The Effects of Acid Rain on Embryonic Development of *Danio rerio*

Skylar Poppe

Union Grove Union High School

Abstract

The experiment was carried out to test the effect of acid rain on the embryonic development of zebrafish. Sulfuric acid, found in acid rain, was used at different pH levels of 4, 5.5, and 7 (control containing no sulfuric acid) in the wells containing zebrafish embryos. The experiment was conducted over a period of five days. Each day the solutions were exchanged with fresh stock solutions and dead embryos were removed. The H_2SO_4 solutions had a dramatic effect on the speed of development and death of embryos. The embryos in the H_2SO_4 solution of pH 5.5 developed more than those in the solution of pH 4, but none of the embryos exposed to sulfuric acid survived. The results of this investigation shows how important preservation of the environment is for the survival of animals and other organisms.

Introduction

Danio rerio, also known as zebrafish, are freshwater fish that are most commonly found in rivers and streams (eol.org). According to Otago University, "The zebrafish – *Danio rerio* – is a tropical freshwater fish originally found in Eastern India's Ganges River and native to the southeastern Himalayan region" (otago.ac.nz). They develop through eight major periods and hatch from their embryos between 48 and 72 hours post fertilization (embryology.med.unsw.edu.au). They are used often in research because zebrafish reproduce large quantities of eggs, are not costly, and develop relatively quickly. Development of zebrafish embryos is very easy to track because eggs develop outside of the mother and are transparent so researchers can easily see changes and structures in the embryo. This allows for embryo development to be able to be tracked with a low-power microscope (yourgenome.org).

Acid rain occurs when sulfur dioxide and nitrogen oxides are emitted to the atmosphere. A majority of these substances come from the burning of fossil fuels. Acid rain has dramatically decreased fish populations in rivers and streams. It has a pH ranging from 4.2 to 4.4 (epa.gov) while natural water in rivers and streams has a pH of about 7.4 (grc.nasa.gov). According to the United States Environmental Protection Agency (EPA), most fish cannot hatch at a pH of 5. While many adults fish can adapt and survive in that pH level of 5, even the adult fish will die off at lower pH levels (epa.gov). In this experiment zebrafish embryo development will be studied at a pH of 4 to display acid rain, pH of 5.5 to display an environment affected by acid rain, and a pH of 7. The pH of 7 will be the control group, displaying natural pH of zebrafish habitat. It is pure instant ocean solution with no added sulfuric acid.

The purpose of this lab is to investigate the effect of sulfuric acid solutions, and therefore acid rain, on the embryonic development of zebrafish. It is hypothesized that the pH of 7 will allow for the fastest developing and most healthy zebrafish embryos because it is most like the natural pH of the zebrafish habitat, rivers. If they are in a pH of 4, there will be more defects, or slowing of development, because zebrafish do not naturally live in that acidic of water. The pH of 5.5 will have less of an effect on the embryos than the pH of 4, but may still cause a slowing of development.

Materials and Methods

Materials List:

- 3 150 mL beakers
- 2 1 L flasks
- 1.84 mL sulfuric acid
- 2 10 mL Graduated Cylinders
- 2 100 mL Graduated Cylinders
- 1.5 L Instant Ocean
- Pipets
- 1 1 L Volumetric Flask
- 3 150 mL Beakers
- 1 Multi-well Plate
- Zebrafish embryos
- Compound Microscope
- Stereoscope
- Methylene Blue
- Stock Solutions
    - 100 mL Instant Ocean
    - 100 mL Sulfuric Acid and Instant Ocean Solution pH 5.5
    - 100 mL Sulfuric Acid and Instant Ocean Solution pH 4

