Growth and Mobility of *Physella acuta* under Individual and Combined Acetaminophen and Salinity Exposure

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Global climate change, driven by rapid population growth and anthropogenic activities, presents an urgent challenge to aquatic ecosystems. Salinity intrusion threatens water quality and availability for human and wildlife populations. At the same time, due to the demand for pharmaceuticals, these pollutants are increasingly detected in freshwater environments, with acetaminophen being one of the most prevalent. This research analyzed the individual and combined effects of salinity (2.5 g/L) and acetaminophen (488 μg/L) on *Physella acuta*. Overall, we observed reduced snail movement under elevated salinity and no effects on growth or movement with acetaminophen alone or in combination with salinity. Our results suggest a complex interaction between stressors and physiological responses. Despite the high tolerance of *P. acuta* to salinity, elevated levels affected its movement. Additionally, there is a need for further research into multiple stressors and their interactions as well as understanding the responses of aquatic organisms to mitigate the impact of pollution and climate change on freshwater ecosystems.

Keywords: *Physella acuta*, *Physa acuta*, aquatic snails, acetaminophen, salinity, freshwater

Introduction

Global climate change is an urgent challenge driven by rapid global population growth (Jiang & Hardee, 2011). Anthropogenic activities such as agriculture, deforestation, manufacturing, transportation, and energy production significantly contribute to this phenomenon (Karl & Trenberth, 2003). Global warming has accelerated the natural melting of sea ice and glaciers, leading to rising sea levels (Sherif & Singh, 1999) and consequent salinity intrusion where seawater moves into freshwater sources. Changes in land use (Zalizniak et al., 2009) and road deicing salt (Novotny et al., 2008) further worsened this issue, leading to changing salinity and the corresponding decline of water quality and availability, critical for human and wildlife populations (Williams, 1987; Chang et al., 2011).

These changes in salinity can harm the growth, survival, metabolism, and movement of freshwater organisms. For example, increased salinity reduces the growth of mayflies (Buchwalter et al., 2019) and affected food metabolism (Garreta-Lara et al., 2018) and movement (Huber et al., 2023) of *Daphnia sp*. Further, the loss of ecosystem services (e.g., tourism) due to increased salinity is expected to threaten up to 4.6% of the human population and annual losses of 0.3% to 9.3% of global gross domestic product in coastal regions by 2100 (Hinkel et al., 2014).

In addition to the challenges caused by climate change, there is an increasing demand for pharmaceuticals for human health (Ferguson et al., 2013; Meyer et al., 2019) and animal agriculture (veterinary drugs; Swabe, 2002). These products can reach aquatic ecosystems through non-point sources like agriculture and point sources such as effluents from drug manufacturers, hospitals, and wastewater treatment plants (Jones et al., 2005). In the United States, over 100 pharmaceuticals were identified in 91% of the streams sampled (Bradley et al., 2020). Similarly, pharmaceuticals like sulfamethoxazole, caffeine, acetaminophen, and others have been found in over 20% of water samples worldwide (Yao et al., 2018), including analgesics, anti-inflammatories, antibiotics, beta-blockers, endocrine disruptors, hormones, steroids, lipid regulators, stimulants, and psychiatric drugs (Whitacre et al., 2010).

Globally, acetaminophen is one of the most used and detected pharmaceuticals. It is sold under the trade names Tylenol® and Panadol® (National Library of Medicine, 2022). Studies have shown its presence in over 80% of samples collected in various countries, including China, Japan, the UK, Spain, Greece (Hoang et al., 2019), and the U.S. (Kolpin et al., 2002; Hoang et al., 2019). Acetaminophen is found in concentrations ranging from ng/L to μg/L in freshwater and marine environments (Bradley et al., 2020). Particularly high levels, exceeding 500 μg/L, have been detected in hospital and municipal wastewater (Kosma et al., 2010; Kumar et al., 2019; Pharms UBA, 2021; Liu et al., 2023).

When acetaminophen reaches aquatic ecosystems, it can adversely affect biota via oxidative stress and neurotoxicity (Almeida & Nunes, 2019). Some of the effects of acetaminophen on aquatic organisms range from interfering with fish liver glycogen and swimming speed (Choi et al., 2018), affecting spawning in mussels (Sole et al., 2010), to changes in respiration, feeding, and behavior of sea snails (Almeida & Nunes, 2019).

