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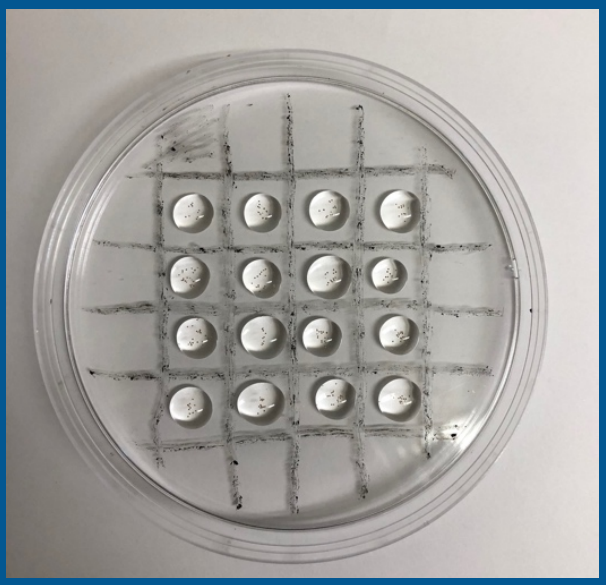

Testing the health of natural runoff streams by comparing hatching rates of *Artemia salina*

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Introduction:

Artemia salina, more commonly referred to as brine shrimp, are one of the more easily accessible model organism to study. This comes from their embryo's ability to become dormant, making it fairly simple to distribute this commonly used food source around the world (Van Stappen, 1996). These dormant embryos, known as cysts, will remain dormant until they are immersed in seawater (Van Stappen, 1996). The cyst swells to become spherical, and after 20 hours (Van Stappen, 1996) the outer membrane of the cyst opens to reveal the embryo still in what is known as the hatching membrane. This membrane quickly ruptures as well and the nauplius instar I is released into the water (Van Stappen, 1996).

In this study, I tested the hypothesis that if *Artemia salina* are sensitive to varying natural water conditions, then the nauplii hatching rates will differ based on the body of water. Extensive research has been conducted to determine the effect of specific toxins on *Artemia salina*. Ammonia and ammonium are the among the most prevalent causes of toxicity in *Artemia salina*, and organic pollutants can also have effect as well (Svensson, Mathiasson, Mårtensson, & Bergström, 2005). A specific study determined that *Artemia salina* was effective in assessing textile dye toxicity (Swarnkumar Reddy & Osborne, 2020). While it is known what specific toxins effect *Artemia salina*, this study aims to see if these findings can be generalized to test whether the hatching rates of *Artemia salina* can be used as an indicator for the general health of a natural body of water.

Additionally, this research proposes a new method of analyzing *Artemia salina* hatching rates. The method involves modifying a petri dish to create small samples of 2-3 water droplets that allow for easy counting of the total number of cysts and the number of hatched nauplii. This procedure, especially with *Artemia salina* cysts being large enough to count with the naked eye, is very accessible and easy to replicate. With very little equipment, this experiment can be performed and the results applied to determine the overall health of a specific body of water.

In this study, I treated hydrated *Artemia salina* cysts with different water samples from the Willamette Valley in Oregon and measured the hatching rates over a 73-hour period. I collected 5 water samples and developed a novel method to create small wells of 2-3 drop samples to accurately count hatching rates with little special equipment.

Materials and Methods:

This experiment involved sampling a variety of water sources in Oregon. All of the samples were taken on the same day to reduce possible weather variables. The samples were collected three days after the last large rainstorm to help ensure that comparable samples of the water were being taken. One 50-mL Falcon tube of water was collected at each location. The water was taken from a part of the stream where the water was moving and the samples were stored at room temperature for one day prior to the experiment starting.

