JOURNAL OF
EMERGING INVESTIGATORS

Article

Differences in the effect of copper sulfate on the mortality rate of Ostracod and *Daphnia*

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SUMMARY

Freshwater plankton habitats and its ecosystems are widely used indicators of chemical pollution. Control and estimation of effects induced by substances used in agriculture on freshwater organisms appear to be an important field of science, providing feedback on use of these chemicals. Our preliminary experiments with growing two species from different groups of freshwater crustaceans, *Daphnia pulex* and Ostracoda, in culture indicated different survival ability related to water quality, overcrowding, and food supply. We hypothesized that copper sulfate ($CuSO_4$), which is a long-established herbicide and pesticide used in pond cleaning, can also have a different mortality rate on these animals. To test this hypothesis, we established and utilized a simple reproducible method via treatment of controlled number of animals with different concentrations of $CuSO_4$. We performed a series of experiments with this chemical and our results indicated that it has different magnitudes of toxicity on two groups of freshwater crustaceans (*Daphnia* and Ostracoda). We conclude that this method can be helpful in ecological toxicity analysis and may be utilized for wider and more precise studies of different pesticides side effects.

INTRODUCTION

Microscopic aquatic organisms are widely used as research models in toxicological, physiological, and other types of studies. They often serve as indicators of environmental stability in different habitats (1-3). Crustaceans are some of the multicellular organisms most sensitive to the quality of water and chemical pollution (4,5). The manufacturing and use of new types of pesticides and herbicides as well as other potentially environmentally harmful chemicals requires strict control of their impact on ecosystems. In this work, we aimed to establish a method for estimating the toxicological effects of different substances by comparing the impact of different environmental pollutants on relatively small aquatic invertebrates that can be easily reproduced in non-laboratory conditions.

In the described studies, we used two different groups of freshwater crustaceans *Daphnia* and Ostracoda) for comparison of the toxicological effect of copper sulfate ($CuSO_4$). *Daphnia* is a widely spread freshwater crustacean genus of Cladocera order, comprising over 200 species with most abundant *D. pulex* and *D. magna*. In our study we used *D. pulex* – a small crustacean, generally 2-3 mm long (6). Ostracoda are very wide class of *Crustaceans* with about 70,000 species identified (7). They are smaller than *Daphnia*, in average about 1 millimeter long.

$CuSO_4$ has been extensively used to control algae growth during the summer months since the early 20th century (2,8). It is also used in nursery catfish ponds to control growth of some invertebrate hosts of fish parasites (2). Concentrations of copper for such treatments may vary depending on hardness and alkalinity of water, but concentrations in the low milligrams per liter of water are recommended (9). In the literature, there are reports showing that $CuSO_4$ has a significant effect on the life and survival of *Daphnia* species; however, very little information on the survival of Ostracods under these conditions is available (3,10-12).

The copper ion is the component of $CuSO_4$ with toxicological properties that can affect the organism. This occurs mainly via increased production of reactive oxidative species (ROS), which leads to lipid and protein oxidation and DNA damage (13). Copper may also be involved in the direct inhibition of proteins and enzymes (12-14). While copper in fishponds is used mainly to prevent the appearance and spreading of fish parasites, it may also lead to food chain and ecological imbalances in these and connected water bodies via impacts on freshwater invertebrates. In a study by Brix *et al.*, the authors analyzed data from two databases as well as a number of studies related to acute copper toxicity and reported the species mean acute value for *Daphnia magna* to be 20 µg/L (15). It has also been quantitatively shown that $CuSO_4$ toxicity could be observed in *Daphnia magna* culture starting from 9 µg/L and that the compound had an LC50 (the concentration that kills 50% of treated animals) of around 36 µg/L (16). A wider study of different *Cladocera* order species showed similar results, with an EC_{50} (the concentration that reduces growth by 50%) on *Daphnia magna* after 48 hours of 26 and 53 µg/L for samples from different water bodies (5).

Based on our long-running observations, we noticed that *Daphnia* and Ostracods have different viability when grown in culture. Temperature, quality of water, food supply and other factors affect the survival of both, but the overall survival rate of Ostracods appears to be higher. The limited number of comparative studies in the literature do not address this phenomenon, but by growing these organisms side-by-side in the same conditions, we observed this effect multiple times. We hypothesized that $CuSO_4$ treatment will mimic the different survival rates and will allow us to quantify possible differences in *Daphnia* and Ostracods growth under varying concentrations of this compound. We expected a higher survival rate for Ostracods compared to *Daphnia* at the same concentration of $CuSO_4$.

