Abstract

This study investigates the age-related impacts of environmental stressors, notably copper and salicylic acid, on the heart rate of *Daphnia magna*. *Daphnia magna* is a model organism widely used in ecotoxicology to measure toxic responses in aquatic species (Koivisto, 1995). *Daphnia magna's* transparency and susceptibility to chemical stressors make it a suitable model for studying how stressors affect physiological systems, such as heart rate, during various phases of life. Understanding the impacts of toxins on juvenile organisms is critical, as younger stages may be more responsive due to immature metabolic and detoxifying processes. The experimental approach involves exposing juvenile and adult *Daphnia magna* to regulated dosages of copper (100 mg/L and 991 mg/L), salicylic acid (100 mg/L and 385 mg/L), and a mixture of the two toxins. Heart rates were measured at baseline, low, and high concentrations to evaluate each toxin's influence. Using a disposable transfer pipette, juvenile and adult *Daphnia* were transferred from a holding beaker (20°C) to 60mm Petri dishes containing approximately 2 grams of Vaseline. After a 5-minute adjustment time, heart rate was monitored under a Zeiss Stemi305 stereomicroscope. The primary findings support the idea that juveniles are more susceptible to various environmental stressors since they showed a larger heart rate decrease in response to each toxin when compared to adults. The heart rate drop was less than the anticipated additive impact, indicating that the combination exposure to salicylic acid and copper did not have any additive or synergistic effects. The conclusion emphasizes the significance of age-specific investigations in ecotoxicology by showing that juvenile *Daphnia* are more vulnerable to stressors such as salicylic acid and copper.

Introduction

The study of ecotoxicology focuses on the relationships between chemical substances and organisms at various ecological stages to understand the distinct impact of each substance on organisms (Zaidi & Imam, 2008). Model organisms are important in toxicology experiments because they provide easy and reliable frameworks, similar to human beings (Hahn & Sadler, 2020). *Daphnia magna* is a commonly used model organism in toxicology experiments due to its reactivity to substances and transparent body (Koivisto, 1995). This allows researchers to easily see the impact of toxins on vital functions such as heart rate (Koivisto, 1995). Copper, salicylic acid, and acetaminophen are important in toxicity research due to their diverse physiological impacts and their accessible availability (Gordon, 2003). Increased levels of copper can cause toxicity and impact the physical processes of organisms (Michalaki et al., 2022). Thus, it is commonly used in toxicology experiments. Salicylic acid is also used in toxicology research because it can alter an organism's physiological and metabolic functions, causing increased heart rate reactions to external stressors (Szabelak & Bownik, 2021). Additionally, acetaminophen is also often utilized in toxicology experiments as it can interfere with regular physiological processes, including heart rate and growth patterns in species exposed to the stressor (Smith et al., 2016).

Previous studies indicate that environmental stressors such as copper and salicylic acid impact physiological processes in *Daphnia magna*, with a notable impact on heart rate. Although previous knowledge of copper's effects is limited, numerous studies highlight copper's physiological impact on *Daphnia magna*, offering insight into how it could affect *Daphnia's* heart rate. "The Effects of Single and Combined Stressors on Daphnids—Enzyme Markers of Physiology and Metabolomics Validate the Impact of Pollution" examines the effects of copper and other toxins on *Daphnia magna*. The paper stated that through fluctuations in enzyme activity and patterns of metabolism, the experimented *Daphnia* underwent severe biological alterations as a result of copper exposure (Michalaki et al., 2022). More specifically, copper altered protective enzyme systems, metabolism in cells, and elevated respiratory stress levels (Michalaki et al., 2022). These are all closely linked to heart rate regulation. This indicates that it had a wide-ranging effect on *Daphnia's* ability to regulate environmental stressors and preserve its normal functions (Michalaki et al., 2022). Exposure to copper in *Daphnia* decreases enzyme function and metabolic stability (Michalaki et al., 2022). This can potentially alter heart rate as the organism's systems respond to increased stress. Furthermore, in the study "Behavioral and physiological responses of *Daphnia magna* to salicylic acid," researchers delved into methods in which another environmental stressor, such as salicylic acid (SA) impacts *Daphnia magna* heart rate. For 3 days, at 3 different times (24, 48, and 72 hours), researchers treated *Daphnia* to different concentrations of SA. This included 5 µg/L, 500 µg/L, 5 mg/L, 50 mg/L, and 500 mg/L (Szabelak & Bownik, 2021). By measuring beats per minute while looking at the *Daphnia* beneath a microscope, heart rates were calculated. The findings indicated that an increase in SA concentrations led to a notable decrease in *Daphnia* heart rate when compared to control groups (Szabelak & Bownik, 2021).