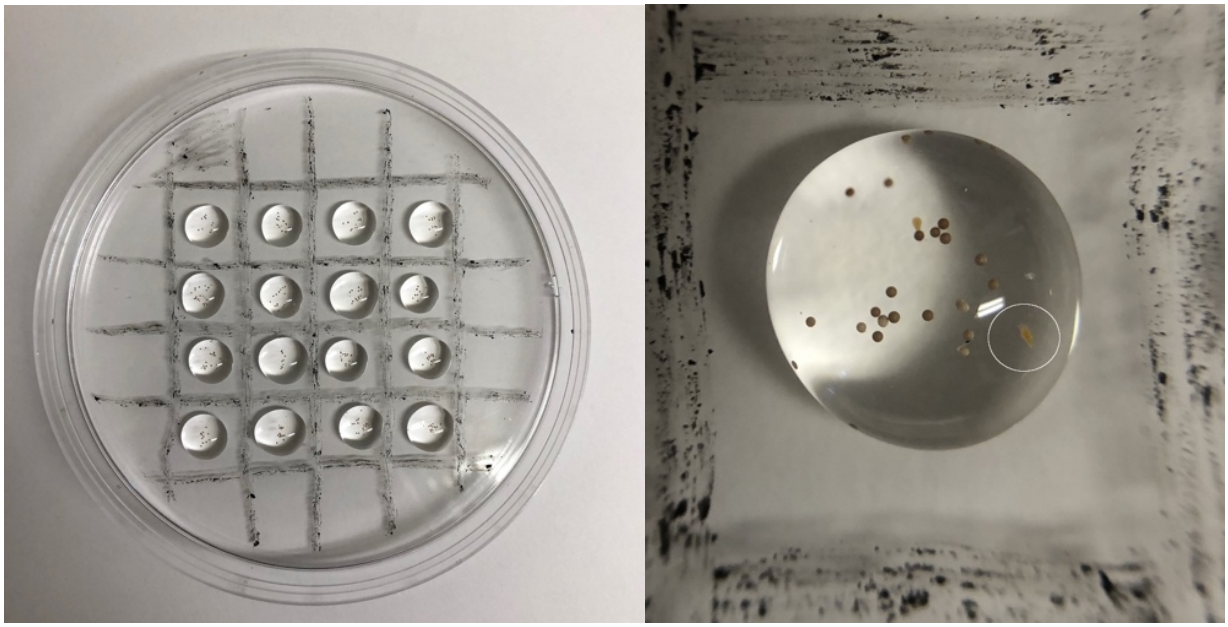

Figure 1. Diagram of the experimental method. In the left image, the modified petri dish is shown with the 16 middle wells filled with countable samples of cysts. Notice how spread out the small amount of cysts are in the drops, making it very easy to count the number of cysts in each well with only the naked eye. In the right diagram, one well is shown in a closer view. The white circle on the image is highlighting a hatched nauplius in the drop, which is clearly distinguishable from the rest of the unhatched cysts in the well.

This experiment required devising a new method to count the number of hatched *Artemia salina* nauplii. These *Artemia salina* cysts were obtained from the Carolina Biological Supply Company (Carolina Biological Supply Company, 2009). As shown in Figure 1, 90 mL petri dishes were modified to create smaller wells. This was done by placing the bottom of the petri dish over a printed 1.25 cm by 1.25 cm grid paper sheet and tracing the grid onto the inside of the bottom of the dish using a black crayon. A black crayon was used because it was dark enough to easily see the grid lines and the wax in the crayon is hydrophobic and useful in keeping the water droplets separated in the respective wells. Given the size of the petri dish and the size of the tracing grid, five lines were drawn in perpendicular directions to create 16 identical square wells in the center of the dish. One dish was made for each water sample collected. For this experiment, with seven different water samples and one control, 8 petri dishes were made.

Because the sample drops were small (2-3 drops of water per well), precautions were taken to prevent evaporation. To accomplish this, a paper towel was cut to fit the top of the petri dish, and

then 2 pieces of tape were placed in an "X" pattern to keep the paper towel from falling onto the samples below. The tape was tightly sealed into the rim of the top of the dish to ensure the top still snuggly fit onto the bottom of the dish. Fresh brine was then added to the paper towels to keep the dishes humidified. The brine was added using a pipette and the drops were counted to ensure all dishes received the same amount. 24 drops of brine were added to the humidifying towel in each petri dish initially, 16 more drops of fresh brine were added to each towel at 24 hours, and 16 drops of brine were added to each towel at 55 hours.

