

EFFECTS OF AMMONIUM NITRATE ON ZEBRAFISH
EMBRYONIC DEVELOPMENT

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ABSTRACT

The purpose of this study was to determine the effects of ammonium nitrate on the embryonic development of zebrafish. Humans have been adding ammonium nitrate to the environment through the burning of fossil fuels and runoff containing nitrogen based fertilizers. Aquatic organisms are especially susceptible to runoff because runoff seeps into aquatic systems that organisms, like zebrafish, thrive in. Zebrafish are small vertebrates used to model human development. Two hundred and forty embryos were divided into 4 treatment groups. The control treatment group had 0 mg/L of ammonium nitrate, the low treatment group had 50 mg/L of ammonium nitrate, the medium treatment group had 100 mg/L of ammonium nitrate and the high treatment group had 200 mg/L of ammonium nitrate. This study used a checklist to determine whether the embryos were on-time, delayed, or dead at 24, 48, 72 and 96 hours. This study's hypothesis was that higher concentrations of ammonium nitrate would lead to higher percentages of delayed and dead embryos. The general results were as the concentration of ammonium nitrate increased so did the percent of delays and deaths in zebrafish embryos. The results of the ANOVA test allowed this study to accept the hypothesis and reject the null hypothesis. The p-value allowed this study to conclude that there was a significant difference between the effects of the different levels of ammonium nitrate at 96 hours.

INTRODUCTION

In this past century, technology has added one and a half times more nitrogen than all natural processes combined (UNESCO & SCOPE, 2007). Since the invention of cars, fossil fuel combustion has greatly increased the amount of nitrogen in the atmosphere. In addition, farmers are utilizing nitrogen-rich synthetic fertilizers to produce more food. Even though nitrogen appears beneficial to the ecosystem, nitrogen has many disadvantages. Ammonium nitrate, a common nitrogenous-based fertilizer, enters aquatic ecosystems through runoff (Burgett et al., 2007). Because of their semi-permeable skin, excess nitrogen affects aquatic organisms, such as zebrafish. Although nitrogen is beneficial to the ecosystem, high concentrations of nitrogenous forms, like ammonium nitrate, will probably affect the embryonic development of zebrafish.

The nitrogen cycle has no beginning, or end; it can start or end at any point of the cycle. When nitrogen is in the atmosphere, nitrogen is in the inert or lifeless form. In order for nitrogen to reach the form when it is active, it has to go through a process called fixation, in which it becomes ammonium. Lightning, lava, and bacteria makes ammonium, found in soil and water. After ammonium is made, the cycle can take one of two paths; it can become both uptake for animals and nutrients for plants, or it can go through a process called nitrification. In nitrification, bacteria break the ammonium down to form nitrites and nitrates. If the ammonium is consumed by organisms, it is then broken down to form organic compounds, such as RNA, DNA, R-N, protein and urine. It can then take one of two paths; it can decay and go through mineralization, or go back to the ammonium stage where it is found in soil and water. During the mineralization stage, insects decompose the organic compounds. In this decomposition, ammonium is made once again. The ammonium then goes through nitrification, and the nitrates and nitrites made from this process go through denitrification. Bacteria are the active bodies in

the process of denitrification, which allow nitrogen to return to the atmosphere in its inert form. Nitrogen can be found in its inert form (N_2), ammonium (NH_4), organic compounds such as RNA and DNA, and as nitrates (NO_2) and nitrites (NO_3).

Human resources are adding excess nitrogen to the environment and unbalancing the nitrogen cycle. (Pidwirny, M. 2006). Nitrogen, particularly in the form of organic compounds, is essential to all forms of life; however, too much nitrogen can be harmful. Excess nitrogen is best for agricultural production rather than vertebrates. Farmers use excess nitrogen in fertilizers to obtain a larger harvest (Pidwirny, 2006). However, the use of fertilizers has increased nitrogen levels in the environment. Excess nitrogen is a danger to the aquatic life due to the fertilizer because it runs off into the rivers and ponds and contaminates the organisms in the water. Excess nitrogen can lead to eutrophication in water habitats, cancer in humans, and respiratory distress in infants (Harrison 2008). Eventually excess nitrogen could affect the drinking water of humans.