The problem of interest is that, while the impacts of environmental stressors such as copper and salicylic acid on *Daphnia* have been collected, minimal data exists that discusses how these effects change over various age groups. Since juvenile organisms may be more vulnerable to these toxins, comparing the tolerance of juvenile and adult *Daphnia* to copper and salicylic acid is crucial for comprehending the greater biological ramifications of *Daphnia* biology. This experiment hypothesizes that juvenile *Daphnia magna* are more susceptible to environmental stressors in comparison to adults. The study of the experiment focuses on whether juvenile *Daphnia* exposed to copper and salicylic acid specifically experience a more prominent change in heart rate compared to adults. The predicted outcome of this experiment is that juvenile *Daphnia* are more biologically sensitive to copper and salicylic acid. The difference in heart rate is likely due to differences in physiological processes in juvenile and adult *Daphnia*. This study examines the impact of salicylic acid and copper in *Daphnia magna*. The experiment focuses on heart rate variations as a crucial factor impacted by environmental stressors. To determine the differences in reactivity across various ages, juvenile and adult *Daphnia* were administered varied amounts of salicylic acid and copper in controlled Petri dishes. Microscopic visuals and careful measurement of the heartbeats were taken to measure the heart rate.

Methods and Materials

Experimental Setup

This experiment used juvenile and adult *Daphnia* magna, which were used for their translucent bodies and easily identifiable changes in heart rate. 3 60mm Petri plates were set up at the experimental station. Using a disposable transfer pipette, juvenile and adult *Daphnia* were transferred from the holding beaker (20°C) to their selected Petri dish. Using a brushed toothpick, roughly 2 grams of Vaseline were added to the bottom of each Petri dish to delicately immobilize the *Daphnia* for observation. The extra water was carefully extracted with a pipette and returned to the holding beaker. 15 mL of culture water (20°C) was then transferred into each 60mm Petri dish using a serological pipette.

Experimental Controls

To evaluate the Time Control group, the initial heart rate was measured at the start of the experiment and then it was measured periodically after every 10 minutes. This was done for 30 minutes. 1.5 mL of culture water was transferred using a P1000 pipette into the respective Petri dish to analyze the Negative Control group. The heartbeat was monitored periodically at 5-minute intervals. This was done for 10 minutes. To assess the Temperature Control group, juvenile and adult *Daphnia* were monitored at various temperatures (~25°C, ~20°C, and ~15°C) to identify the impacts of temperature on the organism.

Copper Exposure Assessment

The heartbeat of the *Daphnia* was measured in 15 mL of culture water. After an adjustment period of 5 minutes, heart rate was monitored after taking out 150 µl of water and adding 150 µl of copper stock solution. The heartbeat was recorded again when the concentration increased to 991 mg/L by adding 1350 µl of copper stock solution and removing 1350 µl of culture water.

Salicylic Acid Exposure Assessment

The heartbeat of the *Daphnia* was measured in 15 mL of culture water. Heart rate was monitored after taking out 750 µl of culture water and adding 750 µl of Salicylic acid solution.

The heartbeat was recorded again when the concentration increased to 385 mg/L by adding 2250 µl of salicylic acid solution and taking out 2250 µl of culture water.

Combined Exposure Assessment

The heartbeat of the *Daphnia* was measured in 15 mL of culture water. Heart rate was monitored after taking out 900 µL of culture water and adding 150 µL of copper stock solution and 750 µL salicylic acid solution. Additive or synergistic impacts from combined toxins were measured and recorded in the LIFESCI 3L03 notebook.

Observation and Evaluation

The Petri dishes were then examined using a Zeiss Stemi305 stereomicroscope. Zen software was used to reduce the frame rate and capture the heart's contraction and relaxation phases. Videos were saved in both CZI and AVI formats.