The *Artemia salina* cysts were hydrated according to directions from the company (Carolina Biological Supply Company, 2009). The cysts were hydrated in a separate petri dish before being transferred into the modified petri dishes. The cysts were hydrated in 10 mL of 1% brine solution made from each of the water samples. For the control water, 240 mL of tap water was treated with 0.60 grams of sodium bicarbonate to neutralize the pH. Testing the pH of the control was beyond the scope of the current study, however a further study could pursue the specific effects of pH on *Artemia salina* hatching rates. A consistent amount of cysts were added to each sample, with enough added to supply each of the 16 wells with 10-20 cysts.

The cysts were added to the wells using transfer pipettes. A 0.2 mL transfer pipette was used for all samples and a clean pipette was obtained for each sample. Cysts were drawn from the outer edge of the water container, as cysts from the concentrated center of the dish may have reduced viability due to anoxia. 10-20 cysts and 2-3 drops of water were added per well. The number of cysts in each well were counted and recorded. Under a bright desk lamp, the cysts were large enough to be counted with the naked eye. One of the unused outer wells was colored in using the black crayon to ensure the orientation of the dish was maintained throughout the experiment. The lids were added to the top of the dish and the dishes were labeled.

Data collection started at 31 hours. Data collection consisted of counting the number of nauplii present in each well. Similar to counting the cysts, under bright light, the nauplii were large enough to count with the naked eye without needing additional equipment as demonstrated in Figure 1. However, a hand lens could have been used for additional clarity. Only moving and active nauplii were counted. Data were collected at 31, 49, 55, 65, and 73 hours after hydration.

Data were analyzed by comparing hatching rates. At the five collection times, the total number of hatched nauplii across all 16 wells were added together. This total was divided by the initial total number of cysts to produce the hatching rate. A line graph using these five hatching rates per sample was created, as shown later in Figure 3.

Images of the general set up were taken with an iPhone 8 camera, and a hand lens was used to increase the magnification. Specific images of an unhatched *Artemia salina* cyst and a hatched nauplii were taken on a foldscope with a 140X magnification ("Foldscope Instruments," 2021). Images were analyzed using Fiji to determine the scale of the images.

Results:

Figure 2 demonstrates the differences between *Artemia salina* cysts and instar I nauplii. This experiment involved counting the hatched nauplii, which are much larger and of different color than the unhatched cysts. The instar I nauplii are somewhat active, and their movement along with the size and color differences were the basis of the counting data collection. With only the naked eye, there were some instances of counting errors due to non-moving nauplii. Therefore, a hand lens should be used in future experiments to reduce error.

