JOURNAL OF
EMERGING INVESTIGATORS

Article

# Increasing $CO_2$ levels in water decrease the hatching success of brine shrimp

**Nadine Greer[1], Dana Johnson[1], Maria Klein[1], Jeffrey Greer[2]**
[1]Clover Hill High School, Midlothian, Virginia
[2]Midlothian Family Practice, VA Physicians, Midlothian, Virginia

## SUMMARY

**Increasing $CO_2$ levels in the oceans due to global warming leads to $CO_2$-induced ocean acidification. When ocean acidity levels rise from additional $CO_2$, it can lead to the endangerment of many crustaceans whose shells may dissolve with the decreasing pH. Through a model of ocean acidification, our study explores the extent to which increasing $CO_2$ levels affect the hatching success of *Artemia sinica*, commonly known as brine shrimp. We hypothesized that as $CO_2$ levels in the tank increased, the hatching success of the brine shrimp would decrease. We added increasing numbers of $CO_2$ tablets to tanks to acidify the water in tanks that each contained ten replicates of brine shrimp eggs, and after four days, we quantified the number of hatched brine shrimp. We found that the brine shrimp exposed to the lowest amount of $CO_2$ had the highest hatching success, and the brine shrimp exposed to the highest amount of $CO_2$ had the lowest hatching success. These data suggest the need to limit both $CO_2$-induced ocean acidification and $CO_2$ emissions to protect all marine life.**

## INTRODUCTION

The current global warming crisis threatens life on Earth; however, this process began over 200 years ago. The Industrial Revolution started in the 1760s, bringing new ideas to manufacturing processes in Europe and the United States (1). The gas production, resulting mainly from the burning of coal, caused an acute rise in atmospheric carbon dioxide ($CO_2$) concentration (1). This seemingly harmless gas trapped in the atmosphere has caused potentially irreversible increases in the global temperature that jeopardize the planet's food chains and physical surroundings (1).

Over time, factory emissions, vehicle exhaust, and other human activities released more $CO_2$ into the atmosphere, where it becomes trapped. As a prominent "greenhouse gas" $CO_2$ blocks a portion of radiated heat from escaping the outer layers of the atmosphere, causing the average temperature of the Earth to rise. Almost 50% of $CO_2$ then dissolves into the oceans after being dispersed into the air (1). The increased amount of dissolved $CO_2$ lowers the pH levels of the oceans due to the production of carbonic acid, a process known as ocean acidification (2). Since the rise in $CO_2$ emissions in the 1700s, the ocean's acidity has increased by 30% primarily due to human activity (1). If emission patterns continue without change, the acidity levels of oceans may rise higher than any point in time in over 10 million years (3).

As the pH of the oceans continues to decrease, the more damage is done to its inhabitants (1). Their preserving efficiency decreases, corroding their defensive characteristics. For example, the coral reefs, an essential ecosystem for many aquatic species, are severely affected by the high acidity levels. The absorbed $CO_2$ in the oceans produces carbonic acid, dissolving the outer layers of the reefs (4). These layers protect the reefs and hold them together, forming a crust. Unfortunately, the coatings have become extremely thin because of the ocean's rising acidity levels. According to recent studies, the outer layers of 22,000-year-old reefs were once estimated to measure approximately 11.5 cm in thickness, but 12,000-year-old reefs were recently measured at 3.5 cm in thickness (5). Therefore, with the rising levels of ocean acidification, younger reefs are no longer able to achieve the same thickness of their protective outer layers as older reefs. At this rate, due to the increases in carbonic acid, the coral reefs will be unable to sufficiently protect themselves in the future.

