International Journal of Social Science and Economic Research

ISSN: 2455-8834

Volume:08, Issue:10 "October 2023"

INVESTIGATING THE COMBINED EFFECTS OF HEAVY METALS COPPER AND ZINC ON OXIDATIVE STRESS LEVELS OF LYMNAEA STAGNALIS

Saanvi Vemareddy

Academies of Loudoun, USA

DOI: 10.46609/IJSSER.2023.v08i10.022 URL: https://doi.org/10.46609/IJSSER.2023.v08i10.022

Received: 2 October 2023 / Accepted: 18 October 2023 / Published: 6 November 2023

ABSTRACT

Biodiversity, the variety of life within a particular ecosystem or habitat, has been a growing concern in freshwater ecosystems. Streams become more polluted as a result of industrial and agricultural runoff, specifically through heavy metals found in industrial waste as well as pesticides. The impact of pollution on stream health could be studied through bioindicator macroinvertebrates, as their health and mortality reflects the quality of their environment. If an a bioindicator organism's environment were in poor health conditions, it would reflect on the organism by creating health issues and defects. This study aims to determine the combined effects of heavy metals Copper sulfate and Zinc nitrate on stream health through investigating the oxidative stress levels of macroinvertebrates *Lymnaea stagnalis*. This involved exposure of *Lymnaea stagnalis* to the following experimental groups: 15 g/L Zinc nitrate, 15 ug/L Copper sulfate, and regular pond water that will be created in the lab. The disparity between oxidative stress levels of each group would be evaluated. This would be done through a thiobarbituric acid reactive substances assay, which measures the creation of lipid peroxidation products that result from oxidative stress. The creation of lipid peroxidation products indicates oxidative stress through producing a pink color, which will be measured through aspectrophotometer. Additional work is needed to corroborate preliminary results and draw final conclusions. This study may be able to support the creation of new water treatment methods through evaluating the extent to which heavy metals play a significant role in water pollution.

Introduction

Macroinvertebrates are miniature creatures that lack a backbone, yet are still large enoughto see with the naked eye. Biomonitoring is the process of measuring the changes in ecosystems, including natural habitats, populations, and species (Bondaruk et al., 2015). As

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macroinvertebrates spend the entirety of their lives in the same habitats, they tend to be good indicators of environmental health, making them a useful tool in biomonitoring streams and other small waterways, specifically freshwater ecosystems. If a stream were polluted by chemicals and toxins, the macroinvertebrates residing within it are bound to face effects. As streams lead into reservoirs, pollution would greatly affect our water supply. Contamination of water supply, most significantly that of drinking water, can lead to critical deterioration of public health (Importance and Benefits of Stream Monitoring, n.d.). The damaging of an ecosystem due to declining stream health would negatively affect humans, as ecosystems clean water, purify air, maintain soil, regulate climate, and provide natural resources (Chivian et al., 2010). Additionally, small streams lead into larger ecosystems, thus the protection of streams preserves the health of the larger ecosystem as well. Therefore, it is essential to biomonitor streams in order to survey stream and ecosystem health. Streams are often subject to pollution from various chemicals and substances, especially from industrial runoff.

Copper is the 29th element on the periodic table. It is a transition metal, found naturally in a metallic state. It is also a good conductor of electricity and heat. Although copper is essential in fundamental biological processes such as energy metabolism, it has been found to be harmful because it can accelerate the generation of toxic reactive oxygen species (Cavaletti et.al., 2022). Reactive oxygen species are highly reactive chemicals formed from oxygen gas. The metal has also been observed to impair growth in both plants and animals. Specifically, copper sulfate will be used in this study. The second heavy metal involved is Zinc nitrate. Zinc is an essential heavy metal in the human body, as it relates to the building of proteins and growth of cells (Read et.al., 2019). Zinc can lead into streams through metal manufacturing and chemical zinc industries.

In large amounts, it can be considered toxic to aquatic life. Zinc and Copper were specifically chosen in this study for their relatively high concentration and effects in streams. Heavy metals are released into streams from industrial or consumer waste, thus leading to stream toxication.

