000). Copper addition and pollution both change the chemical makeup of the water, creating new conditions in the environment. The Cu^{2+} in the ecosystem can be taken up by organisms in the water, affecting their ability not just to thrive but to survive (ATSDR 2024). Living organisms need copper to survive, but high quantities can be dangerous

(ATSDR 2024). Excess copper in humans can cause mild symptoms like the inability to take up other key nutrients (ASTDR 2024). In extreme cases, copper toxicity can cause organ damage and eventual failure (ASTDR 2024).

Copper leaching can be incredibly dangerous to the organisms that inhabit impacted waters. Many aquatic organisms develop externally, meaning they absorb chemicals through the water they are exposed to during development. This, like exposure to other chemicals, can cause damage to a variety of aquatic, vertebrate, and invertebrate species (ATSDR 2024). A popular model vertebrate organism that can be used to determine the dangers of $CuSO_4$ in waterways is *Danio rerio*, more commonly known as the zebrafish.

Zebrafish are a long-established vertebrate model and common research organism (Hill et al, 2005). These vertebrates have extensive genetic data available and have been used to study a variety of fields, including toxicology, genetics, and neurology. For example, to help uncover the impact of heavy metal and microplastic pollution on fish gills, Santos et al. (2022) exposed *Danio rerio* to microplastics and copper in conjunction, discovering higher levels of oxidative stress in the fish exposed to higher concentrations of copper and microplastics. Hernandez et al. (2006) applied copper concentrations to *Danio rerio's* neuromasts, which are important sensory hair cells that allow the fish to sense the flow of water around them. These neuromasts are similar to the sensory hair cells found in all vertebrates. Hernandez et al. (2006) found that concentrations of copper as low as 1μM can have effects including reduced neuromast regeneration and reduced neuromast growth, indicating a possibility that copper exposure causes damage to sensory hair cells in other species of vertebrate as well.

Zebrafish are often used as a model for the human visual system (Link and Collery, 2015). In 2007, Parker and Connaughton found that nicotine exposure as low as 20 μM reduces the eye diameter growth of zebrafish, providing further evidence of negative impacts that nicotine may have on humans. In 2021, Ganzen et al. determined that carvedilol, a beta-blocking drug, could be a possible treatment for retinitis pigmentosa, a retinal degeneration affecting every 1 in 4,000 people. Ganzen et al. tested the drug on zebrafish with artificially-induced retinal degeneration and found that carvedilol could be effective in helping humans with the disease to retain or even regain their vision, as the drug effectively helped zebrafish with a similar condition. Zebrafish are also used to determine how the eye naturally develops, as when Linda Nevin et al. (2008) studied whether activity was necessary to form certain stratified layers in the eye and found that this was not the case.

Zebrafish develop quickly, with their visual recognition being fully developed in 72 hours post fertilization (hpf; Schmitt & Dowling 1999). At 11.5 hpf, the eye begins to form, with the eye cup being well developed at just 24 hpf. The retina then nearly doubles in size from 24 hpf to 36 hpf. Ganglion cells, one of the retinal cell layers responsible for transmitting visual information to the brain, can first be found at 32 hpf, and continue to develop until 40 hpf. Synapses begin forming in the inner nuclear layer of the eye at 50 hpf. The entirety of retinal development occurs between 28 and 76 hpf (Schmitt & Dowling1999). Zebrafish are capable of responding to an optokinetic response OKR behavioral bioassay by day 5 (Neuhauss 2003).

The quick visual development of *Danio rerio* allows for fast-paced experiments. Due to the development of zebrafish being external, the fish can also be easily exposed to chemicals like $CuSO_4$ through the water they reside in, creating an accessible method of application. The fish's visual systems are advanced, with a highly conserved eye comparable to humans and other vertebrates (Zimmermann et al. 2018). Since it would be unethical and dangerous to run chemical studies in human beings' eyes, zebrafish serve as a model that creates comparable results (Link and Collery, 2015).

One method of studying zebrafish visual response is the optokinetic response (OKR) behavioral bioassay (Brockerhoff 1995). The OKR is a behavior assay that helps determine whether zebrafish react to visual stimulus. Larvae are immobilized in methylcellulose, then placed in the center of a rotating drum with alternating color stripes. Eye flicks, or saccades can be recorded as a measure of how well the larvae can see. Successful ORK outcome suggests that the visual system of the larvae (lens, retina, optic nerve and visual processing portions of the brain), as well as the muscles that move the eye, have all developed properly (Brockerhoff 1995; Schmitt & Dowling 1999).