Sulfuric Acid (H_2SO_4) is corrosive to eyes and skin. Goggles were worn at all times while working with it. Hands were also be washed to avoid ingestion of H_2SO_4. Once done working with sulfuric acid, the lab space and materials were be thoroughly washed. A solution with a pH of 1 was prepared in a 1 L volumetric flask by mixing 1 L of instant ocean with 1.84 mL of 18M H_2SO_4. 100 mL graduated cylinders were used to measure 10 mL of pH 1 solution and 90 mL of instant ocean. In a 150 mL beaker, 10 mL of the pH 1 solution were added to 90 mL of instant ocean to make a stock solution with a pH of 5.5. 100 mL graduated cylinders were used to measure 15 mL of pH 1 solution and 85 mL of instant ocean. In another 150 mL beaker, 15 mL of the pH 1 solution were combined with 85 mL of instant ocean to make a solution with a pH of 4. 100 mL of pH 4 solution, pH 5.5 solution, and pH 7 solution (instant ocean) were stored as stock solutions in 150 mL beakers and covered with parafilm.

The next day (recorded as Day 1) embryos were collected from instructor. A multi-well plate was also collected from instructor. Each well was labeled depending on which solution was added to well, pH 4A, pH 4B, pH 4C, etc. Three wells were used for each pH level. Each well was filled with 3 mL of the labeled solution and one drop of methylene blue was stirred into each solution, serving as an antifungal for the embryos. Embryos were inspected under a stereoscope and dead embryos were discarded in a separate beaker. Seven embryos were placed in each well of the multi-well plate. The developmental stages of the embryos were recorded. The plate was covered and left in an incubator overnight. The incubator was set at 28.5°C.

On day 2, embryos were again examined under stereoscope. The numbers of hatched, live, and dead embryos were recorded, along with developmental stages of embryos, and dead embryos were again discarded. Solutions in each well were replaced with stock solutions of each pH level using pipets and 10 mL graduated cylinders. This was repeated for two more days and

data was recorded. Embryos were also inspected under compound microscopes for clearer, more defined views of them. Compound microscopes were used to determine if opaque embryos were dead or alive. On the last day the living fish were placed in a separate beaker. Embryos that remained unhatched and leftover stock solutions were disposed of. All beakers, graduated cylinders, and the multi-well plate were all cleaned well and put away.

Results

Acid rain is a significant environmental issue. This experiment was carried out to exhibit the effects that acid rain has on the development of zebrafish embryos. The pH of 7 was the control group, displaying natural pH of zebrafish habitat. Therefore, the other pH level results were then compared the trials of pH 7. The independent variable was the pH levels of sulfuric acid and instant ocean solutions that embryos are placed in, while the dependant variables were speed of development of embryos and health or defects of embryos. The presence of sulfuric acid greatly affected the embryonic development in the zebrafish, as shown in the data below.

Data Presentation

Day 1:

Day 1 Observations: All embryos were alive and in the developmental period of 0-6 hours post fertilization (hpf).

Day 2:

Table 1:

Day 2	Living Unhatched	Living Hatched	Dead
pH 4A	7/7	0/7	0/7
pH 4B	7/7	0/7	0/7
pH 4C	7/7	0/7	0/7
pH 5.5A	11/11	0/11	0/11
pH 5.5B	7/7	0/7	0/7
pH 5.5C	4/5	0/5	1/5
pH 7A (Control)	7/7	0/7	0/7
pH 7B (Control)	9/9	0/9	0/9
pH 7C (Control)	7/7	0/7	0/7

Day 2 Observations: All embryos in pH 4 were yellow and seemed to be in 2.5-4 hpf stage, except 5. 5/7 embryos in pH 4A were clear and seemed to be in the 10-14 hpf stage. The embryos in the pH 5.5 and control solutions seemed to be in the 10-16.5 hpf, and almost all of them were seen moving in their embryos.

Day 3:

Table 2:

Day 3	Living Unhatched	Living Hatched	Dead
pH 4A	5/7	0/7	2/7
pH 4B	0/7	0/7	7/7
pH 4C	0/7	0/7	7/7
pH 5.5A	8/11	0/11	3/11
pH 5.5B	6/7	0/7	1/7
pH 5.5C	3/5	0/5	2/5
pH 7A (Control)	7/7	0/7	0/7
pH 7B (Control)	9/9	0/9	0/9
pH 7C (Control)	6/7	1/7	0/7

Day 3 Observations: Yellow embryos in all pH 4 trials had not developed past 2.5-4 hpf and declared dead. The 5 clear embryos in pH 4A developed to 16.5 hpf. The embryos in pH 5.5 trials were all between 19 and 48 hpf, but looked more opaque than those in the control. All embryos in the control solutions were clear with black stripes and eyes. They were all at 48 hpf.