Similar past research relating to stream health has looked into the diversity of aquatic benthic macroinvertebrates, specifically through alpha, beta, and zeta diversities of regional and community streams. Stochastic and niche assemblies were also compared, with the overall purpose of identifying patterns in the ecological monitoring of streams (Simons et al., 2019).

This is important because excessive chemical concentration would affect the diversity of these ecosystems. Additional research investigated the impact of land use on microbial diversity and stream health. Macroinvertebrates were again used as indicators of ecosystem health, classifying it as either good, fair, poor, and very poor. It was found that microbial diversity differed among watersheds with relatively high use of the land, either agricultural, urban, or forested. The community similarity also decayed with increasing geodesic distance across the study region

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(Laperriere et al., 2020). Furthermore, ongoing research in Ghana has been examining the health of the lower basin of the Volta river through sampling macroinvertebrates. While referencing a set accepted water quality value, the aim was to determine the association between macroinvertebrate distribution and physiochemical and environmental variables within the stream. It was found that pesticide concentrations and nutrient concentration levels were generally low. Additionally, physicochemical parameters and abundance of macroinvertebrates were heavily affected by the seasons (Kwakye et al., 2021). It is evident that extensive research has been conducted relating to the pollution of streams. However, most of the studies have focused on one specific factor, rather than multiple.

The model to be used is *Lymnaea stagnalis*, also known as the great pond snail. They are a common model in environmental biology related research due to their well-known anatomy and embryonic development process. They are mostly used in neurobiology, but can also be usedto study host-parasite interactions, ecotoxicology, evolution, genome editing, human disease modeling, and more. Pond snails are naturally found throughout Northern America, Europe, Asia, and Australia. They are generally herbivores and fall prey to leeches and crayfish. During this study, focus will mainly be placed on their embryos. Pond snail embryos develop in cocoons of about 60 to 90 eggs. They lay approximately two to three egg masses per week, each of which contain 100 to 150 eggs. These eggs are transparent, allowing for visible tracking of the embryonic development (Fordo, 2020). As pond snails are macroinvertebrates, they could beused as a model to biomonitor the effects of specific elements leading to stream toxication.

Oxidative stress is a condition during which there is an imbalance between antioxidants and reactive oxygen species in the body (Pizzono et.al., 2017). Antioxidants are substances in the body that protect against free radicals, which is a molecule with an unpaired electron, thus making it highly reactive. Reactive oxygen species are free radicals. When there are no longer a sufficient amount of antioxidants to protect the body from free radicals, it can result in oxidative stress. This is harmful because it can break down cell tissue, cause damage to DNA, and much more. Oxidative stress also leads to lipid peroxidation, a process during which the free radicals attack fatty acids in the body (Ayala et.al., 2014). Lipid peroxidation products create MDA, which can be calculated. This was chosen as a dependent variable because it can be caused by pollution and exposure to industrial chemicals and pesticides.

As previously stated, a significant amount of research has already been done relating to the pollution of streams, specifically due to a single heavy metal. This study differs by looking into the combined effects of two pollutants: the heavy metals Zinc nitrate and Copper sulfate. It is possible that although they might affect pollution one way individually, their combined effects could be different. This will be done by exposing *Lymnaea stagnalis* to varying concentrations of each pollutant in their water. The resulting changes in oxidative stress levels will be studied.

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Changes in the macroinvertebrate correlate to changes in stream quality, as they are typically used as a model for biomonitoring related studies. If significant results are found, they could be used to further understand how streams get toxicated. This information could potentially be used to develop a method of water treatment, in order to maintain the quality of freshwater ecosystems.