Among the organisms affected by acetaminophen are freshwater snails, which are used in ecotoxicological research due to their high pollution tolerance and rapid reproduction (McCarthy et al., 2000; Jayachandran et al., 2022). One of these snails is *Physella acuta*, which is prevalent throughout North America (Dillon et al., 2002; Morningstar & Daniel, 2023) and a primary consumer and decomposer (Newman et al., 1996), making it an important indicator species. *Physella's* range and abundance are a direct response to human activity (Cieplok & Spyra, 2020; Jayachandran et al., 2022) and its high invasive potential (Wethington et al., 2009; Saha et al., 2016; Vinarski, 2017).

Researchers use shell length and movement to assess the effects of pollutants on snail populations. These metrics are effective in providing standardized and reproducible (Dillon & Jacquemin, 2015; Irniger et al., 2017) indicators of environmental stressors (Zalizniask et al., 2009; Bernot & Justice, 2014; Kumari et al., 2023) in field studies or settings with limited resources (Camargo & Alonso, 2017; Spyra et al., 2019).

While many authors have addressed multiple stressors (e.g., Altshuler et al., 2011; Schuler & Relyea, 2018; Naqash et al., 2020), we have not fully explored the combined effect of salinity and acetaminophen on freshwater organisms with limited research on *P. acuta*. This research aimed to evaluate the individual and combined effects of salinity and acetaminophen on *P. acuta*. We hypothesized that elevated salinity would lead to reduced growth and movement due to increased energy requirements for osmoregulation (Hart et al., 1991; Rivera-Ingraham & Lignot, 2017; Buchwalter et al., 2019). Additionally, exposure to acetaminophen, either alone or in combination with elevated salinity, was predicted to further decrease growth and movement, potentially through mechanisms involving oxidative stress, neurotoxicity (Almeida & Nunes, 2019), and synergistic or antagonistic interactions between stressors (Coors & Meester, 2008).

Methods

Snails (*Physella acuta*) were collected from the Tar River (Rocky Mount, NC) and kept for 14 days before the start of the experiment in a 40 L glass aquarium filled with aerated and dechlorinated tap water at 20±1°C. Snails were fed spinach and fish flakes (Tetramin®). Snails used for the experiment were kept in 25 mL test tubes (20 mL treatment solution) for 14 days (*sensu* Bernot & Lamberti, 2008; Brown et al., 2012). Snail shell length mean (±SD) was 7.7 ± 1.34 mm, ranging from 5.6 mm to 11.4 mm. Treatments with eight replicates each included control, acetaminophen (Ace, 488 µg/L), elevated salinity (Sal, 2.5 g/L), and combined acetaminophen and elevated salinity (Sal x Ace). Each snail was randomly assigned to a treatment group. Weekly water changes were conducted, and snails were fed fish flakes during each change.

Salinity stock solution was prepared using Instant Ocean®, a commercial sea salt used in marine aquariums. Aliquots from the stock solution were added to test units to reach a final concentration of 2.5g/L. Water quality parameters for the control treatment were tested for salinity: 80 mg/L, electric conductivity (EC): 150.6 uS/cm, pH: 7.04, and total dissolved solids (TDS): 108 ppm. Water quality parameters for the elevated treatment were tested for salinity: 1250 mg/L, electric conductivity (EC): 2500 uS/cm, pH: 6.68, and total dissolved solids (TDS): 1770 ppm.

