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Synergistic Effects of Temperature and Pollution on *Artemia salina*

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Abstract

Environmental conditions play a vital role in the ability of organisms to properly function and survive in their ecosystems. Negative conditions that can impact ecosystems and organisms' survival include temperature change and pollution. Specifically, increasing ocean temperatures due to climate change has an impact on aquatic organisms because these organisms need an optimum external temperature for them to efficiently carry out essential biological processes. Other stressors, such as pollution, have a negative impact on the development and vitality of aquatic organisms. On the contrary, some organisms exhibit a high tolerance and adaptation towards changing environmental conditions such as sub-optimum temperature conditions, polluted environments, and decrease in food sources. Our study focused on the synergistic effect of two factors; atrazine and increased temperature level to gain insight on whether a combination of both stressors had an effect on the survival of *Artemia salina (A. salina)*. We found that there was an effect of temperature on A. salina survival, however, pollution had non-significant effect on their survival. Furthermore, all interactions had no significant effect on their survival rate. Overall, our results showed there was not a significant toxic synergistic effect on *A. salina*. This preliminary experiment could be the starting point for future studies on other environmental conditions that have a synergistic effect on organisms and their ability to function and survive.

Introduction

Recent changes in global climate have driven increases in ocean temperatures (Vose et al., 2012). Therefore, it is imperative to study the impact of increasing ocean temperatures because there are many consequences that may arise from it (Bellard et al., 2012). As ocean temperatures are essential to the proper functioning in aquatic organisms—significant temperature fluctuations will affect these biological functions, and in turn, affect biodiversity (Forsman et al., 2016; Eissa & Zaki, 2010). Furthermore, other environmental stressors—such as pollution—have a similar role affecting aquatic habitats. For example, empirical evidence has shown that inorganic nitrogen pollution can impair the survival, growth and reproduction of aquatic animals (Camargo & Alonso, 2006, Chiu et al., 2017). Many organisms in their natural settings tend to live in sub-optimal conditions, sometimes with severe environmental stress (Holmstrup et al., 2010). However, the combination of two environmental stressors, such as water temperature increases and pollution can have a greater impact to the organism than either stressor alone (Holmstrup et al., 2010).

A synergistic effect emerges between two or more factors where upon interacting; the overall effect is greater than the sum of their individual effects. Synergistic toxicity describes how the toxicity of a substance is increased by the presence of another factor. The environmental effects of a synergistic interaction between temperature increase and pollution on aquatic organisms include a decrease in reproduction and a reduction of population size (Gajardo et al., 2012). In recent studies, researchers found that increasing temperatures trigger an increase in the growth rate of some aquatic organisms, but only to an optimum. After going past an organism's tolerance ranges, their growth rate declines (Dallas et al., 2015). Other studies have shown that atrazine, a commonly used pesticide, also has an impact on aquatic crustacea invertebrates. Atrazine inhibits growth for specific species of crustacea who feed on algae because their food source is reduced (Graymore et al., 2001).

Water temperature is one of the most important factors that deeply affect aquatic organisms. Increase in water temperature can impact different aspects such as growth rates, behavior and survival (Dallas et al., 2015). All organisms have a preferred range of temperature, known as an optimum temperature, in which they can thrive and successfully carry out normal biological processes. For *A. salina*, the optimum temperature range varies between 20 °C and 25 °C. In this experiment, the water temperatures of the habitat of *A. salina*—a crustacean species—will be increased to test its outcome on their population.

The toxicant utilized in this study is atrazine, an effective herbicide commonly used to control unwanted weeds. Atrazine is utilized in agricultural fields and the surface runoff of it mostly ends up in aquatic systems, which further impacts aquatic ecosystems and community function (Graymore et al., 2001). It is reported from previous studies that atrazine has a more direct effect on plant species such as weeds and algae, rather than on aquatic animals. Earlier studies showed that a concentration of 500 µg/l of atrazine causes a reduction of algal production. An atrazine concentration as minor as 20 µg/l can have a direct effect on species of phytoplankton and macrophytes. Indirect impacts on aquatic animals come from limited food sources due to the affected plants species. However, the full impact of atrazine on both aquatic animals and ecosystems are better studied over long periods of exposure (Graymore et al., 2001). Since atrazine inhibits the growth of other species in aquatic habitats, it may affect *A. salina* as well. This paper mainly focuses on the direct effects of atrazine on *A. salina* species, which has not been acutely studied. Some studies have found that atrazine caused no significant effects in multiple crustacean species, while others have found certain species to be affected, namely at high concentrations (Graymore et al., 2001). More concrete conclusions are expected from this experiment as to the effects atrazine has on *A. salina*, independent of the effects it would have on their food source.

In this study, we will use *A. salina* to test the synergistic effects of both temperature increases and pollution in aquatic habitats. They are aquatic crustaceans that inhabit shallow ends of salt water where the majority of pollutants and increased temperatures are concentrated. Furthermore, they are a significant food source to other animals such as fish and birds, so their survival is essential. Equally important, *A. salina* are known to be well-adapted to harsh, ever-changing environmental conditions. This includes changes in salinity of aquatic environments, temperature change, and droughts (Gajardo & Beardmore, 2012). *A. salina's* resilience to environmental changes can provide important insights on how synergistic toxicity affects their rate of survival.