The independent variable in this study is the concentration of Zinc and Copper in *Lymnaea stagnalis* environments. The dependent variable is the oxidative stress within snails, specifically the MDA concentration. The controls include snail care procedures, including their food, tanks, light/dark schedule, and water change schedule. It was hypothesized that if the snails were exposed to either Copper or Zinc in their environment, they would express greater levels of oxidative stress. This seems logical because heavy metals are pollutants and will likely have a negative effect on the health of *Lymnaea stagnalis.*

Materials and Methods

Lymnaea stagnalis Care Protocol

All snail populations were grown from previous lab generations. They were fed small leaves of lettuce once every two days. Once a week, the water in each tank was changed. This involved pipetting out debris through the use of a serological pipette and disposing the waste down a drain. For larger tanks, a siphon method was used. A suction was created within a tube using a batch of pond water, which was then let go into the tank. This would act as a vacuum andcollect all debris as maneuvered. Once the debris was cleared, a new batch of pond water was created by adding 1.55g of Calcium carbonate and 10g of Baking soda to 5 gallons of distilledwater.

Data Collection Protocol

In order to measure the effects of heavy metals on *Lymnaea stagnalis*, it was first required to determine the concentration of each metal at which the snails would not immediately die. This ensures that the metal would be able to take effects that would not be immediately lethal. A toxicity assay would be run to confirm the concentrations of Copper sulfate and Zinc nitrate. Starting at 100 ug/L the following procedure was repeated by lowering the concentrations at equal increments each time until more than 80% of the snails were alive. From a laboratory group of healthy snails, around 10 just-hatched juvenile snails were collected and placed in two petri dishes with the respective concentration of heavy metal in each. After an exposure period of 48 hours, the mortality of the juvenile snails was counted under a microscope. This process was repeated until successful results were achieved at 15 ug/L of Copper sulfate and Zinc nitrate.

Having confirmed the concentration of each heavy metal, it was possible to move on to the

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exposure period. This study consisted of three groups: the control group, 15 ug/L of Copper sulfate, and 15 ug/L of Zinc nitrate. Concentrations in the tanks were created by making a stock solution for each heavy metal and adding the corresponding amount to pond water in the tank. The calculated amount of stock solution was added each time water was changed in a tank. Each tank, corresponding to an experimental group, consisted of four snails. The exposure period lasted two weeks.

After the completion of the exposure period, it was possible to run the TBARs assay. The thiobarbituric acid reactive species assay studies the creation of lipid peroxidation products within a tissue sample. Lipid peroxidation, which is the result of oxidative stress, creates Malondialdehyde (MDA). When MDA is combined with a TBA solution, it creates a substance known as TBARs, which can be measured through the assay. A TBARs assay kit was purchased, consisting of Thiobarbituric Acid Assay Reagent, TBA Acetic Acid, 3.5M Sodium Hydroxide Assay Reagent, TBA Malondialdehyde standard, TCA Assay reagent, and a 96-well solid plate. Prior to conducting the assay, the TBA Acetic acid and Sodium hydroxide were diluted in 40 ml of distilled water. A color reagent was then created by combining 106 mg of TBA, 10 ml of diluted TBA Acetic acid, and 10 ml of diluted Sodium hydroxide. It was next needed to prepare the samples that would be analyzed. Each snail would produce two samples. First, the snail was dissected and 25mg of tissue was weighed out and added to a 1.5ml microcentrifuge tube. This was repeated for three snails from each experimental group, leading to 18 samples total. Next, 250uL of RIPA buffer was added to each tube and then homogenized. The 18 microcentrifuge tubes were then centrifuged at 1600 xg for 10 minutes at 4C. The supernatant was collected from each tube and stored at -80C. These samples would be used to run the assay.

Having prepared all materials, it was then possible to run the TBARs assay. First, 18 microcentrifuge tubes were labeled with their experimental group and sample number. Then, 100uL each sample was added to its respective labeled tube. Next, 100uL of TCA Assay Reagent was added to each tube and swirled to mix. This was followed by adding 800uL of color reagent to each of the 18 tubes. The microcentrifuge tubes were then vortexed and placed in a foam tube-holder. They were then placed in vigorously boiling water for one hour. It was necessary to be on stand-by, as the tube lids frequently popped off. After one hour, the tubes were taken out ofthe boiling water and incubated in an ice bath for 10 minutes in order to stop the reaction. They were then centrifuged for 10 minutes at 1600xg and 4C. Then, 200uL of sample was taken from each tube and transferred to an organized clear plate for colorimetric analysis. The plate was run under a plate reader and the absorbance was read at 535 nm. The absorbance values could later be used to determine MDA concentration in each sample.