The hypothesis for this study supposed that increasing levels of $CuSO_4$ would have an increasingly negative effect on the visual development of larval *Danio rerio*.

Methods

Larval *Danio rerio* were ordered from Carolina Biological Supply and received at approximately 13 hours post fertilization (hpf). The zebrafish were separated into eight petri dishes, which were 35 mm x 10 mm. Two replicate dishes of 16-17 larvae each were used for 0 mg/L $CuSO_4$, 0.25 mg/L $CuSO_4$, 0.75 mg/L $CuSO_4$, and 1.25 mg/L $CuSO_4$. The concentrations were determined based on lethal limits found in Takemara et al. (2024). The concentrations of $CuSO_4$ were created with solid $CuSO_4$ powder and 'blue water,' which is an anti-fungal mix made with methylene blue, marine salt, and reverse osmosis (RO) water. Each petri dish was filled with 20 mL of the given concentration mixture.

The petri dishes were placed into an incubator set to 28.4°C. They received water changes, mortality counts, debris removal, and feeding every 24 hours. Larvae were treated with $CuSO_4$ from 13 to 85 hpf (72 hours), encompassing the period of visual development (Schmitt and Dowling, 1999). Following this, all groups were transitioned to 'blue water' for the duration of the experiment.

Data was collected at 10dpf. The fish were placed in groups of four in 35 x 10 mm petri dishes which were lined with methylcellulose to immobilize the fish (Brockerhoff 1995). These small petri dishes were then placed into the OKR. The fish were filmed while the OKR drum spun 30 seconds clockwise and 30 seconds counterclockwise at a rate of 120 stripes per 60 seconds (Brockerhoff 1995). The fish were then photographed for later analysis of total length and to note deformities. A total of 22-24 larvae were analyzed and photographed per treatment.

The OKR videos were reviewed to count saccades of each zebrafish in both the clockwise (CW) and counterclockwise (CCW) directions. Each fish's CW and CCW saccades were summed and averaged by treatment.

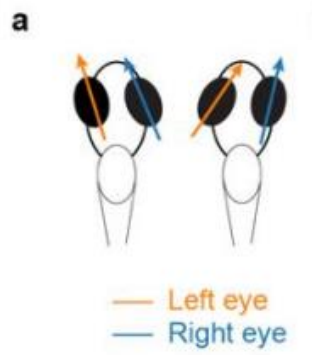

Figure 1: An example of a saccade. There is a visual movement of the eye from the left to right, which “flicks” back to its original position and repeats (Choudhary et al 2023).

The saccades were analyzed using a one-way ANOVA and a *post hoc* multiple comparisons test. The length measurements were compared by photographing under a Leica SGD Greenough stereo microscope under 10x magnification, with ImageJ being used for analysis. A one-way ANOVA and a *post hoc* multiple comparisons test were run on the data.

Results

There was a significant decrease in the mean summed saccades with the increase of $CuSO_4$ concentration (Figure 1). The 0.75 mg/L and 1.25 mg/L showed significantly lower mean summed saccades than the control group of 0 mg/L. The 0.25 mg/L treatment group was lower; though not significantly different from the control, the saccade number was significantly greater than the two highest dosages.

Percent mortality did not yield any significant results; however, quantitative differences were measured. The highest percent mortality was in the 0.25 mg/L treatment, and the lowest mortality rate was in the 0 mg/L treatment (Figure 2).

Increasing $CuSO_4$ concentration resulted in decreased standard length measurements. The 1.25 mg/L treatment group expressed significantly lower lengths than the 0 mg/L, 0.25 mg/L, and 0.75 mg/L treatment groups. The 0.75 mg/L was significantly shorter than the 0.25 mg/L, but not the 0 mg/L group (Figure 3).