Along with the coral reefs, organisms such as pteropods and phytoplankton are also at risk. As aqueous $CO_2$ concentrations increase, available carbonate ion concentrations will simultaneously decrease as they bond with excess hydrogens from carbonic acid. As a result, marine calcifying organisms, such as pteropods, will have further difficulty using the carbonate ions to form calcium carbonate, which is essential to shell development and maintenance (6). Additionally, increasing amounts of carbonic acid can threaten to corrode the protective shells of pteropods due to the water's high acidity (7). Studies have shown that a decrease in oceanic pH to between a 7.8 and 7.9 resulting from an increase in aqueous $CO_2$ concentrations would be sufficient to reduce the amount of carbonate ion concentrations required for shell maintenance by 50% (1). If the current trend of decreasing pH levels continues, the oceans are predicted to dissolve the outer layer of calcium carbonate shells within 48 hours by the year 2100 (7).

Phytoplankton, on the other hand, serve as the microscopic organisms that form the base of the oceanic food chain, supporting organisms from small crustaceans to the blue whale. They require $CO_2$ dissolved in the upper levels of the ocean to carry out the essential process of photosynthesis, but ironically, their productivity is also decreasing (8). One reason for this phenomenon is that increased atmospheric temperatures resulting from greenhouse gasses have altered an ocean current called the Atlantic Meridional Overturning Circulation (AMOC) (8). The AMOC is crucial for circulating the nutrients that phytoplankton eat throughout the layers of the ocean, but the current's circulation has weakened in recent years. Some scientists suggest that this is due to Greenland's ice melting from rising atmospheric temperatures, which

https://doi.org/10.59720/23-201

then alters the equilibrium of freshwater and saltwater in the AMOC needed for proper circulation (8). Therefore, since the AMOC has experienced a decrease in productivity, the phytoplankton are unable to filter out absorbed $CO_2$ in the ocean, thus compounding the problem for the pteropods.

Research on the effects of ocean acidification on different species can help determine how this phenomenon may impact the ocean ecosystem. It has been shown that crustaceans exposed to acidic water experienced detrimental effects on their behavioral prey defenses (9). Specifically, as water temperatures and $CO_2$ concentrations increased, the crustaceans experienced neurological interferences that affected essential protection mechanisms, such as shell thickening and evasive swimming speed. These neurological interferences can be attributed to shifts in ionic gradients that can lead to neuronal depolarization (9). Another study examined the effects of ocean acidification on the survival of the larval blue crab. The authors found that larvae grown in acidic water were 10% smaller in size and experienced a 23% decrease in survival rate compared to controls (10). Another study looked at the effects of $CO_2$-driven seawater acidification on the crustacean brine shrimp. The study examined three pH levels as a result of increased $CO_2$, the control (8.2), the first level (7.8), and the second level (7.6). They found that the hatching rate, growth rate, and survival success were all reduced with increasing $CO_2$ levels. The genetic material in the brine shrimp was also altered, along with essential enzyme activities (11). While this research examined some of the same variables as the 2015 study, our study both corroborates their results and adds to the body of knowledge that demonstrates the drastic consequences of ocean acidification on crustaceans.

Given the negative effects of rising $CO_2$ levels on calcium carbonate and the performance of other organisms, we suspected that brine shrimp would suffer a similar fate. The purpose of this study was to determine the effect of increasing $CO_2$ levels on brine shrimp hatching success by observing them in their early larval stage. We hypothesized that as more $CO_2$ was added into the tank, the hatching success of the brine shrimp would decrease. Our results suggest that continuing ocean acidification will have life-threatening effects, such as the dissolving of eggs or protective shells, on crustaceans in oceanic environments.

## RESULTS

Our study examined $CO_2$ tablets in numbers of zero, two, four, and six added to their respective tanks of brine shrimp eggs daily. For each tablet amount, we conducted 10 replicates with equal amounts of brine shrimp eggs, securely fastened in jars and placed in one of the four tanks (**Figure 1**). We observed each replicate for a total of four days, added tablets initially to each tank with the brine shrimp eggs, and subsequently at the same time over the course of the study.