Day 4:

Table 3:

Day 4	Living Unhatched	Living Hatched	Dead
pH 4A	0/7	0/7	7/7
pH 4B	0/7	0/7	7/7
pH 4C	0/7	0/7	7/7
pH 5.5A	0/11	0/11	11/11
pH 5.5B	0/7	0/7	7/7
pH 5.5C	0/5	0/5	5/5
pH 7A (Control)	4/7	3/7	0/7
pH 7B (Control)	5/7	2/7	0/7*
pH 7C (Control)	0/7	7/7	0/7

*Two of the embryos were lost in the exchanging of solutions so the total number was taken from nine to seven because it is unknown if they were alive or dead.

Day 4 Observations: When looked at under a stronger microscope, a compound microscope, the clear embryos in pH 4A were declared dead because of their black color and lack of further growth. Although the embryos in pH 5.5 still looked alive, yet somewhat opaque under the stereoscope, they were clearly seen as dead and black in the compound microscope and all in pH 5.5 were declared dead. One either hatched or burst in 5.5A, but was not seen in the well and must have disintegrated in the solution. The embryos that had not yet hatched in the control solutions were still in 48 hpf.

Day 5:

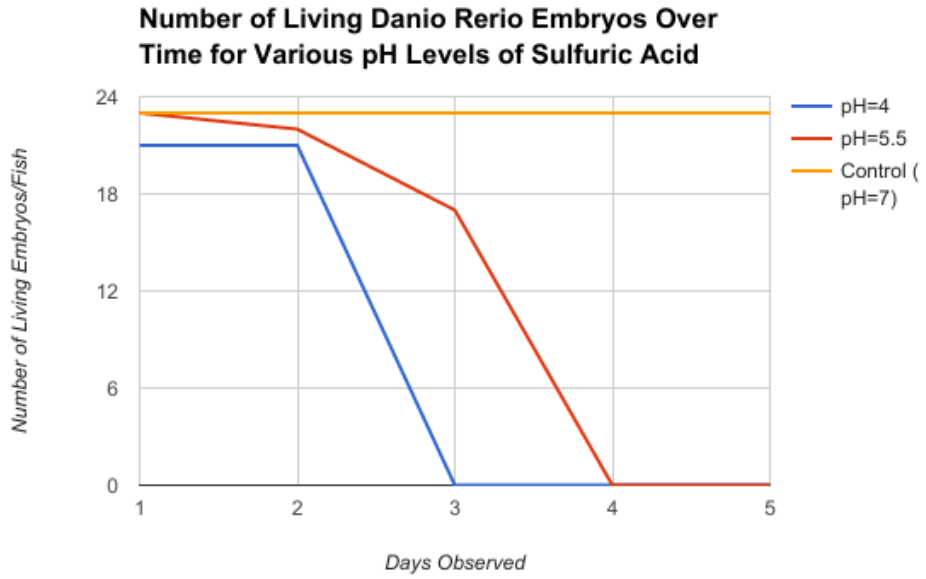
Table 4:

Day 5	Living Unhatched	Living Hatched	Dead
pH 4A	0/7	0/7	7/7
pH 4B	0/7	0/7	7/7
pH 4C	0/7	0/7	7/7
pH 5.5A	0/11	0/11	11/11
pH 5.5B	0/7	0/7	7/7
pH 5.5C	0/5	0/5	5/5
pH 8A (Control)	2/7	5/7	0/7
pH 8B (Control)	1/7	6/7	0/7
pH 8C (Control)	0/7	7/7	0/7