Here, we will test if the increase of water temperature, addition of atrazine as a pollutant, or a combination of both have a negative effect on *A. salina* populations. This experiment can bring about different outcomes: an increase of temperature is the only factor to have an effect on the survival of *A. salina*, the presence of atrazine is the only factor to have an effect on the survival of *A. salina*, or both the increase of temperature and the presence of atrazine have an effect on the survival of *A. salina*. We hypothesize that the presence of both temperature change and pollution will have a negative effect on their survival; although *A. salina* are known to have a high tolerance to temperature increase, the presence of two stressors may have a magnified effect on their survival rate.

Materials & Methods

Organisms and stock culture conditions

We purchased *A. salina* eggs from Carolina Biological Supply (#142242) and maintained them in an instant ocean salt mix that was obtained to stimulate a natural environment. Before starting the actual experiment, the eggs had to be hatched over a period of 36 hours. There were a total of 18 petri dishes and 30 *A. salina* eggs were placed in each petri dish. After counting 30 eggs in each petri dish, 25 milliliters of the salt solution was added and all petri dishes were put in an incubator that was set to 29 °C. Within the hatching period, the *A. salina* cysts had a 26.67%-76.67% success rate.

Experimental design

Once the hatching process was complete, each petri dish, containing 8-23 newly hatched *A. salina,* was given a one-time supply of spirulina flakes. This is blue - green algae that served as the source of nutrition for the *A. salina* throughout the entirety of the experiment. A mortar and pestle was used to grind the flakes into a fine powder small enough for the *A. salina* to feed on. The spirulina flakes were chosen because they were already dead, eliminating the possibility of atrazine having an impact on it. This way, any effects of atrazine on the *A. salina* would directly be from the atrazine, and not a secondary consequence of starvation. Afterwards, the *A. salina* were subjected to the treatments and controls.

We used three temperature conditions; 24, 29, and 34 °C

in temperature controlled chambers. We measured the variation of the chamber and found that the chamber temperature varied plus and minus one degree. Thus, the accurate effective temperatures of our experimental conditions were 23-25, 28-30, and 33-35 °C. 24 °C was used as their optimal temperature, while the 5 and 10 °C increases were the levels used to test the extent temperature increase would negatively affect the *A. salina*.

As a pollutant, a concentration of 50µl of atrazine per 25 mL of instant ocean solution was added to half of the petri dishes in the experimental group. The two pollution conditions were with or without atrazine.

In order to obtain adequate experimental data, our experimental design consisted of three replicates of the treatments and controls for a total of 18 petri dishes. The replicates were numbered 1-3 per treatment type to keep track of the progress of *A. salina* in each individual dish. Our control was when the *A. salina* were in their optimal temperature (24 ?), without atrazine.

The study was conducted over a six-day period and the survival rate of the *A. salina* was investigated two times throughout the six-day process. On Day 1, the *A. salina* was fed with atrazine-polluted food. Atrazine was added to nine out of 18 of the assigned dishes, and arranged in the incubators. On Day 4, the *A. salina* were taken out of their incubators and the number of those that died, stayed alive, and those that did not hatch were counted under a microscope. After the counting, the petri dishes were placed back in the incubators. The final treatment was completed on Day 6 and the number of A. salina that died and survived were again, recorded.

Microscopic analysis

Since the *A. salina* eggs were so minuscule, we used a microscope to first count the cysts and then to observe them and gather necessary data. To count the 30 that we needed in each petri dish, we poured some out on a petri dish with the salt solution. Under a microscope, we slowly pipetted each egg into another petri dish, until there were precisely 30 eggs in it. This was repeated until all 18 petri dishes had 30 *A. salina* eggs in them.

When their hatching period ended, we observed them under the microscope to make sure that the majority of the eggs hatched. We noted that they were swimming rapidly around the petri dish. On Day 4 and Day 6, we used a microscope to count the *A. salina* under the microscope. Using a pipette, we collected those that were alive, counting and keeping tally marks of each one that was taken out. Then, the ones that died were counted as they were pipetted out. Finally, we totaled those that did not hatch, being certain that everything added up to 30 for each petri dish that data was collected from.

Statistical analysis

We tested for assumptions of the General Linear Model (repeated measures ANOVA) using Shapiro-Wilk's normality test and Bartlett's test of homogeneity of variances. Our data did not violate those assumptions, so we proceeded with the repeated measures ANOVA using temperature and pollution as predictors, and time as a repeated measure to accommodate the countings of Day 4 and Day 6.