In this study, the increasing number of $CO_2$ tablets added to each tank negatively affected the number of brine shrimp eggs that hatched successfully after the given amount of time (**Figure 2**). We used the measure of mean to display central tendency for each set of replicates. The mean number of hatched brine shrimp for the control, 2 tablets added, 4 tablets added, and 6 tablets added were 17.05 ± 4.98, 9.35 ± 3.11, 6.25 ± 1.90, and 3.64 ± 1.51 respectively. The mean

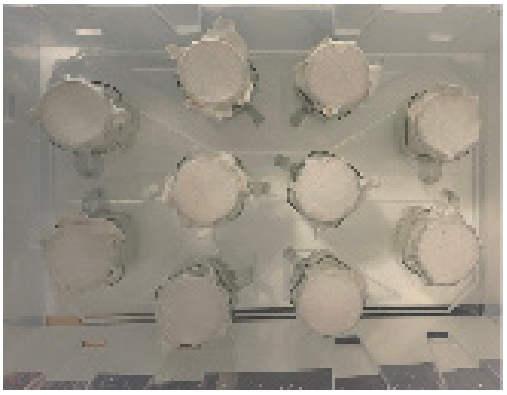

**Figure 1: Picture of the arrangement of jars with brine shrimp egg replicates.** Ten jars (replicates) of brine shrimp eggs in control bucket. Each jar secured with cheesecloth to ensure equal water distribution and security of eggs.

number exhibited a gradual decline in the number of hatched brine shrimp as we added more $CO_2$ tablets (**Figure 3**).

The measures of variation were range and variance for each set of replicates. The measures of range for the control, two $CO_2$ tablets, four $CO_2$ tablets, and six $CO_2$ tablets were 19.0, 7.5, 6.0, and 4.0 respectively. The measures of variance for the control, 2 tablets added, 4 tablets added, and 6 tablets added were 24.802, 9.669, 3.625, and 2.288 respectively. We tested the null hypothesis of no significant difference using a one-way ANOVA test with a 0.05 level of significance. As a result of the ANOVA test, the p value measured < .00001 for all three comparisons of the experimental group vs. the control.

The average number of hatched brine shrimp over a total of four days when exposed to different levels of $CO_2$ was assessed. The number of successfully hatched brine shrimp decreased after the addition of more $CO_2$ tablets; the control, with zero tablets added, had the highest number of successfully hatched brine shrimp (**Figures 1 and 3**).

**Figure 2: Magnified picture of six hatched brine shrimp from control group.** Hatched (circled) vs. unhatched brine shrimp. Due to the presence of a carotenoid pigment, esterified astaxanthin, the newly hatched brine shrimp in the first larval stage (nauplii) appear bright orange. The dark brown eggs are unhatched. Image captured through a 30x magnifying glass.

https://doi.org/10.59720/23-201

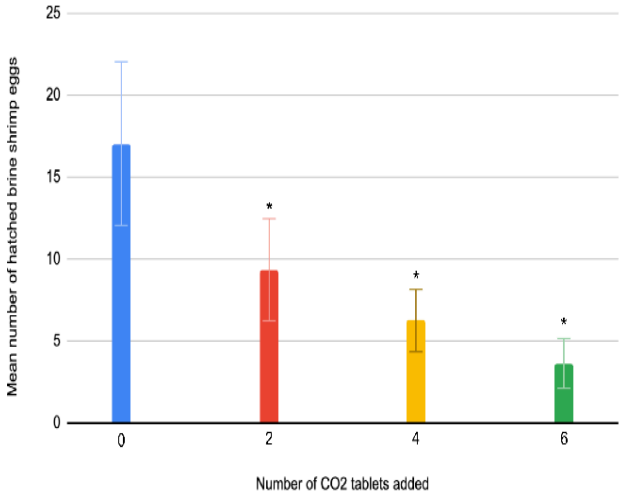


**Figure 3: The effect of increasing $CO_2$ levels on brine shrimp hatching success.** Graph showing mean number of hatched brine shrimp in all four tanks. Brine shrimp eggs were hatched in tanks with either no additional $CO_2$ added, two $CO_2$ tablets added, four $CO_2$ tablets added, or six $CO_2$ tablets added. One-way ANOVA was statistically significant at $p < .00001$ for all three comparisons of the experimental group vs. the control (zero tablet). A * indicates the statistically significant values.