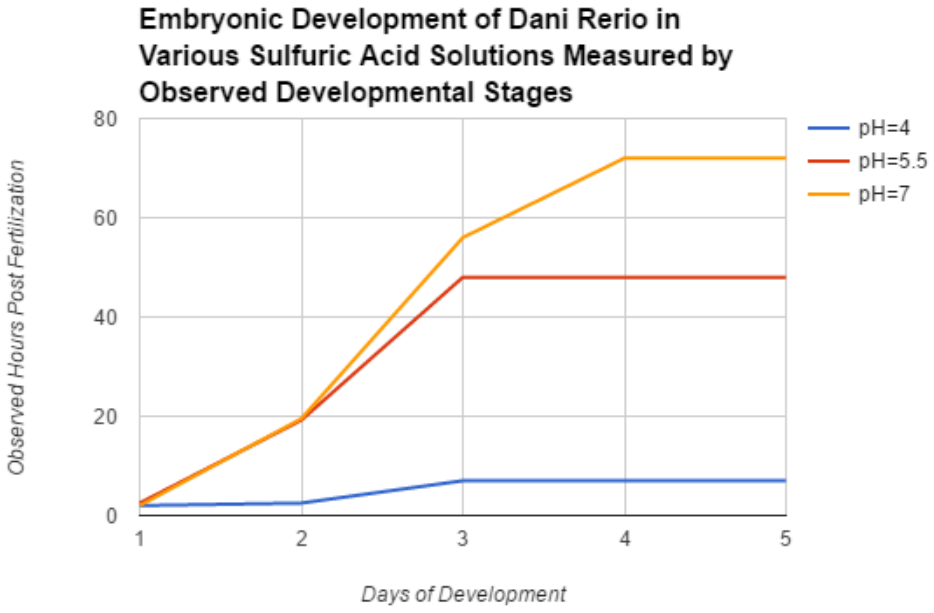
Day 5 Observations: Only three of the twenty-one embryos in the control groups had not yet hatched. It was decided that if they had not hatched yet, they never would, and were discarded.

Data Analysis

Graph 1:



Graph 2:



The first graph shows all of the living fish, hatched and unhatched, throughout the five day experiment. All of the fish in the pH 4 solution died very quickly, within the first three days. All of the fish in the pH 5.5 solutions kept developing until day four, but also died much sooner than the control group. Not one of the embryos exposed to sulfuric acid solutions had hatched during the experiment, however the fish exposed to the pH 5.5 sulfuric acid solution developed farther than those of the pH 4 solution. None of the fish in the control group died, but two were still unhatched on the last day of observations.

The second graph displays the average perceived stage of development the embryos had grown to over the course of the five day experiment. The stages of development were measured in hours post fertilization (hpf). From the data represented it is clear that the acidic solutions slowed down development by a large margin when compared to the control group. The most developed zebrafish in the pH 4 solutions only grew to 16.5 hpf and most in pH 4 only grew between 2.5 and 4 hpf. The most developed zebrafish in the pH 5.5 solutions only grew to 48 hpf, while the zebrafish in the control group hatched and kept developing to 72 hpf and over by the end of the experiment.