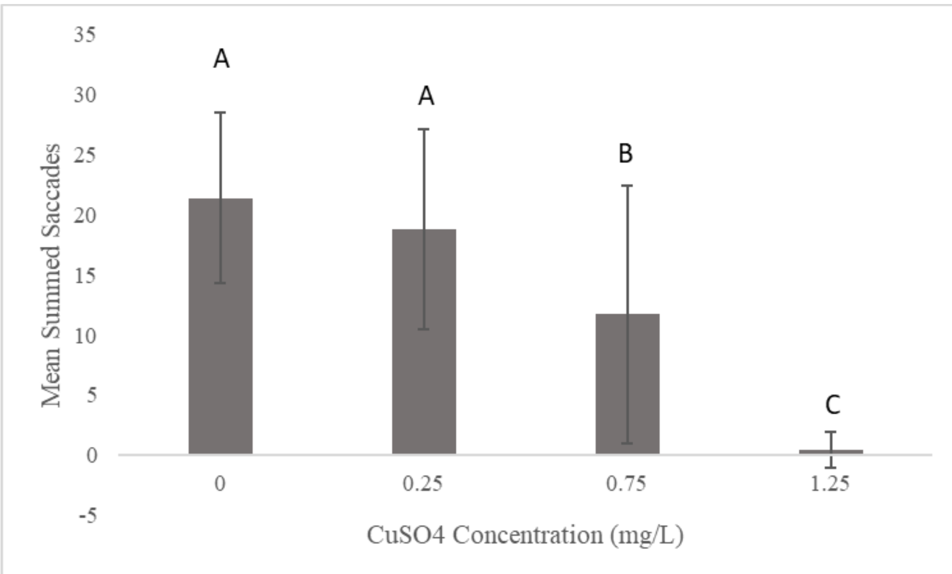

Figure 1: The effect of $CuSO_4$ exposure between 13 and 85 hpf on the mean summed saccades in larval *Danio rerio* (n=22-24). Saccades summed from 30 second clockwise and counterclockwise run in an OKR rig at 9 dpf. Letters are used to indicate significance ($p < 0.05$), and bars indicate standard deviation.

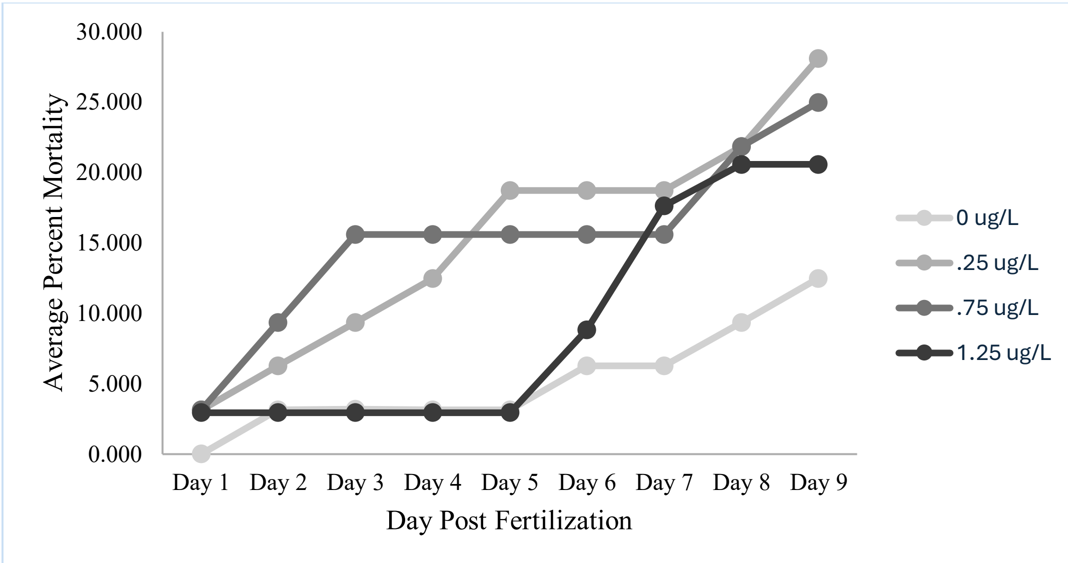

Figure 2: The effect of $CuSO_4$ exposure between 13 and 85 hpf on percent mortality in larval *Danio rerio* (n=40-42). Mortality counts were taken every day over the span of 9 days post fertilization.