## DISCUSSION

The purpose of this experiment was to determine the effect of increasing $CO_2$ levels on the hatching success of brine shrimp. To determine the effects on the brine shrimp, we introduced the same number of eggs into water with various levels of $CO_2$. After four days, we recorded the number of hatched brine shrimp in their early larval stage. We found that as the eggs were exposed to more $CO_2$, their hatching success decreased. In the control group, we found an average of 17.05 hatched brine shrimp, while the third group with 6 tablets dissolved only produced an average of 3.64 hatched brine shrimp in the same period of time. Our initial research hypothesis stated that as we added more $CO_2$ into the tank, the hatching success of the brine shrimp would decrease. Our findings supported the research hypothesis as fewer brine shrimp between the levels hatched when exposed to more $CO_2$.

In a similar 2015 study, hatching rate, growth rate, and survival success of brine shrimp were all reduced because of the process of ocean acidification, caused by the lowering of pH from additional $CO_2$ (11). The authors concluded that not only were the brine shrimp negatively affected, but all crustaceans could be at risk for similar consequences of ocean acidification. In this experiment, we similarly showed that a brine shrimp's hatching success decreases with the addition of $CO_2$. Since the added $CO_2$ caused the water to increase in acidity, the outer shell of eggs most likely began to dissolve. Thus, based on previous studies, the developing eggs would have lacked protection from the acidic water, which caused their hatching success to decline as the water increased in acidity (11). While the dissolving eggshells were not observed directly, it could explain the decrease in hatching success.

To increase the accuracy of our experiment, we could have regulated the temperature to stay consistent, instead of a general range of "room temperatures." Since the temperature was subject to change based on the time of day, the temperature was not always consistent, fluctuating around a 5° F range. We placed all tanks in the same room, equidistant to light and heat sources, so all temperature fluctuations remained constant, but the fluctuating temperatures could have affected the productivity of the hatching brine shrimp. Growth and development of crustaceans, specifically shown in a study of brachyuran crabs, can be delayed with lower temperatures, and increased with optimal (higher) temperatures, so temperature fluctuations could have led to variations in hatching success (12). However, since we only calculated the total number of brine shrimp hatched, we did not directly observe fluctuations in hatching based on temperature changes. Keeping the temperature constant throughout would allow for the brine shrimp to continue hatching at a constant rate.

In addition, we could have used a type of water to mimic seawater instead of tap water. Seawater is optimal to produce a higher brine shrimp hatching success since it is closer to the conditions of the brine shrimp's natural habitat. Adding salt to the tap water could have changed the results of this experiment because, while it may not change the pH of the water due to salt's neutral properties, it could have fostered a higher hatching success overall. However, the tap water conditions remained constant throughout all levels of $CO_2$ exposure, so adding salt would not account for inter-level differences in hatching success. Therefore, the results of this experiment are significant in that hatching success decreased as the brine shrimp were exposed to $CO_2$. Further research could determine whether the conditions of the water could alter the assumed decrease in pH with the addition of $CO_2$. In doing so, future studies could examine the pH of tap water in comparison to the pH of seawater, which would then likely change as $CO_2$ is added.

Brine shrimp are particularly adaptable to water and external variables and are often grown as pets by young children. Brine shrimp, also known as "sea monkeys", are grown in small plastic containers filled with tap water and are occasionally supplemented with other nutrients. The resilience of brine shrimp means that a majority of the "sea monkeys" will hatch. In comparison, coral reefs require a narrower range of variables for optimal growth, making them a poor subject to replicate an experiment on ocean acidification. However, in laboratory conditions, the brine shrimp were significantly affected by the increase of $CO_2$, suggesting that the implications of ocean acidification could be much more severe on other, less adaptable, marine life, solidifying ocean acidification as a key threat to the health of marine life.