Aliquots of acetaminophen stock solution (Spectrum MFG Corp) were added into test units to reach a nominal treatment concentration of 488 µg/L. This concentration is below the calculated LC50 (96 hours) for *P. acuta* of 248.8 mg/L (Sobrino-Figueroa, 2015). Environmentally relevant concentrations of acetaminophen (488 µg/L) and salinity (2.5 g/L) were chosen to represent concentrations found in contaminated lotic ecosystems where organisms are exposed to pharmaceuticals (Pharms UBA, 2021) and saltwater intrusion (Stockwell et al., 2011). Exposing snails to these stressors for 14 days provides adequate time to observe potential effects. For example, Kefford and Nugegoda (2005) and Zalizniak et al., (2009) observed changes in *P. acuta* growth after 14 days exposure to 1000 µS/cm electrical conductivity (salinity was expressed as electrical conductivity). Other studies have used different durations, from 10 days (e.g., Ma et al., 2010) to 28 days (e.g., Bernot and Brandenburg, 2013) to assess the long-term effects of pollutants. Therefore, our 14-day study period is a starting point for identifying changes in growth and movement that may indicate the lowest observed effect concentrations endpoints.

ImageJ software measured shell length (mm) (*sensu* Johansson et al., 2016; Najar, 2022; Balph & Krist, 2023). Before and after treatment, photos were taken with an iPhone 12 (Apple Inc., Cupertino, CA) fixed to a mount to avoid angle changes that could affect the length of the software. Biomass was not recorded due to feasibility and resource limitations.

Movement was measured for *P. acuta* in response to treatment exposure for 14 days. Individual snails were placed in a glass aquarium (50.8 x 27.9 x 30.5 cm) with a 1 cm^2 square grid paper underneath (Elias & Bernot, 2017). Dechlorinated aerated tap water was added to the glass aquaria to a height of 5 cm. The snail was placed with the aperture down on the bottom of the aquarium in the center of the grid paper. Once the snail attached itself to the bottom, a timer was set for 2 minutes and 10 seconds to measure the number of squares crossed. Any distance traveled during the first 10 seconds was discarded as the first 10 seconds were allotted for the snail to acclimate (Elias & Bernot, 2017).

Data Analysis

The effect of treatments (acetaminophen and salinity) on snail movement and growth was evaluated using Analysis of Variance when data were normally distributed or the Kruskal-Wallis test when data were not normally distributed. Shapiro-Wilks test was used to assess normality. Post-hoc analysis was completed if there was a statistically significant effect. Pairwise comparisons for normally distributed data were analyzed with a Tukey test, while non-normally distributed data were analyzed with Dunn's test. Analysis was conducted in R 4.2.2 (R Core Team 2022), and the Dunn test was used as part of the FSA package (Ogle et al. 2023). Alpha was set at 0.05 for all analyses.

Results

Data was not normally distributed (control: $W = 0.566$, $p < 0.01$; treatments $p < 0.05$). Therefore, the Kruskal-Wallis test was used to evaluate an overall effect in this study. Overall, snail shell length growth decreased across treatments (Figure 1). Specifically, snail growth

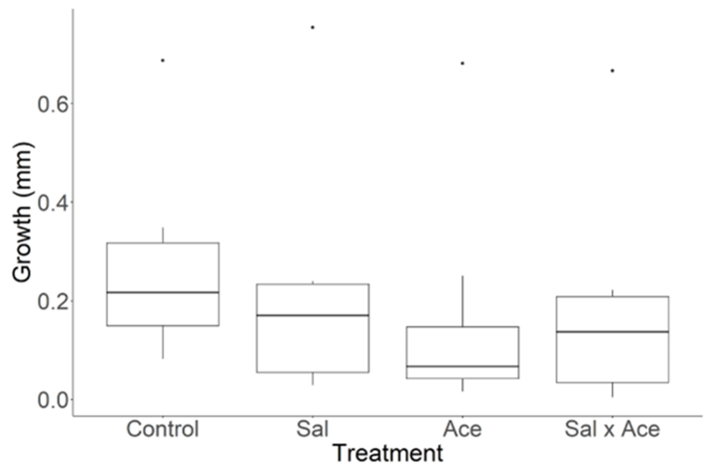

Figure 1: *Physella acuta* growth (mm) exposed to normal salinity (0.11 g/L; Control), elevated salinity (2.5 g/L: Sal), acetaminophen (488 µg/L; Ace), and combine elevated salinity (2.5 g/L) and acetaminophen (488 µg/L). Dots represent outliers (•).