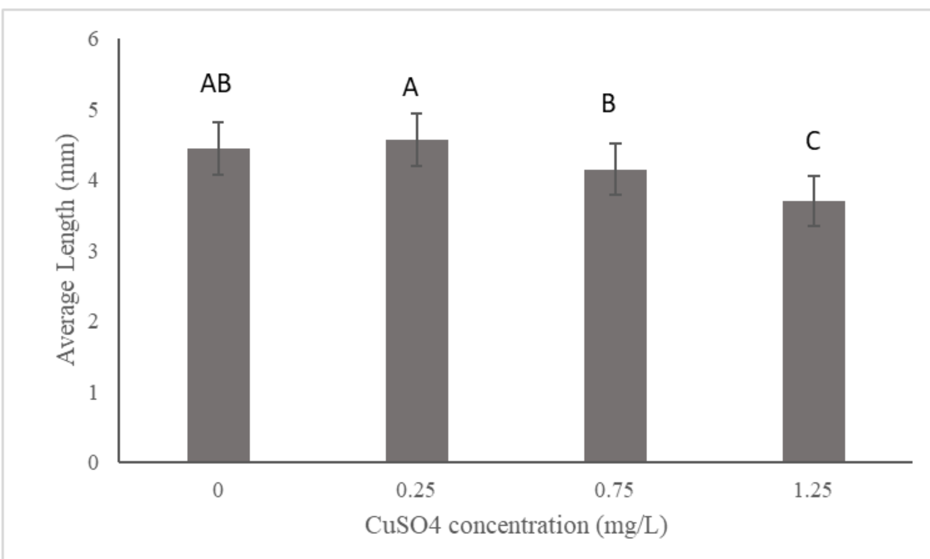

Figure 3: The effect of $CuSO_4$ exposure between 13 and 85 hpf on average standard length in mm of larval *Danio rerio* (n=22-24). Fish were photographed under 10x magnification and

measured using ImageJ on day 9 post fertilization. Letters are used to indicate significance ($p < 0.05$) and bars are used to indicate standard deviation.

Discussion

The focus of this experiment—the mean summed saccades— provided interesting results that largely supported the hypothesis. The hypothesis assumed that an increase in $CuSO_4$ concentration would cause a decrease in visual development in larval *Danio rerio*. Including a dose-dependent group, a significant decrease in the number of saccades was observed between the 0 ug/L, 0.75 ug/L, and 1.25 ug/L treatments. However, the 0.25 ug/L treatment group did not have significantly less saccades than the control, though the number was greater than the two highest dosages. The cause of the 0.25 ug/L results could be that the low dosage does not have an effect on the vision of zebrafish, indicating that low doses of $CuSO_4$ may not be a problem and only higher doses should be monitored.

There was not a noticeable difference between clockwise and counterclockwise saccades in any treatment, as it varied heavily by fish. Notably, many of the fish in the 1.25 mg/L treatment group had 0 total saccades, indicating a possible complete lack of eye function and recognition of movement. Saccade total also varied heavily from fish to fish, as saccades within the same treatment group would vary up to 37 saccades. Fish in the 1.25 mg/L group were most likely to have no response at all to the rotation of the drum.

The mortality counts did not give a strong indication of variation among the treatments. The highest mortality rate was in the 0.25 mg/L treatment group, which was unexpected due to the lower concentration than the other two treatment groups. It was predicted that the 0.75 mg/L and 1.25 mg/L groups would have a higher mortality rate due to the higher concentration of $CuSO_4$.

The highest average length was in the 0.25 mg/L treatment, and though this was not significantly different from the control group it was greater than the two higher dosage groups. The lowest average length was found in the 1.25 mg/L treatment, as predicted.

Copper has a well-documented effect on fish. Martin Grosell (2011), in his book titled *Fish Physiology*, dedicated a chapter to copper toxicity. Waterborne copper toxicity is known to impact a variety of functions, including cellular, protein, and organ, such as the visual system. The results of this study further corroborate the existing literature on copper as a toxic chemical to fish.

Pilehvar et al. (2020) determined through their study using two types of behavioral assays that copper has a negative impact on the learning ability and brain function of zebrafish. Specifically, fish response within the study's reaction time increased with higher copper concentrations when reacting to different color visual stimulus. The results of the Pilehvar et al. study correlate with the results of this study that indicates copper has a negative impact on recognition of visual stimulus.