It is important to recognize that due to the continuing rise of ocean acidification in the oceans, the results of our study could predict the future reality of oceanic environments for not only brine shrimp, but all crustaceans. The goal of this work is to simplify the conditions of ocean acidification to represent the dangers of $CO_2$ in our oceans, thus showing that crustacean life could be severely threatened if no action is taken towards reducing global $CO_2$ emissions.

https://doi.org/10.59720/23-201

## MATERIALS AND METHODS

### Experiment Preparation

On the first day of the experiment, four 56.8 L buckets (serving as tanks) and 40 glass jars were washed with warm tap water and Dawn Dish Soap. Then, each tank was filled with exactly 30 L of tap water using a glass measuring cup, so the water surpassed the tops of the glass jars. Following this, 80 squares of cheesecloth were cut into 10 cm by 10 cm pieces. After that, ten glass jars were assigned to each bucket, creating ten replicates per level of $CO_2$ exposure (**Figure 3**). The tanks were uncovered and placed on a table in a temperature-controlled room with one window for sufficient sunlight. The experiment took place over four consecutive days.

To distribute the brine shrimp eggs (San Francisco Bay), jars were removed from the water-filled buckets and filled approximately 50% with tap water. The water came from each jar's assigned bucket, which ultimately kept the water levels constant across each level of $CO_2$ exposure. To disperse shrimp eggs, a wooden dowel measuring 0.5 mm in diameter was dipped into the water of a single jar, then dipped into the brine shrimp eggs until the shrimp eggs fully covered the dowel from tip to a mark at 0.5 cm. Then, the dowel was taken out of the egg jar and mixed into the original jar. The dowel was stirred around the jar's water, then dried off with a paper towel. Measuring eggs with the marked dowel allowed for a small number of eggs to be accurately placed into each jar (without the use of an advanced scale or other measuring tool). While the dowel does not provide an exact number of eggs, it ensures roughly equivalent amounts were added to each jar.

This process continued for every jar until all 40 jars (10 replicates per number of tablets added to tanks) had equal amounts of brine shrimp eggs added to them. After that, two layers of cheesecloth were placed on top of each jar and secured with a single rubber band around the rim of the jar. Then the jars were placed into their assigned bucket. Each bucket was left uncovered with equal exposure to light.

### Tank Introduction to $CO_2$

On the same day as the setup, the $CO_2$ tablets (ISTA Water Plant $CO_2$ tablets) were also added to the tanks. In the first bucket, two tablets were added, both on opposite sides of the bucket. In the second bucket, four tablets were added, with similar placement to the first bucket. In the third bucket, six tablets were added. Each $CO_2$ tablet was comprised of sodium bicarbonate and citric acid and weighed approximately 1 g. On the second and third days, the same number of $CO_2$ tablets were added as on the first day of the experiment.

### Data Collection

On the fourth and final day, the brine shrimp were counted using a random sampling technique. Their number was counted from a portion of brine shrimp swimming in the jars. First, each jar was taken out of its bucket, and the cheesecloth was removed. Then, using an extra lid from the jars, a single jar was lightly shaken for about two seconds. The lid was removed, and a dropper gathered 10 mL of the water. The water was deposited into a clean petri dish, and the hatched brine shrimp eggs were counted using a magnifying glass. Due to the presence of a carotenoid pigment, esterified astaxanthin, newly hatched bring shrimp in the first larval stage (naupli) will appear bright orange, allowing us to differentiate between hatched and hunhatced brine shrimp (14) (**Figure 2**). The number of hatched brine shrimp eggs was recorded, then the contents of the petri dish were deposited back into the original jar. The closed jar was then shaken, and a second measurement of the brine shrimp was gathered, then the two numbers were averaged together. After the measurement of one jar was complete, the petri dish was rinsed with soapy water in one bowl, then rinsed with clean water in the second bowl. The cleaned petri dish was then used again to measure the next jar, until all 40 jars were measured.