Results: Text

The copper and salicylic acid administration experiments were designed to see how environmental stressors impacted the heart rate of *Daphnia magna*. The experiment's main goal was to identify patterns and variations between juvenile and adult organisms. The purpose of the mixed experiment was to look at the mixed impact of copper and salicylic acid on *Daphnia magna* heart rate and analyze whether the administrations caused additive or synergistic effects. Heartbeats of *Daphnia* were recorded at baseline, 100 mg/L, and 991 mg/L for the copper experiment and baseline, 100 mg/L, and 385 mg/L concentrations for the salicylic acid experiment. For the copper experiment, adult *Daphnia* had a calculated baseline heart rate of 456.18 beats per minute, whereas juveniles had a heart rate of 492.10 beats per minute (**Figure 1**). The adult and juvenile heart rates dropped to 405.53 and 478.56 beats per minute, respectively, following the administration of 100 mg/L of copper. This suggests that there is a minor decrease in cardiac activity. When the concentration was increased to 991 mg/L, the adult heart rate decreased to 318.64 BPM, and the juvenile organism's heart rate decreased to 441.53 BPM. The noticeable alterations imply that exposure to copper causes a decrease in the total heartbeats counted, as juveniles experience a greater change in comparison to adults. For the salicylic acid experiment, adult *Daphnia* had a calculated baseline heart rate of 439.47 beats per minute, whereas juveniles had a heart rate of 309.25 beats per minute (**Figure 1**). The adult and juvenile heart rates dropped to 346.96 and 364.96 beats per minute, respectively, following the administration of 100 mg/L of salicylic acid. This suggests that there is a moderate decrease in cardiac activity. When the concentration was increased to 385 mg/L, the adult heart rate slightly increased to 351.43 BPM, while the juvenile heart rate decreased significantly to 256.13 BPM, indicating that juvenile organisms are more sensitive to salicylic acid exposure than adults. For the combined mixture experiment, adult *Daphnia* had a baseline heart rate of 428.10 BPM, while juveniles had a rate of 540.22 BPM (**Figure 1**). Upon exposure to 100 mg/L of each pollutant, the adult heart rate was 433.92 BPM, and the juvenile rate dropped to 506.10 BPM, indicating a moderate reduction in cardiac activity. At higher concentrations (991 mg/L for copper and 770 mg/L for salicylic acid), the adult heart rate further dropped to 363.42 BPM, and the juvenile heart rate to 349.49 BPM. This suggests that combined exposure does not amplify the effects by much.