Due to the extensive procedure, many safety concerns were involved. It was important to always don personal protective equipment, including gloves, lab coats and goggles. During sample

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collection, dissecting instruments were used. Since they can be sharp, it was essential to handle the snail and the instrument carefully, in order to avoid cuts. Furthermore, it was essentialto be careful around the boiling water and keep a constant eye on the samples. It was possible to burn fingers while trying to press the lid back onto the microcentrifuge tubes while they were boiling.

Data

Table 1: The Absorbance Reading at 535 nm for Snails of Each Experimental Group

Control	Absorbance Reading	Copper	Absorbance Reading	Zinc	Absorbance Reading
1A	0.641	1A	0.564	1A	0.199
1B	0.183	1B	0.128	1B	0.312
3A	1.123	2A	0.440	2A	0.434
3B	0.255			2B	0.379

Each number, ranging from one to three, represents the individual snail from that specific experimental group. The letters, either A or B, represent the number of the sample extracted from that snail. Each snail provided two samples. The inconsistencies within the values were due to errors and the loss of samples during data collection.

Results

In order to calculate the amount of MDA in each sample, it was required to conduct a colorimetric analysis. It was first needed to calculate the average absorbance of Standard A, this was provided on the plate reader as 0.034nm. This value was subtracted from itself and all other values, including the absorbance values of the standards. This provides the corrected absorbance value. For example, the absorbance for Standard B was 0.035. When subtracted by 0.034, the corrected absorbance for this standard was found as 0.001 nm. This was repeated for all

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remaining standards and samples.

Table 2: Corrected Absorbance Values of All Samples and Standards

Sample	Absorbance (nm)	Corrected Absorbance (nm)
A	0.034	0
B	0.035	0.001
C	0.036	0.002
D	0.037	0.003
E	0.038	0.004
F	0.039	0.005
G	0.051	0.017
H	0.046	0.012
Control 1A	0.641	0.607
Control 1B	0.183	0.149
Control 3A	1.123	1.089
Control 3B	0.255	0.221
Copper 1A	0.564	0.530
Copper 1B	0.128	0.094
Copper 2A	0.440	0.406
Zinc 1A	0.199	0.165
Zinc 1B	0.312	0.278
Zinc 2A	0.434	0.400
Zinc 2B	0.379	0.345

The corrected absorbance values were then plotted against the provided MDA concentration in each of the given samples, in order to create a colorimetric standard curve.