Results

Survival rate

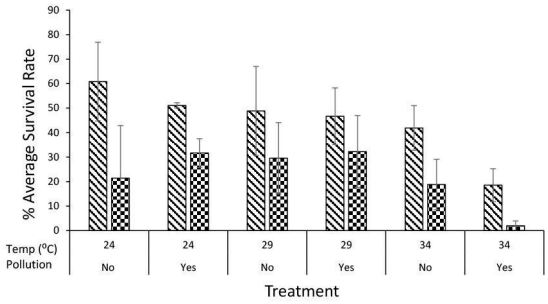

Figure 1. Average percent survival rate of A. salina under temperature and pollution treatments. The plot shows the mean values along with the standard error bars for each treatment. The lined bars represent Day 4 Treatment and the checkered bars represent Day 6 Treatment for each of the treatments.

Day 4 showed the survival rate of *A. salina* decreased with increasing temperature and pollution (Figure 1). For the effect of temperature alone over A. salina survival, a downward trend was displayed with each increasing temperature level—24 °C had the highest percent survival rate followed by 29 and 34 °C. Day 6 showed mixed results where 29 °C unexpectedly showed the highest survival rate, rather than 24 °C. However, the temperature level for 34 °C showed the lowest percent survival rate compared to the other two temperature levels. The significance of these observations was tested through repeated measures ANOVA while considering temperature and pollution as the main factors. The p-value of repeated measures ANOVA testing the significance of temperature on the recorded survival rate

was 0.04924. This showed that the probability of the effect of temperature on survival rate being due to random chance is very low. In other words, there was a significant effect of temperature on the survival rate of *A. salina.*

Pollution as an independent factor did not cause a significant decrease in the survival of *A. salina* overtime, across all temperature values. Temperature levels 24 °C and 29 °C had higher percent survival rates in the treatment with pollution than without pollution. 34 °C had the lowest percent survival rate for treatments with pollution versus those without pollution for Day 4 and 6. The significance testing through repeated measures ANOVA for pollution as an independent factor gave a p-value of 0.37551. This value is greater than 0.05 implying that there is a high probability that the observed effect of pollution on the survival rate of *A. salina* is due to random chance.

When comparing day 4 and day 6 between each other, the overall results show that 24 °C, with and without pollution similarly had the highest percent survival rate; this was followed by 29 °C with and without pollution, 34 °C without pollution, and 34 °C with pollution as the lowest survival. The repeated measures ANOVA test for the crossed interaction of temperature and pollution showed a p-value of 0.76213. This means that there was a high probability the crossed effect of temperature and pollution on the survival rate of *A. salina* was due to random chance. Overall, the interaction between temperature and pollution did not have any significant effect on the survival rate of *A. salina*.

Discussion

In this study, we tested whether or not the interactive effect of temperature and pollution had stronger effects on the survival rate of *A. salina* rather than either effect alone. Our data did not support our prediction; we found that there was not a significant synergistic effect of temperature change and pollution on the survival of *A. salina*. In contrast, our results showed that the strongest negative effects on A. salina survival were driven by temperature alone, under the highest temperature (34 °C) A. salina had the lowest survival rate. This response may be explained by Gajardo's 2012 study on the *Artemia* species and their adaptation to critical life conditions. *Artemia* can survive in extreme habitats that range from 5 °C to 40 °C, but when their upper limit is reached, there are drastic effects. This results in a major reduction of the *Artemia* populations (Gajardo et al., 2012). It was predicted that with increasing temperature levels above the optimum (24 °C) would affect the biological functions of the *A. salina*. Hence, it was hypothesized that the most extreme conditions would yield the lowest rate of survival, but it was not expected that the 24 °C with atrazine treatment would have similar results to the control. This hypothesis may be explained by Chiu et al's., 2017 study on the response of aquatic ecotoxicity and pesticide contamination. Pesticide pollution is a serious concern of global aquatic ecosystems, impacting its populations. The effects involves changes in an organism's maturation, emergence, and survivorship (Chiu et al., 2017). As shown in Figure 1, the increase in temperature had an effect on the survival rate of *A. salina*, but pollution did not have such drastic impact since many of the *A. salina* thrived under presence of atrazine.

This seems unique to our study since in a previous study, *Artemia nauplii*—aquatic organisms similar to *A. salina*—were used to examine adverse outcomes of increasing silver nanoparticle concentrations on *A. nauplii* (Chinnasamy, 2014). The test groups with higher levels of particle concentrations had the highest mortality rates.

For future studies, it will be much more efficient to have larger sample sizes of *A. salina* as well as completing more trials so that the possibility of data being due to chance is decreased significantly. Also, techniques for hatching *A. salina* eggs need to be refined in order to yield a higher success rate of hatching. Larger amounts of *A. salina* in equal proportions across all trials would yield more concrete results for analysis. Other ways to improve efficiency would be to hold as many outlier variables constant throughout the experiment such as light exposure and temperature fluctuations.

This research study maintains the assertion that temperature has an effect on organisms' biological function and survival. However, our results bring about questions of whether synergistic toxicity truly has an added effect on the survival of various organisms. Even though what we came across contradicts previous studies cited, we believe further experimentation would help us come to a definitive answer. For now, our hypothesis was rejected in favor of a result that states there was no synergistic effect of temperature and pollution on *A. salina*.

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