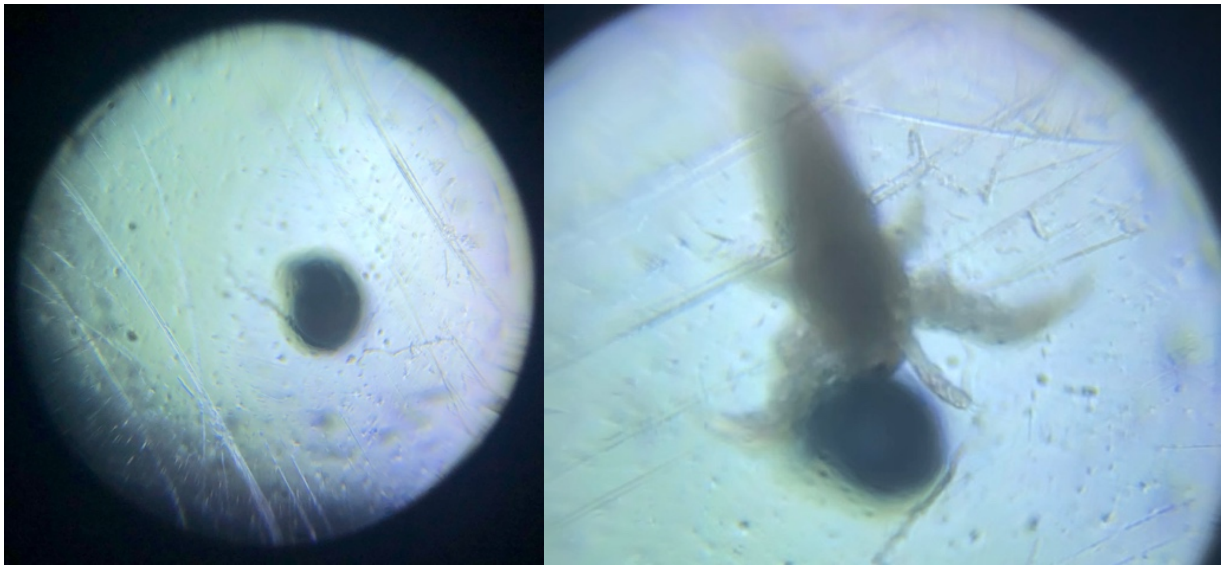

Figure 2. View of *Artemia salina* cysts and instar I nauplii under a foldscope microscope. The left image shows an unhatched cyst that is 170 micrometers in length. The right image shows a hatched instar I nauplii in comparison to the same cyst in the left photo. Notice how much larger the nauplii is than the cyst and how clearly differentiable the two developmental stages are. The nauplii was measured at 430 micrometers.

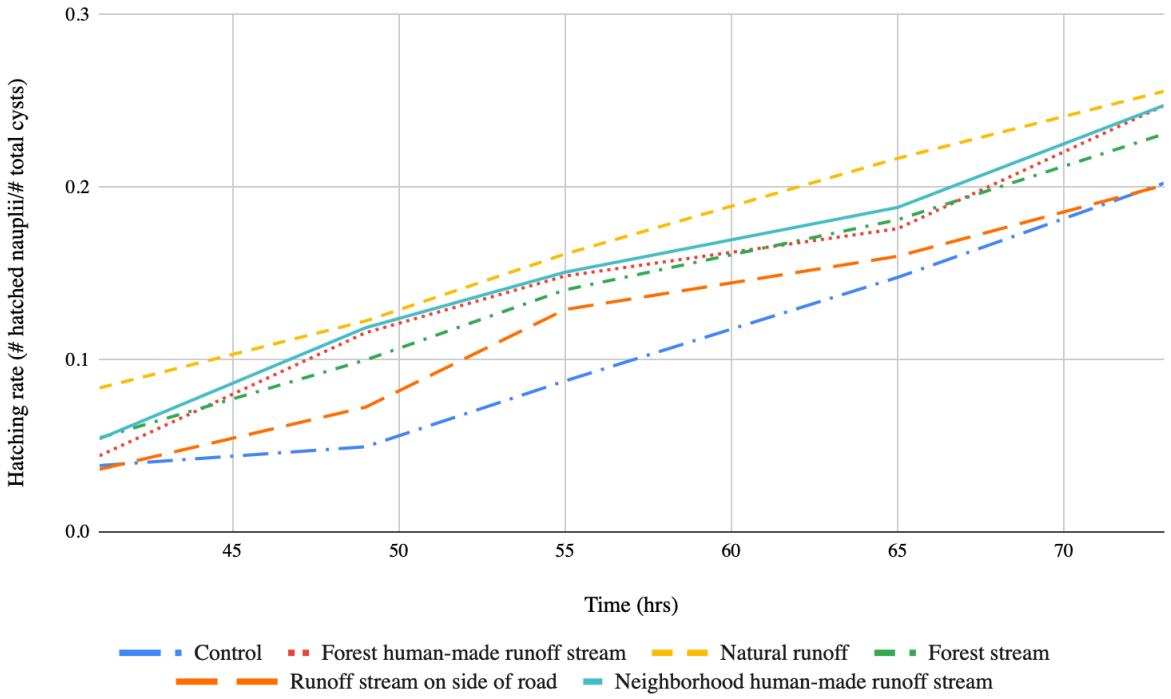

Figure 3. Overall hatching rates of *Artemia salina* in water. The graph demonstrates the hatching rates at the five collected times. Notice how although all samples demonstrate similar slopes across the entire 73-hour period, there are two main trends in specific hatching rates.

The samples were collected from a variety of natural water sources, as briefly described in Figure 3. Two human-made runoff streams were sampled, one from a forested area and one that ran through a suburban neighborhood. A natural forest stream was sampled, as well as two semi-permanent runoff streams that form after large rainstorms. One of these streams formed by the side of a busy road, and the other formed in the middle of a dense forest.