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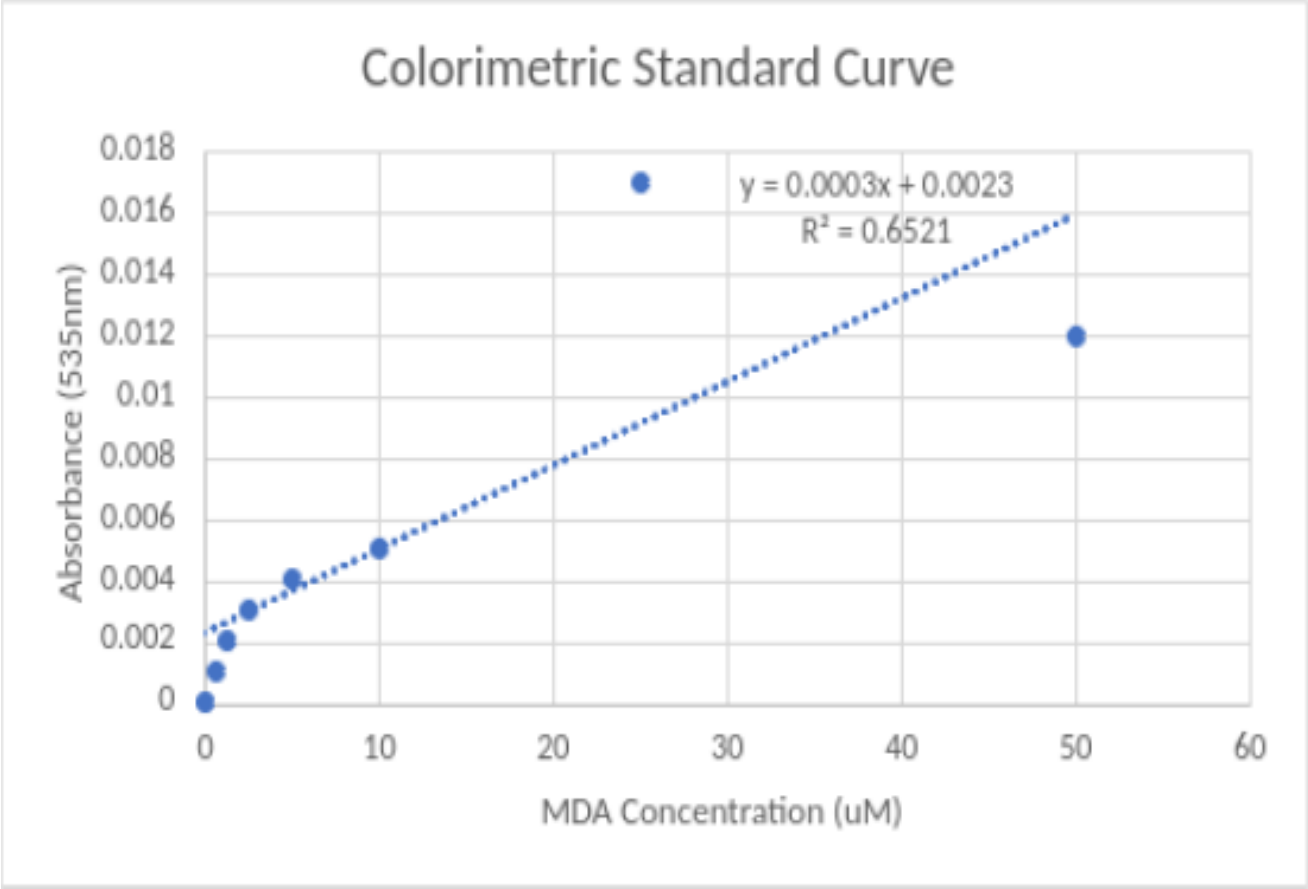
Figure 1: MDA Concentration in Standards vs. Corrected Absorbance



In order to calculate MDA concentration in the collected samples, the following formula was used:

$$\text{MDA } (\mu M) = \left[\frac{(\text{Corrected absorbance}) - (\text{y-intercept})}{\text{Slope}}\right]$$

The corrected absorbance values were previously calculated. The y-intercept and slope values can be found within the equation provided on the graph. The slope is 0.0003 and the y-interceptis 0.0023. The MDA concentration in sample Control 1A would be ((0.607-0.0023)/0.003), which is equal to 2015.667 uM. In this equation, 0.607 is the corrected absorbance, 0.0023 is the y-intercept, and 0.0003 is the slope. This calculator was repeated for all remaining samples.