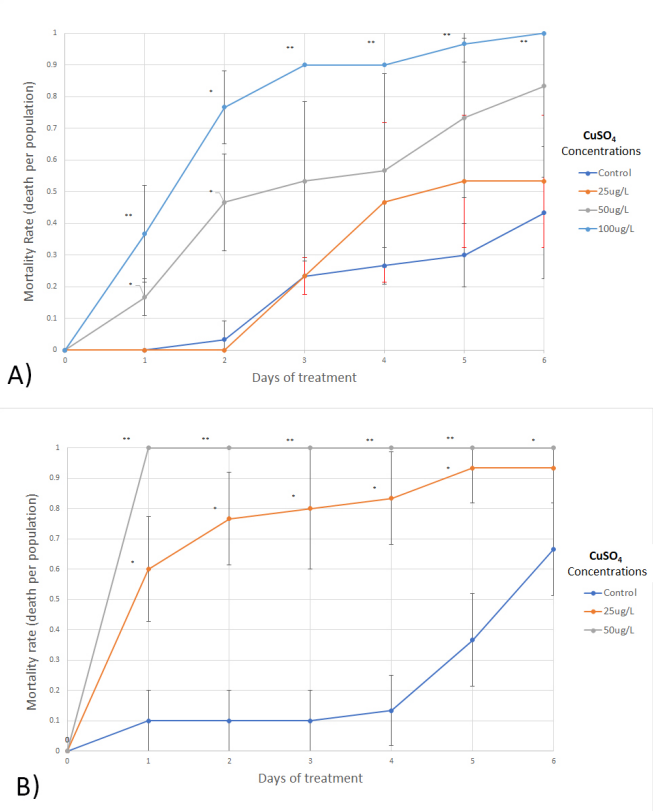

Figure 1: Mortality rate with different concentrations of $CuSO_4$. A) Ostracods and B) *Daphnia*. We used 10 animals with 3 replicates per condition. The mortality rate was calculated as the ratio between the number of dead animals and the total number of animals in the group. Standard deviation and *p*-value labeled for each point (* $p < 0.05$, ** $p < 0.01$).

Our results confirmed significant difference in mortality rate between two groups of used crustaceans. 100 µg/L of $CuSO_4$ has completely eliminated all live *Daphnia* after 24 hours of incubation, while over 50% of Ostracods survived. Similar pattern was observed with lower doses - no visible effect on Ostracods at 25 µg/L of $CuSO_4$ after 24 hours, while over 50% of Daphnia was dead.

RESULTS

Our previous work showed that concentrations of $CuSO_4$ above 200 µg/L exhibit very high toxicity in either class of crustacean and that all animals die during the first 36 hours (data not shown). In response to these findings, we decided to explore survival of *Daphnia* and Ostracods at lower $CuSO_4$ concentrations and compare our findings with published results from experiments with *Daphnia* (5,15,16).

We expanded cultures of *Daphnia pulex* and unidentified species of Ostracods in aquariums to obtain sufficient number of offspring at a synchronized stage. We placed animals in groups of equal number and placed them into individual containers with different concentrations of $CuSO_4$ and kept them at room temperature. To make the experimental and control conditions comparable, the experiment was carried out without feeding. This is because $CuSO_4$ acts both on animals and plants. If algae (the usual food used in crustacean culture) were used to feed the *Daphnia* and Ostracods, it would create unequal conditions in the control and experimental groups, as the presence of copper ions effects the algae as well (the control algae presence and growth would remain unaffected). We applied a range of $CuSO_4$ concentrations from 25 to 100 µg/L. As a control, we used water without any additives. Animals were kept in plastic containers up to six days and live ones were counted daily.

We can see the expected dose response to $CuSO_4$ among all treated samples with *Daphnia* significantly more sensitive than Ostracoda (**Figure 1**). All used concentrations above 50 µg/L were lethal during first 24 hours. We also noticed a striking difference in survival of these two groups of Crustaceans from Day 1, especially at higher concentrations of $CuSO_4$. At 50 and 100 µg/L all *Daphnia* were dead (mortality rate = 1), while for Ostracods it was about 0.17 and 0.38, respectively. Lower concentration of 25 µg/L had a similar pattern – zero mortality rate for Ostracods and 0.77 for *Daphnia*. This difference between two animals' groups were statistically significant ($p < 0.05$, unpaired two sample t-test). After two days, the mortality rate for Ostracods is about 0.47 when treated with 50 µg/L of $CuSO_4$ (**Figure 1A**) while all *Daphnia* at this concentration were already dead, meaning mortality rate equals 1.0 (**Figure 1B**). Results with 100 µg/L for *Daphnia* mirrored those seen with 50 µg/L. Slight difference between mortality rate in control groups of *Daphnia* and Ostracods was not statistically significant, for example on day 3 mortality rate in control group of Daphnia was 0.1 and in control group of Ostracoda 0.23 (p-value 0.133) . The main difference in $CuSO_4$ action happened during first three days. A rough estimation of the LC_{50} showed Ostracods (above 50 µg/L) had a LC_{50} approximately twice as high than in *Daphnia* (less than 25 µg/L) during first two days. This finding is in accordance with our mentioned observation of higher viability of Ostracods.