Results: Figures and Tables

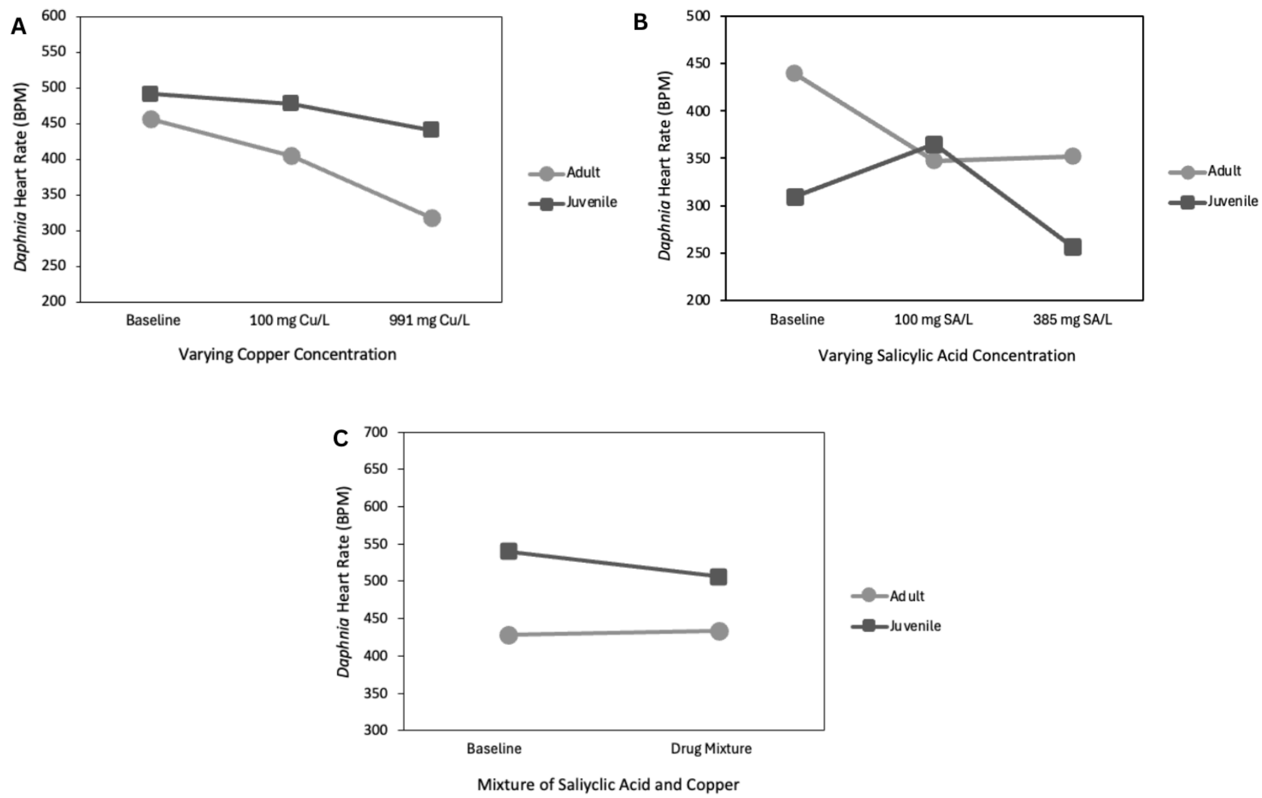

Figure 1. Adult and juvenile *Daphnia magna* heart rates in response to copper (A), salicylic acid (B), and a combination of the two stressors (C). In the copper experiment (A), adult and juvenile heart rates decreased as concentrations increased from baseline (456.18 and 492.10 BPM) to 100 mg/L (405.53 and 478.56 BPM) and 991 mg/L (318.64 and 441.53 BPM), indicating less cardiac activity, particularly in adults. In the salicylic acid experiment (B), adult heart rates reduced from baseline (439.47 BPM) to 100 mg/L (346.96 BPM) but marginally increased at 385 mg/L (351.43 BPM). However, juvenile heart rates decreased substantially, indicating enhanced responsiveness. In the mixed experiment (C), baseline heart rates (428.10 BPM for adults, 540.22 BPM for juveniles) decreased to smaller values (433.92 BPM for adults, 506.10 BPM).