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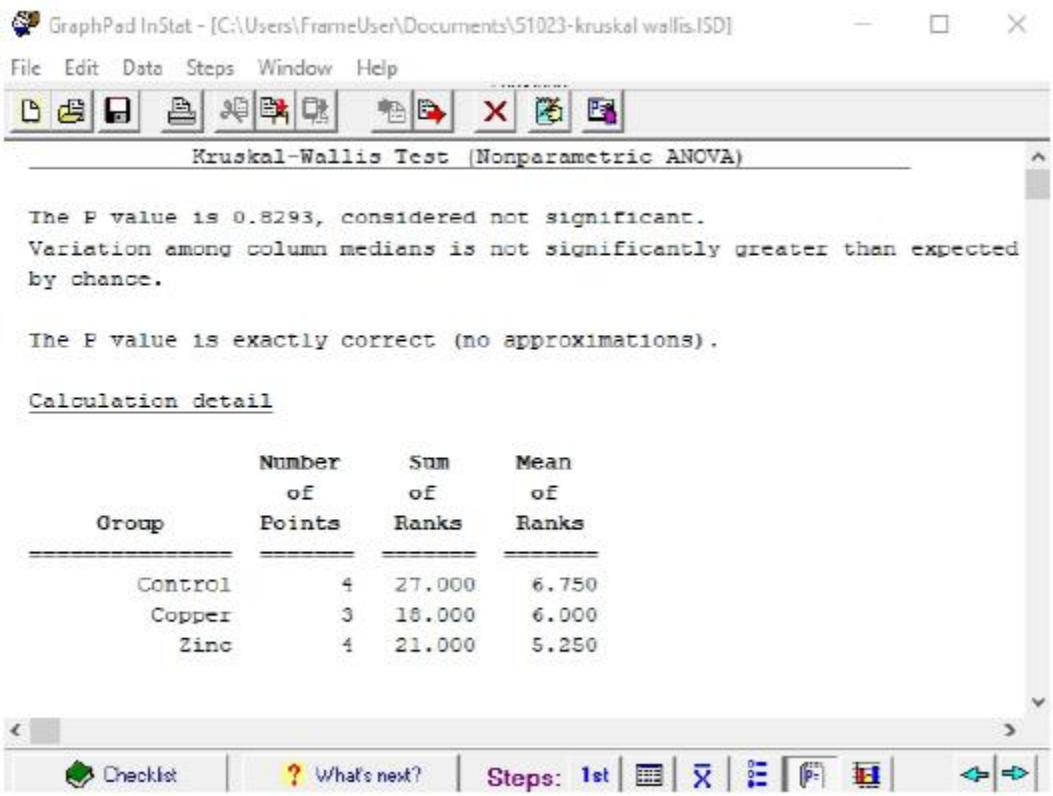
Table 3: MDA Concentrations Within Experimental Samples

Sample	Control 1A	Control 1B	Control 3A	Control 3B	Copper 1A	Copper 1B	Copper 2A	Zinc 1A	Zinc 1B	Zinc 2A	Zinc 2B
Corrected Absorbance	0.607	0.149	1.089	0.221	0.530	0.094	0.406	0.165	0.278	0.400	0.345
MDA (uM)	2015.7	489.0	3622.3	729.0	1759.0	305.7	1345.7	542.3	919.0	1325.7	1142.3

The greater the concentration of calculated MDA within the sample, the more oxidative stress the snail was. In order to analyze these results, a Kruskal Wallis nonparametric statistical test was conducted. This test was chosen because it specifically compares the medians of multiple groups. Using the Instat software, the following results were obtained.

Figure 2: InStat Statistical Analysis Results



The results of the Kruskall Wallis test can then be used to analyze the results in context of the overall objective.

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Discussion

The Kruskal Wallis nonparametric test compares the means of different groups to determine if they are statistically different. A p value determines the statistical significance of adata set. If the p-value is greater than the significance of 0.05, the data is considered not statistically significant, meaning that all groups had essentially equal means. The p-value for this data set was 0.8293, which is greater than the significance level of 0.05. This means that the data was not statistically significant, and all groups had similar means. In context, the three experimental groups each exhibited similar amounts of MDA concentration, meaning that snails from all three groups were approximately equally oxidatively stressed. This indicates that neither heavy metal, nor copper or zinc, had any significant effect on the oxidative stress levels of *Lymnaea stagnalis*.

It was originally hypothesized that *Lymnaea stagnalis* exposed to Copper sulfate and Zinc nitrate would exhibit greater levels of oxidative stress. This was proven incorrect because the statistical analysis showed that snails exposed to Copper and Zinc contained a similar amount of MDA in their tissue as the control group, which was exposed to neither heavy metal. Since all groups had similarly low levels of MDA, and accordingly oxidative stress, it can be concluded that the heavy metals Zinc and Copper do not significantly pollute streams and freshwater ecosystems.