decreased by 22% at higher salinity, 41% in acetaminophen, and 33% in combined acetaminophen and higher salinity relative to the control. However, there was no statistical difference in snail growth across treatments ($\chi^2 = 4.301$, df = 3, $p = 0.23$; Figure 1). There was a reduction of movement after snails were exposed to treatments (Table 1) of higher salinity (37%), acetaminophen (22%), and combined acetaminophen and higher salinity (29%) relative to the control treatment (0.082 cm/s). The movement study had a statistical overall effect ($\chi^2 = 8.978$, df = 3, $p = 0.03$). Pairwise comparisons indicated that the control group significantly differs from the salinity only group ($Z = 2.912$, $p_{adj} = 0.022$; Figure 2). There were no effects of individual acetaminophen and combined treatment (Figure 2).

Discussion and Conclusions

Our study measured the individual and combined effects of salinity and acetaminophen on *Physella acuta* growth and movement. Salinity was chosen as a treatment to address the effects of saltwater intrusion on freshwater ecosystems and anthropogenic activities (Chang et al., 2011; Tully et al., 2019). The acetaminophen treatment addresses the presence of pharmaceuticals in freshwater ecosystems due to population growth and demand for these drugs (Love et al., 2008; Adeleye et al., 2022). Although many studies have been done on the effects of multiple stressors (e.g. Heugens et al., 2001, Völker et al., 2014, and Elias & Bernot, 2017), there is not enough research done on the combined effects of salinity and acetaminophen.

Physella acuta is thought to have a high salinity tolerance (Kefford & Nugegoda, 2005; Zukowski & Walker, 2009). Further, Zalizniak et al., (2009) indicated that ionic composition is a better predictor of the effects on growth than salinity. Specifically, lack of calcium (Ca^{2+}) was associated with negative growth and reduced shell strength (Zalizniak et al., 2009). In our experiment, we used Instant Ocean®, and its composition has similar proportions of calcium to seawater (400 mg/L; Christy & Dickman, 2002). Thus, because of *P. acuta* tolerance to salinity and appropriate calcium levels, we did not observe changes in shell growth at the concentration chosen. However, this could be due to a small sample size and high variability. The percent change based on point estimates suggest an effect could be occurring with individual and combined treatments. Future studies should use a larger sample size than this study to confirm.

We observed reduced movement of snails exposed to elevated salinity (2.5 g/L) and no effects when exposed to combined acetaminophen (488 µg/L) and salinity (2.5 g/L) or acetaminophen alone. Our results are not consistent with the findings of Sackey-Ansah et al., (2023). Their research showed increased movement when snails

Table 1: *Physella acuta* mean and standard deviation between parentheses for growth (mm) and movement (cm/s) exposed to normal salinity (0.11 g/L; Control), elevated salinity (2.5 g/L; Salinity), acetaminophen (488 µg/L), and combine elevated salinity (2.5 g/L) and acetaminophen (488 µg/L).

Treatment	Growth (mm)	Movement (cm/s)
Control	0.27 (0.19)	0.082 (0.02)
Salinity	0.21 (0.24)	0.052 (0.01)
Acetaminophen	0.16 (0.22)	0.064 (0.02)
Salinity x Acetaminophen	0.18 (0.23)	0.058 (0.02)

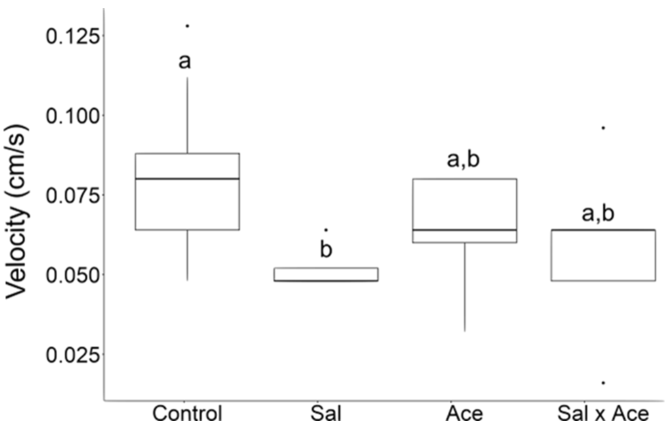

Figure 2: *Physella acuta* movement (cm/s) exposed to normal salinity (0.11 g/L; Control), elevated salinity (2.5 g/L: Sal), acetaminophen (488 µg/L; Ace), and combine elevated salinity (2.5 g/L) and acetaminophen (488 µg/L). Different letters represent significant differences between treatments. Dots represent outliers (•).

were exposed to combined salinity (0.68 g/L) and acetaminophen (500 µg/L). Likely, the salinity concentration used in Sackey-Ansah et al., (2023) may not have induced changes in physiological mechanisms that manage osmotic balance (Hart et al., 1991; Rivera-Ingraham & Lignot, 2017).