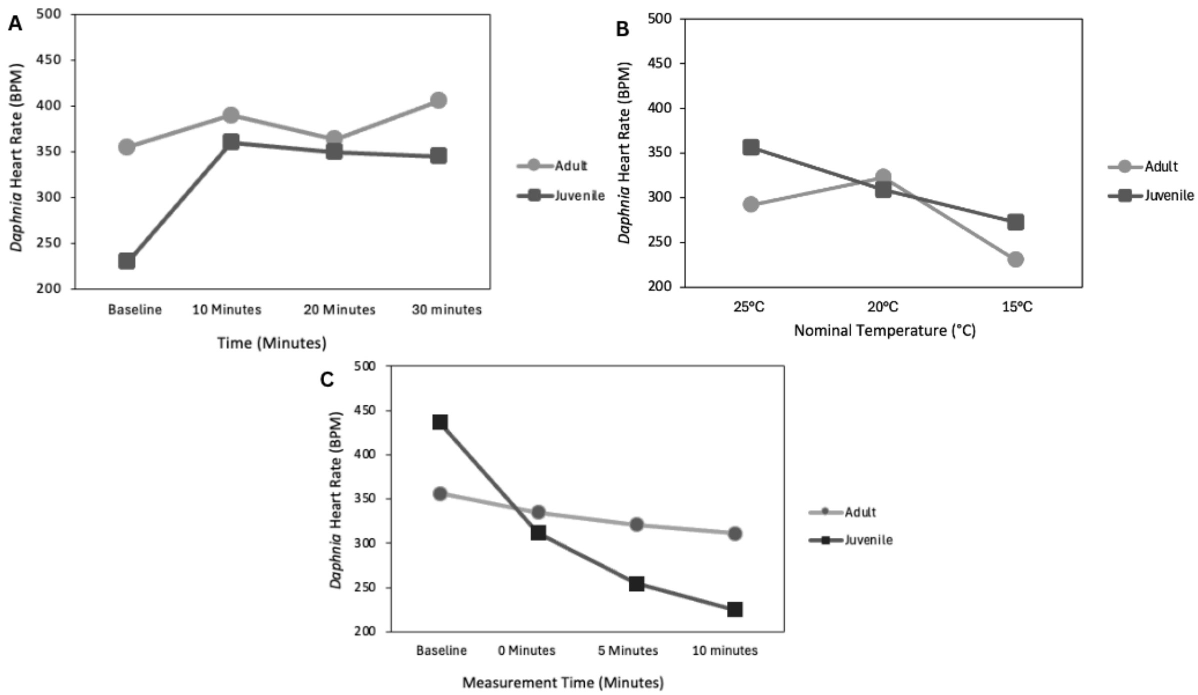

Figure 2. Heart rate of adult and juvenile *Daphnia magna* at different time intervals (A), temperatures (B), and measurement periods (C). In the time-varying investigation (A), adults' and juveniles' heartbeats were evaluated at baseline, 10, 20, and 30 minutes, revealing changes with adults generally sustaining higher rates. The temperature study (B) reveals heart rate responses at 25°C, 20°C, and 15°C, with juveniles showing a reduction at lower temperatures and adults remaining steady in their values. In the measurement study (C), the heartbeats fell in both groups over 0, 5, and 10 minutes, with juveniles showing a more significant decrease. This indicates greater responsiveness for juvenile groups.

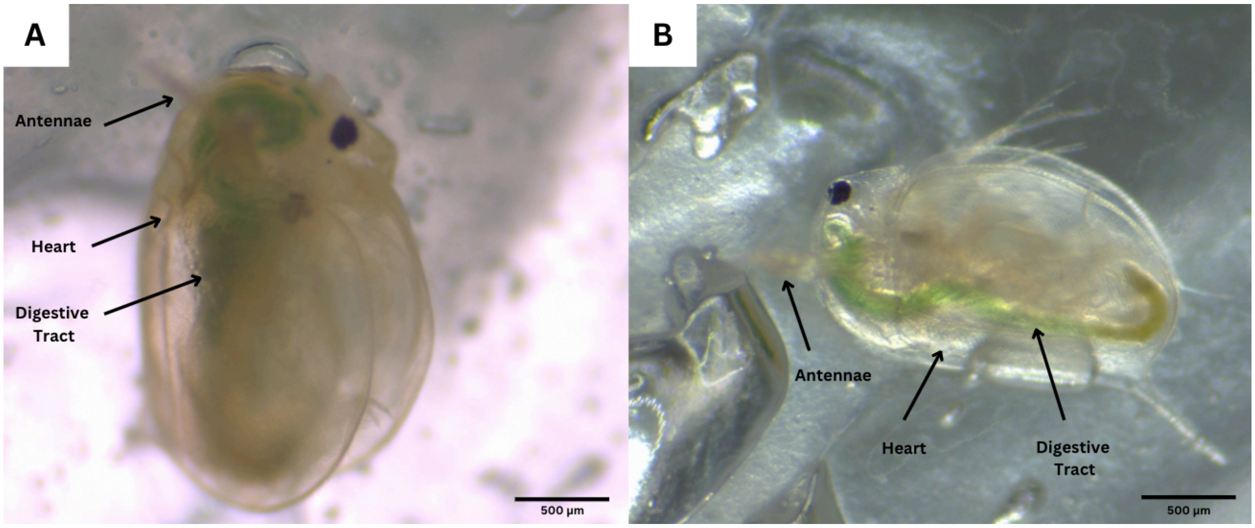

Figure 3. Anatomical structures of adult (A) and juvenile *Daphnia magna* (B). Adult *Daphnia* magna (A) shows key structures: the antennae, which aids in movement and sensing. The heart is visible in both panels. The digestive tract is visible, and it is full of a greenish substance within the *Daphnia*. Juvenile *Daphnia* magna (B) has similar structures. The heart is positioned behind the antennae, and the digestive tract extends along the body, compactly arranged within the transparent exoskeleton. Scale bars represent 500 µm in both images.

Discussion

The findings of this study show that juvenile *Daphnia magna* are more vulnerable to environmental stressors, especially copper and salicylic acid. This is evidenced by the lower heart rates in juvenile species compared to adult organisms. This finding supports the hypothesis that young *Daphnia* would react more strongly to these toxins compared to adult species. Juvenile heart rates decreased more significantly in both the copper and salicylic acid tests. The combined mixture amplifies this finding. These findings indicate that younger species are less resistant to environmental stressors due to physiological and metabolic processes. The heightened responsiveness of juvenile *Daphnia* could be attributed to immature detoxifying mechanisms and higher metabolic needs of the immature species, which make it more difficult for them to cope with the toxins. When exposed to copper and salicylic acid together, the juvenile *Daphnia magna*'s heart rate decreased from 540.22 BPM to 349.49 BPM, which was less than the expected additive impact. The findings imply that salicylic acid and copper do not have a synergistic or additive effect on *Daphnia magna* heart rate. The fact that the observed effect is less than the anticipated additive impact suggests that the effects of these stressors might not be reliable in stating if they are additive or synergistic.