These results may be attributed to multiple sources of error. First, it is likely that the concentrations of the heavy metals were too low to take effect. The concentrations used in this study were significantly low, in order to ensure the mortality of the snails. However, this may have also led to there being no impact on the oxidative stress levels. Furthermore, many challenges were encountered during the multiple TBARS assay trials. There were at least three times during one trial during which samples had to be transferred within the tubes, which may have led to the loss of sample accuracy. During the boiling of the samples, the lids of the microcentrifuge tubes tended to pop off and had to be pressed back on. If too many were to pop off at once, the foam tube holder tended to lose balance and sink into the beaker of boiling water. During this process, two samples were lost to the boiling water. Finally, a considerable amount of samples were lost due to an accident in the lab, during which a filled colorimetric plate was dropped. This caused the inconsistency between sample labels in the data presented above. These difficulties may have led to the incongruity of the data, which likely altered the final results.

Additional trials could be conducted in the future using higher concentrations of each heavy metal in order to validate these results. The study could also be repeated with different macroinvertebrates in order to confirm the minimal effect of stream health.

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References

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-Hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity*, *2014*, 1–31. https://doi.org/10.1155/2014/360438

Cavalletti, E., Romano, G., Palma Esposito, F., Barra, L., Chiaiese, P., Balzano, S., & Sardo, A. (2022). Copper Effect on Microalgae: Toxicity and Bioremediation Strategies. *Toxics*, *10(9)*, *527*. https://doi.org/10.3390/toxics10090527

Fodor, I., Hussein, A. A. A., Benjamin, P. R., Koene, J. M., & Pirger, Z. (2020). The unlimited potential of the great pond snail, Lymnaea stagnalis. *ELife*, *9*. https://doi.org/10.7554/elife.56962

Jungclaus, G., Avila, V., Hites, R. (1978). Organic compounds in an industrial Wastewater: a case study of their environmental impact. *Environmental Science and Technology, 12(1), 88-96.* https://doi.org/10.1021/es60137a015

Laperriere, S. M., Hilderbrand, R. H., Keller, S. R., Trott, R., & Santoro, A. E. (2020). Headwater stream microbial diversity and function across agricultural and urban land use gradients. *Applied and Environmental Microbiology*, *86*(11). https://doi.org/10.1128/aem.00018-20.

Mazur, R., Shubiao, W., Szoszkiewicz, K., Bedla, D., & Nowak, A. (2016). A Lymnaea stagnalis embryo test for toxicity bioindication of acidification and ammonia pollution in water. *Water*, *8*(7), 295. https://doi.org/10.3390/w8070295

Muszkat, L., Lahav, D., Ronen, D., Magaritz, M. (1993). Penetration of pesticides and industrial organics deep into soil and into groundwater. *Insect Biochemistry and Physiology, 22(3), 487-499.* https://doi.org/10.1002/arch.940220314

Onwona Kwakye, M., Peng, F.-J., Hogarh, J. N., & Van den Brink, P. J. (2021). Linking macroinvertebrates and physicochemical parameters for water quality assessment in the Lower Basin of the Volta River in Ghana. *Environmental Management*, *68*(6), 928–936. https://doi.org/10.1007/s00267-021-01535-1

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., & Bitto, A. (2017). Oxidative stress: Harms and benefits for human health. *Oxidative Medicine and Cellular Longevity*, *2017*, 1–13. https://doi.org/10.1155/2017/8416763

Read, S. A., Obeid, S., Ahlenstiel, C., & Ahlenstiel, G. (2019). The role of zinc in antiviral immunity. *Advances in Nutrition*, *10*(4), 696–710. https://doi.org/10.1093/advances/nmz013

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Simons, A. L., Mazor, R., Stein, E. D., & Nuzhdin, S. (2019). Using alpha, beta, and Zeta diversity in describing the health of stream-based benthic macroinvertebrate communities. *Ecological Applications*, *29*(4). https://doi.org/10.1002/eap.1896