DISCUSSION

Planktonic freshwater Crustaceans is a widely used model in the field of toxicology and ecology. Some species from this huge subphylum (like *Daphnia*) studied much more than the others We hypothesized that ability to survive in polluted water bodies may differ in different taxonomic groups of plankton and developed a method to test mortality rate in two groups of Crustaceans – *Daphnia* and Ostracoda.

From our results, we can infer that CuSO4 was toxic to our culture of Ostracods and *Daphnia*. In or experiments we used *Daphnia pulex* and homogeneous Ostracods culture (exact species unknown) developed from samples taken in local pond. We were also able to show that a higher concentration of $CuSO_4$ causes a higher mortality rate, with a much higher effect on selected *Daphnia* than Ostracods species. In our hands, the mortality rate for $CuSO_4$ after two days of treatment with 25 µg/L was not noticeable in Ostracods but was about 0.77 in *Daphnia*. A similar trend was found for concentration of 50 µg/L - 0.47 for Ostracods and 1 (all animals dead) for *Daphnia*. Approximate LC_{50} values obtained from these results (below 25 µg/L for *Daphnia* and above 50 µg/L for Ostracods) are in line with what has been shown before on different species. *D. magna* in culture has an LC_{50} of about 36 µg/L (16), and different *Cladocera* species from various water bodies showed LC_{50} values that spanned from 5.3 to 70.6 µg/L (5). The variability of results in some of our experiments can be explained by insufficient replicates and slight variability of

Figure 2: *Daphnia pulex*. Example of *D. pulex* at 10X magnification used in our experiments.

developmental stages of used offspring. Nevertheless, our results showed a statistically significant ($p < 0.05$) trend. For more accurate determination of copper-induced mortality rate and LC_{50}, and to gain a better understanding of the difference between the families of *Daphnia* and Ostracod, we are planning more experiments with a wider range of species and concentrations of $CuSO_4$. One of the reasons that may lay behind the difference between survival rates of *Daphnia* and Ostracods could be the pelagic life of *Daphnia* versus the benthic life of Ostracods. However, we need more data and wider analyses to make this conclusion.

Environmental studies provide us with necessary information demonstrating how human activity impacts nature. It will be more and more imperative to strictly control possible pollution from old and newly manufactured chemicals. For this purpose, the scientific community needs multiple approaches and methods. Here, we established and utilized a method for assessing how a pollutant affects two groups of microorganisms and tested our hypothesis of different sensitivities of freshwater crustaceans to $CuSO_4$. This research can lead to a better understanding of mechanisms underlying environmental toxicity and aquatic organisms' physiology. It may help to choose and finely tune chemical water treatments or even herbicide and pesticide concentrations used in agriculture.

We conclude that our experimental model is sufficiently informative and reproducible to continue our research of different polluting environmental factors as well as other chemical compounds. We plan to perform these further experiments along with a series of extended physiological and behavioral experiments related to the phototactic ability of *Daphnia* and Ostracods in the presence of $CuSO_4$.

MATERIALS AND METHODS

We defined *Daphnia pulex* using the following features described by Lynne Witty: stout middle pecten with 5-7 teeth, ocellus present, smooth helmet, ventral margin of head deeply concave, shell spine <¼ valve length (short), densely pubescent (i.e. "hairy") abdominal processes (17). It is a small crustacean and is generally 2-3 mm long. *Daphnia* is a member of the order *Cladocera* and is one of the several small aquatic crustaceans commonly called water fleas because their swimming style resembles the movements of fleas. The anatomy of this organism is made easier to discern by the fact that most of its outer covering is clear, showing most of the internal organs at work, including the heart (**Figure 2**).

Ostracods are a wide class of crustaceans with about 70,000 species identified (7). They are small animals, having an average length of about 1 millimeter with a bi-valved carapace in which the animal is suspended. The head contains two antennas which the organism uses to swim. We were not able to define the exact species of Ostracods collected but based on the carapace shape compared with description from Rodriguez-Lazaro and Ruiz-Munoz (18), we think it is of the genus *Cyprinotus* (**Figure 3**). They were expanded in culture from a few identical animals.