The findings are consistent with prior research on the effects of copper and salicylic acid on *Daphnia magna*. The findings support the concept that environmental stressors have a major impact on physiological functioning, including heart rate. Michalaki et al. (2022) indicates that copper exposure impairs enzyme performance and cellular metabolism in *Daphnia*, resulting in physiological stress (Michalaki et al., 2022). The experiment conducted advances this understanding by highlighting that copper exposure lowers heart rate, particularly in juveniles (Michalaki et al., 2022). This implies that copper interferes with normal cardiac and metabolic activities (Michalaki et al., 2022). Similarly, the results on salicylic acid exposure are consistent with Szabelak and Bownik's (2021) findings that salicylic acid reduces *Daphnia magna* heart rate by impacting physiological systems (Szabelak & Bownik, 2021). The previous study discovered that prolonged salicylic acid administration resulted in higher heart rate reductions (Szabelak & Bownik, 2021). This supports the effects observed on juveniles in the current experiment. Szabelak and Bownik used longer exposure times (24, 48, and 72 hours), which could have exacerbated the effects. In comparison, the current study used an acute model with shorter intervals. Thus, the findings are consistent with previous the study, demonstrating that even short-term exposure to salicylic acid impacts physiological stability. The study found no indication of a synergistic or additive effect between copper and salicylic acid since the combined exposure resulted in a heart rate drop that was less than the projected additive impact. This discovery contradicts previous studies, such as Michalaki et al. (2022), which implies that many pollutants together enhance physiological stress by affecting metabolic and enzyme processes (Michalaki et al., 2022). Likewise, Szabelak and Bownik (2021) find that salicylic acid independently impacts heart rate by modifying physiological processes, raising the possibility that simultaneous exposure would amplify these effects (Szabelak & Bownik, 2021). The absence of such an interaction in our study means that reactions to copper and salicylic acid may not always pair predictably. One limitation is that immobilizing *Daphnia* with Vaseline may cause excess stress that disrupts the heart rate. Another limitation is that the experiment only examined short-term exposure, which would have obscured the data that would have been achieved through the long-term effects of salicylic acid and copper on *Daphnia magna*.

Future studies should consider alternative, less stressful immobilization techniques, like those used by Taylor and Scroggins (2013). Their methods included using extended exposure durations to better understand the long-term effects on *Daphnia magna*. Additionally, they incorporated realistic environmental conditions that would provide a comprehensive view of toxin impacts across developmental stages (Taylor & Scroggins, 2013). In conclusion, this study demonstrates *Daphnia magna's* age-dependent responsiveness to environmental contaminants. Juvenile species experience a larger heart rate drop in response to copper and salicylic acid exposure in comparison to adults. Contrary to expectations, combined exposure did not produce synergistic or additive effects, implying that these contaminants do not always interact reliably. These findings stress the need to take developmental stages into account when analyzing toxicological consequences. This approach would lead to a deeper understanding of age-dependent responses in ecotoxicology.

References

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Appendix

Appendix A: Sample Calculation 1

Calculate the amount of $CuSO_4$ needed for a Cu^{2+} stock solution at the given concentration and volume:

Molecular weights:

Cu: 63.546 g/mol

S: 32.065 g/mol

O (4 atoms): 15.999 g/mol × 4 = 63.996 g/mol

Combined weight: 63.546 + 32.065 + 63.996 = 159.607g/mol

The Data Provided:

Desired concentration = 375 mg/L = 0.375 g/L

Volume = 150 mL = 0.15 L

Moles of Cu^{2+}= 0.375g/L / 63.546g/mol =0.005899mol/L

0.005899mol/L × 0.15L (150mL) =0.00088485mol

Mass: 0.00088485mol × 159.607g/mol = 0.1413g

Rounded: 0.141 g or 141 mg

Appendix B: Sample Calculation 2

Info provided:

529 µL = 0.000529 L

415 mg/L (initial concentration of triclosan in Dish A)

5000 µL = 0.005 L

Use formula:

$C^1V^1 = C^2V^2$

$C^2 = C^1 \times V^1 / V^2$

$C^2 = 415\text{mg/L} \times 0.000529\text{L} / 0.005\text{L}$

$C^2 = 43.9$ mg/L