Our findings suggest that elevated salinity levels (2.5 g/L) can directly affect *P. acuta* movement, regardless of acetaminophen concentration. Cellular damage might have occurred due to changes in permeability and osmotic balance (Barrios Figueroa & Urbina, 2023). However, the absence of individual and combined acetaminophen effects indicates a complex interaction between stressors and physiological responses (Cañedo-Argüelles et al., 2019). When the organisms were exposed only to acetaminophen, movement decreased by 22% and 29% when exposed to the combined treatment. This decrease in movement might be explained by changes in digestive enzyme function, which subsequently impacts snail energy intake and reduction in energy reserves (Kumari et al., 2023).

Physella acuta plays an important role in the ecosystem as a consumer, prey in food webs, host of parasites, and contributes to nutrients cycling through egestion (Bronmark et al., 1992; Turner & Montgomery, 2009; Elias & Bernot, 2017; Chodkowski & Bernot, 2017). Specifically, *P. acuta* are consumers of algae, bacteria, and fungi (Newman et al., 1996). They fall prey to organisms such as crayfish, sunfish, and turtles (Bernot & Brandenburg, 2013). Thus, reduced movement can increase the time snails remain exposed to predation risk and reduced grazing (Lefcort et al., 2015).

Physella acuta are intermediate hosts to disease-causing parasites (e.g., *Halipegus eccentricus, Echinostoma trivolvis,* and *Fasciola hepatica*; Wojdak et al., 2013). Due to their egestion, snails are important in the food web and play a key role in nutrient cycling, specifically nitrogen and phosphorus. Excreted nutrients support primary producers' growth and development (Vanni et al., 2017). Because of their critical role, it is important to understand how pollution affects these aquatic snails (Newman et al., 1996; Perrotta et al., 2020).

Multiple stressor effects are observed when organisms are exposed to chemical mixtures (Eggen et al., 2004) or non-chemical stressors (e.g., salinity, UV-irradiation, predation, parasitism). Chemicals with similar mechanisms and targets have an additive effect, where their combined effect equals the sum of their individual effects (Eggen et al., 2004). In synergistic interactions, the combined effects of individual stressors are greater than the sum of their individual impacts (Darling & Cote, 2008). For example, *Daphnia sp*. exposed to carbaryl and under parasitic stress synergistically affected its immune response (Coors & Meester, 2008). In contrast, antagonistic effects reduce the combined effect of the stressors below the expected simple additive effect (Didham et al., 2007).

Our hypotheses were partially supported as we observed reduced snail movement when exposed to elevated salinity. While we did not observe a significant effect on the combined treatment, there was a reduction in movement. A likely antagonistic relationship between acetaminophen and salinity might have influenced snail movement. Acetaminophen can affect biological responses to salinity, including antioxidant homeostasis (Correia et al., 2016). Overall, snails with decreased locomotive functioning due to water contamination may encounter difficulties escaping predators, accessing food sources, and reproducing. These challenges can alter food web dynamics, population changes, and nutrient cycling.

This research focused on the effects of multiple stressors (i.e., salinity and acetaminophen) in response to the increasing usage of pharmaceuticals and global climate change. It is important to continue assessing multiple non-chemical stressors and the underlying mechanisms driving these responses to better understand the ecological implications on aquatic organisms.

Notes

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Data Availability Statement: The data that support the findings of this study are available in https://docs.google.com/spreadsheets/d/1UamUOOCq_o6fPFWf_xnBLF_FtZiK2gtP/edit?usp=drive_link&ouid=107040984011745022067&rtpof=true&sd=true

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