A statistical test was performed to determine if the results were significant, using a one-way ANOVA test format. The test was conducted using an online calculator with a significance level of $p < 0.05$ (13).

### Experimental Risk

The risks for this experiment were loss of consciousness and suffocation due to the presence of $CO_2$ tablets. To prevent this from occurring, all people left the room as soon as the tablets were placed in water for approximately two hours, or until the tablets had fully dissolved. The room also had ample ventilation to ensure there was a sufficient flow of air.

**Received:** July 19, 2023
**Accepted:** March 05, 2024
**Published:** January 07, 2025

## REFERENCES

1. "Ocean acidification." Encyclopædia Britannica, 2020.school.eb.com/levels/high/article/ocean-acidification/570924.
2. Christie-Blick, K. "Ocean acidification investigation." *Science Scope*, vol. 42, no. 5, 2019. https://doi.org/10.2505/4/ss19_042_05_42
3. Riebesell, et al. "Enhanced biological carbon consumption in a high $CO_2$ ocean." *Nature*, vol. 450, no. 7169, 2007, pp. 545-548. https://doi.org/10.1038/nature06267
4. "Coral reefs at risk." *Science and Children*, vol. 45, no. 7, 2008.
5. "Great Barrier Reef 'glue' at risk from ocean acidification; Geologic 'glue' thickness is an accurate measure for historic pH." ENP Newswire, 2020, www.sydney.edu.au/news-opinion/news/2020/09/03/Great-Barrier-Reef-microbialite-glue-at-risk-from-ocean-acidification.html.
6. Lippsett, L. "Beyond warming the planet, buildup of $CO_2$ threatens the shells of ocean organisms." Oceanus, vol. 44, no. 3, 2005.
7. Orr, J., et al. "Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms." *Nature*, vol. 437, no. 7059, 2005, pp. 681-686. https://doi.org/10.1038/nature04095
8. Koenig, E. "Ocean Productivity Falls as the Planet Heats Up." Oceanus, vol. 54, no. 2, 2019.
9. Clements, J. C., and Comeau, L. A. "Behavioral defenses of shellfish prey under ocean acidification." *Journal of Shellfish Research*, vol. 38, no. 3, 2019. https://doi.org/10.2983/035.038.0324

https://doi.org/10.59720/23-201

10. Giltz, S. M., and Taylor, C. M. “Reduced growth and survival in the larval blue crab callinectes sapidus under predicted Ocean acidification.” J*ournal of Shellfish Research*, vol. 36, no. 2, 2017. https://doi.org/10.2983/035.036.0219
11. Zheng, C.-Q., et al. “Detrimental effect of $CO_2$-driven seawater acidification on a crustacean brine shrimp, Artemia sinica.” *Fish & Shellfish Immunology*, vol. 43, no. 1, 2015, pp.181-190. https://doi.org/10.1016/j.fsi.2014.12.027
12. Azra, Mohamad N., et al. “Effects of climate $CO_2$-induced water temperature changes on the life history of Brachyuran Crabs.” *Reviews in Aquaculture*, vol. 12, no. 2, 2019, pp. 1211–1216. https://doi.org/10.1111/raq.12380
13. “One-Way ANOVA Calculator, Including Tukey HSD.” Social Science Statistics. www.socscistatistics.com/tests/anova/default2.aspx.
14. “The Pigments of Artemia.” *Proceedings of the Royal Society of London. Series B. Biological Sciences*, vol. 152, no. 946, 1960, pp. 118–136. https://doi.org/10.1098/rspb.1960.0028