Previous studies have found other physiological effects on zebrafish due to copper exposure. As previously mentioned, Santos et al. (2022) found high levels of oxidative stress in zebrafish exposed to copper. Oxidative stress can lead to decreased growth and apoptosis, eventually causing organ damage or failure. These findings coincide with the reduced visual development found in this experiment. It is possible that oxidative stress could be responsible for the decreased visual response in the zebrafish, or at least partially the cause of the lack of response. Hernandez et al. (2006) found that copper concentrations can damage the neuromasts in zebrafish, an important part of the sensory system. If both neuromasts and the visual system are damaged by copper, zebrafish are in danger of not being able to respond to the environment around them, including responses to food sources and predators. Reduced reactivity puts the fish's ability to survive in jeopardy.

There are several potential sources of error that could have occurred throughout this experiment. While water changes consistently occurred daily, they occurred at irregular times of the day due to personal time constraints. There were two days when some petri dishes received water changes earlier in the day than others, which could lead to longer amounts of time being spent with debris in the water, causing a possibility of increased harm to the fish.

One petri dish also had the majority of its water spilled out at one point during the study. While all of the fish remained inside the dish, the spill could have led to a stress response that decreased their growth and visual development. Additionally, petri dishes had to be removed from the incubator daily for around an hour and a half, meaning that there was a temperature variation in the water that could have impacted growth or visual development since the fish were not in ideal conditions.

As *Danio rerio* are often used as a model for humans, especially for eyes (Link and Collery, 2015), the results of this study have broader implications than negatively affecting fish and wildlife. Since this experiment found that $CuSO_4$ may cause harm to eye development, it could be furthered to indicate that pregnant women should pay careful attention to the copper concentrations in the water they consume and submerge in, since copper concentrations could impact the visual development of the baby in their wombs. It is worth noting that zebrafish are not a perfect model for human development, as more complex brain function and patterns in humans are difficult to replicate and certain chemicals may have different impacts on humans than they do on zebrafish (Babin et al. 2014).

The results of this experiment have implications for the future use of $CuSO_4$ and possible concerns about the chemical. This study may help promote research into alternatives for $CuSO_4$ and other copper compounds. If more people are aware of the potentially harmful impact that copper may have on both wildlife and humans, communities are more likely to invest in alternatives. For example, awareness about copper toxicity could help promote a new algacidal treatment instead of spreading $CuSO_4$ in lakes to kill harmful algal blooms. The results of this study could also make people more conscious of the copper they use, such as in boat paint, and encourage awareness surrounding the impact individuals have on the natural environment.

Conclusion

This experiment was conducted in the effort to gain an understanding of the impact copper sulfate has on the visual development of larval *Danio rerio*. Copper is used for a variety of functional purposes throughout the world, including the construction of pipes, batteries, and car tires, and copper sulfate is used in boat paints and algicidal treatments. Copper is an important chemical of life for organisms, but it can be dangerous in large quantities. Overexposure to copper is known to cause organ failure and to lead to eventual death. This study found that copper has a negative impact on the visual development and length of zebrafish, indicating problems for zebrafish survival. In this study, zebrafish serve as a model for any fish species which might be exposed to copper toxicity in the wild. If wild fish species are impacted as the zebrafish in this study, they may not be able to visually locate food sources or predators. Decreased visual acuity would decrease survival and reproduction, potentially leading to a decline in population size, which may lead to an increase in the presence of a species' food sources and cause an overabundance that could alter an entire ecosystem. A decreased fish population might also have a cascading effect on a species' predators, which would lose access to a food source and in turn decrease in population, furthering negative impacts on the ecosystem.

The results of this study further affirmed that there is a need for increased attention on copper regulation. While the current EPA limits stay at 2 mg/L, this study suggests that there may be a need for reevaluation of these limits and closer attention to what harm can take place within current limits.

Future research could include what part of the visual pathway of larval *Danio rerio* is being damaged by the $CuSO_4$. There is a possibility that a specific layer of the eye is damaged, or that the brain itself is harmed by the chemical exposure. If research is conducted to understand what part of the visual system is harmed, work can be done to help prevent or even reverse impacts of the $CuSO_4$ exposure. Further studies could also lead to an increased understanding of why copper is so dangerous and what specific properties make it harmful, which would help environmentalists to more successfully target and reduce dangerous copper pollution.

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