The hatching rates across all six water types demonstrated growth over the entire sampling period, as shown in Figure 3. However, the figure also shows two general trends. The first trend of consistent growth over the entire sampling period was demonstrated in both the control and natural runoff samples. The second trend of varied growth in a more jagged upward slope was demonstrated by the other four water samples.

Discussion:

The hypothesis of if *Artemia salina* are sensitive to varying water conditions, then the nauplii hatching rates will differ based on the body of water is generally supported from the results of this preliminary study. Testing five different experimental waters resulted in two general trends.

The control and natural runoff samples showed a consistent growth over the entire sampling period. Given that this group included the control, we assume that this trend is the expected trend for *Artemia salina* hatching rates. Additionally, because the natural runoff sample was collected from a forested runoff stream that resulted from a recent rainstorm, it is possible that this sample is relatively free of toxins other than those from the forest soil.

The other four water samples conveyed similar trends of more scattered increasing hatching rate. This trend could be because of a chemical present in the water that caused inconsistencies in the hatching rates across the sampling period. Because these waters were collected from either human-made runoff streams or in areas of increased human activity, it is very possible that these waters contain chemicals that resulted in the observed scattered growth.

However, all water samples demonstrated hatching, and the final hatching rates across all water samples were very similar. Because testing the waters for specific toxins was beyond the scope of the current study, it is impossible to conclude that these three trends are a direct result of a specific toxin or nutrient found only in water samples of a specific trend. Based on the results of the Oregon Department of Environmental Quality's Oregon Water Quality Index from the site closest to the sites being sampled in this experiment, the water status of that closest sample was very poor ("OWQI Sampling Site Report - 2019," 2021), meaning it is possible there are chemicals present in the water sample that are resulting in these trends.

While no specific toxins were noted in the OWQI report, research has been conducted on the response of *Artemia salina* to specific toxins present in runoff water. Chloride ions are commonly found in runoff from landfills, but however do not affect *Artemia salina* to a large extent because the organism is found in salt lakes where there is an abundance of exposure to chloride (Svensson et al., 2005). This study also found that ammonia and ammonium are the most prevalent cause of toxicity of *Artemia salina* present in leachate water from landfills (Svensson et al., 2005). Organic pollutants can also affect the organism's hatching rates, while metal ions do not seem to have an effect on *Artemia salina* hatching rates without being in the presence of other pollutants (Svensson et al., 2005). Based on the widespread prevalence of ammonia from both industrial uses and as a natural byproduct of bacterial processes in soil, it is possible that ammonia present in the water samples could be a cause of the two distinct trends (Health, 2011).

Another possible toxin that could be affecting hatching rates are oxidized multi-walled carbon nanotubes. Carbon nanotubes are used in many industrial applications, and can easily infiltrate rainwater and runoff from contaminated soil and air (Zhu et al., 2017). Zhu's study found that the carbon nanotubes directly affected ROS formation in the *Artemia salina* (Zhu et al., 2017). Reactive oxygen species (ROS) are a product of oxidative metabolism in the mitochrondria, and an imbalance of ROS causes oxidative stress (Ray, Huang, & Tsuji, 2012). Carbon nanotubes were shown to increase oxidative stress in the *Artemia salina*, affecting hatching rates (Zhu et al., 2017).

Additionally, some toxins have been found to accumulate in nauplii without affecting their hatching rates, so basing the health of a body of water on just the hatching rates is not effective in analyzing the overall health of the water. Nickel oxide and cobalt oxide have been found to

accumulate in the digestive track of *Artemia salina* as a result of filter feeding (Ates, Demir, Arslan, Camas, & Celik, 2016). Therefore, a further study could look into the effect of different water samples on the development of *Artemia salina*, rather than just hatching rates.

The new method of analyzing nauplii hatching rates developed in this study can be further applied to research analyzing the effects of specific toxins, nutrients, or chemicals on hatching rates of both *Artemia salina* and other organisms that develop in a similar way. The use of a hand lens to increase counting accuracy can be implemented to improve the method. One practical application of this research is that it can be conducted with limited materials and the method can be generally applied to many future areas of study.

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