Both *Daphnia* and Ostracods were collected in June of 2020 from a local pond near Potters Bar, UK and kept in 5 L plastic bottles and 4 L spheric plastic tanks with Ashbeck English Natural Mineral Water (Tesco) and Essential still natural water (Waitrose). Feeding occurred once a day with 10 ml of "green water" — a mixture of single-cell algae from the same pond where animals were taken from, filtered and grown in separate tanks. They were kept at room temperature on a windowsill receiving natural light daily and avoiding direct sunlight. Once a month, half the liquid in the bottles was exchanged with fresh water.

To synchronize stages of development and body size of *Daphnia*, we did the following: A week before the experiments, about 100 fully developed *Daphnia* were transferred to a fresh 5 L bottle, fed daily with "green water", and left for 2 days to reproduce. After 2 days, parents were removed based on their size, and the new *Daphnia* were used in our experiments 2-3 days later.

Ostracods were simply collected from a separate tank. Offspring synchronization similar to *Daphnia* was not possible due to the size of Ostracods. The only parameter we could

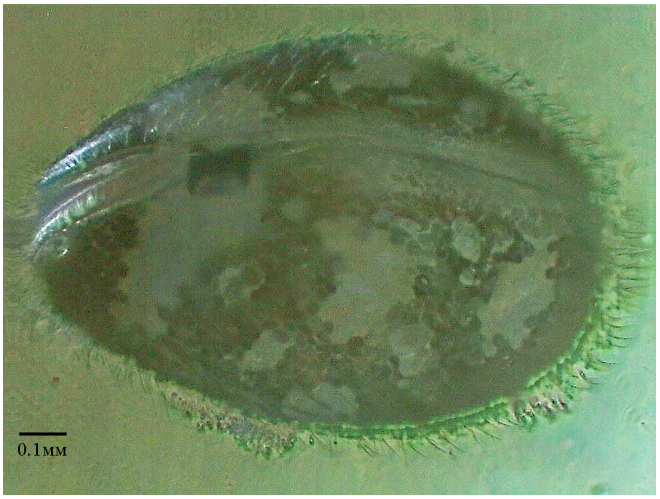

Figure 3: Ostracod. Example of Ostracod at 20X magnification used in our experiments.

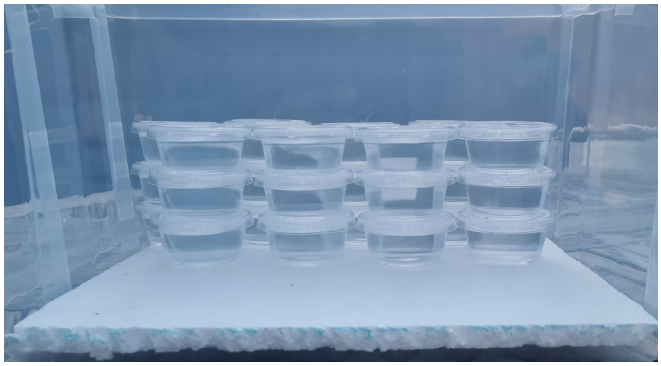

Figure 4: Photograph of the experimental setup. Stacks of 50 mL plastic containers were held in a plastic box to keep surrounding conditions equal for all samples.

utilize for choosing Ostracods was a similar size. This may have an additional effect on survival rate as animals can have different sensitivity to copper at different developmental stages, and this potential bias should be addressed in future experiments.

The survival experiment was carried out in 50 mL plastic transparent containers with lids (Amazon) containing 40 mL of pure water or different concentrations of $CuSO_4$. The lid was left loose to provide air access. We used three replicates per condition. First, we transferred 10 animals from culture tanks into clean plastic cups with fresh pure water to make 20 ml in total. We then added 20 ml of $CuSO_4$ (copper sulfate pentahydrate (APC Pure, re-crystallized in deionized water to produce pure $CuSO_4$ crystals), creating final working concentrations of 25, 50, 75, and 100 µg/L. In the case of control samples, we added 20 mL of pure water. The containers were kept at room temperature and left out for 24 hours in a big transparent plastic box (**Figure 4**). The living crustaceans were then counted to determine how many had died in each solution. We considered an animal to be dead if it was not swimming even after soft pushing with a water stream from a Pasteur pipette. This step was repeated until all of them were dead or for six days. The number of dead animals was converted into the mortality rate as a ratio of dead at a certain time-point to the total number of animals. From the data collected, analyzed in Excel, graphs were plotted. Statistical analysis was performed using an unpaired two-sample t-test.

Received: September 30, 2021
Accepted: July 17, 2021
Published: December 17, 2021

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