Figure 1

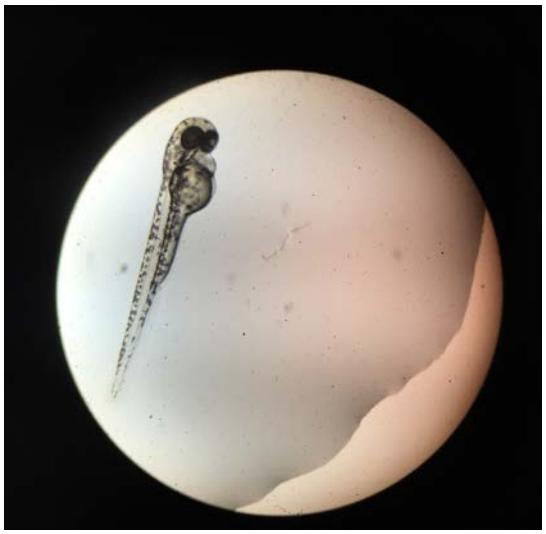


Figure 2

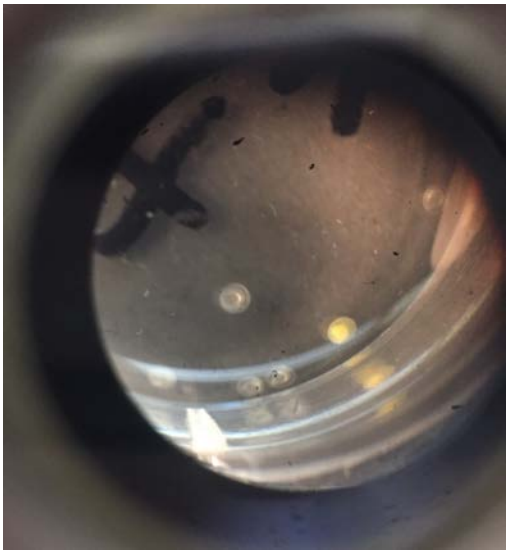


Figure 3

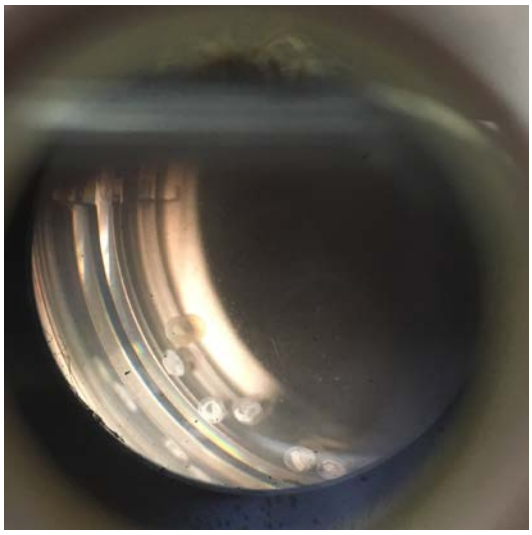


Figure 4

Figure 1 shows a hatched embryo from the control group C from day 3. Figure 2 is a picture of the embryos in well pH 4A. It shows both yellow and clear embryos from day 2. Figure 3 is a picture that was taken on day 4 of a dead embryo in pH 5.5. Lastly, figure 4 shows the embryos developing in a well of pH 5.5 solution on day 2.

Discussion

The experiment investigating the effect of sulfuric acid on embryonic development of *Danio rerio* provided substantial results showing that sulfuric acid, found in acid rain, can significantly lower life expectancy. All of the zebrafish exposed to different pH levels of sulfuric acid died within 4 days of the experiment. The hypothesis stating that if the zebrafish embryos were introduced to the acid, it would slow their development was supported. The zebrafish in the pH 4 solutions did not develop past 16.5 hpf, and the zebrafish in the pH 5.5 solution developed to 48 hpf, but did not hatch. The zebrafish exposed to H_2SO_4 were also observed to be more opaque in color when compared to the control.

Heart rate and embryo movement could have been measured more accurately to define some of the developmental problems caused by sulfuric acid. Compound microscopes should have been used before day 4 to make results more accurate and to see how H_2SO_4 affected zebrafish organs and function. We also could have done more trials of different pH levels to show a stronger correlation between acidic environment and embryonic defects and to show at what pH the acid really starts to affect embryonic development. Further experiments can investigate different pH levels of H_2SO_4, the effect sulfuric acid on other organism development, and look for ways to decontaminate bodies of water subject to acid rainfall.

This experiment shows the importance of environmental awareness and keeping water sources unpolluted. The fact that the sulfuric acid present in these solutions had that much of an impact on the zebrafish, killing all of those exposed, shows that acid rain really is a prevalent issue involving the safety of animals and other organisms living in freshwater environments. We need to find other ways to obtain energy than burning fossil fuels in order to preserve life in water sources and other natural environments. If more environmentally friendly energy sources

are not developed soon, species that rely on their uncontaminated, natural environments for life will slowly die off and become extinct.

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