The focus of this study is to see the impact ammonium nitrate has on the development of vertebrates using zebrafish embryos. Zebrafish are small tropical fish native to warm waters in India. Zebrafish are good test subjects because zebrafish development is similar to humans. Also, zebrafish embryos develop in 96 hours (Kimmel et al, 1995) so data can be obtained quickly. Once the zebrafish is fertilized, its 96 hour development starts. Zebrafish endure six stages of development. The first stage is the cleavage stage, which is the rapid division of cells until a solid ball of cells has formed (Raven and Johnson, 1996). The second stage is the blastula formation during which the outer blastomeres join to form a ring around all others cells (Gilbert, 1997). At the sixteen cell stage, the cells begin to pump in sodium, and water is drawn into the center, increasing cell mass. In gastrulation, the third stage, the outer wall pushes inward forming the gastrula (Raven and Johnson, 1996). This creates the main axis of the body (Raven

and Johnson, 1996). During the stage of neurulation the development of the nervous system, including the neural tubes and notochord begins (Miller and Levine, 2004). The tubes make up the spinal cord and brain, and the notochord makes up the spine (Raven and Johnson, 1996). After neurulation, cell migration starts to take place, and the cells begin to move into their proper place in the body (Gilbert, 1997). Organ development and the majority of the growth occurs during the last stage, organogenesis (Raven and Johnson, 1996). All this takes place within the chorion, which is a protective membrane around the embryo that protects the embryo from some environmental threats until the embryo hatches (Raven and Johnson, 1996).

Many organisms have been affected by the excess nitrogen production, even humans. Based on a previous study conducted on wood frog tadpoles, ammonium nitrate can have both lethal and sublethal effects on aquatic wildlife (Burgett et al., 2007). Burgett et al studied development and behavioral impacts of ammonium nitrate. Premature deaths and delayed development in wood frog tadpoles were observed in the Burgett study. During the study on wood frog tadpoles, four different concentrations of ammonium nitrate were used. Those included a control (0 mg/L ammonium nitrate solution), low (50 mg/L ammonium nitrate solution), medium (100 mg/L ammonium nitrate solution), and high (200 mg/L ammonium nitrate solution). The data from the study on tadpoles suggested that higher levels of ammonium nitrate resulted in a higher rate of delayed development and lower rate of survivorship in wood frog tadpoles (Burgett et al., 2007).

The purpose of this experiment is to determine the effects of ammonium nitrate on the embryonic development of zebrafish. The group's hypothesis is that higher concentrations of ammonium nitrate will lead to higher percentages of delayed and dead embryos. The group's

null hypothesis is that higher concentrations of ammonium nitrate will not lead to higher percentages of delayed and dead embryos.

METHODS

This study was conducted at Frostburg State University, FSU, from June 26th 2008, to July 4th 2008. The experiment was based on the study *Impact of Ammonium Nitrate on Wood Frog Tadpoles: Effects on Survivorship and Behavior* by Burgett et al. Similar to the previous study; this study contained four treatment groups: control, low, medium and high. The goal of this study was the same as that of Burgett's, but the group decided to use zebrafish instead of tadpoles. Because both wood frogs and zebrafish are vertebrates, zebrafish were expected to display similar effects to ammonium nitrate.

The group began by making the treatment solutions on Thursday, June 26th. A stock salt solution was used to make egg water to nourish the embryos, and then varying amounts of ammonium nitrate were added to produce the four treatment groups. The stock salt solution was made by adding 40 grams of Instant Ocean Sea Salts from Aquarium Systems to 1 L of distilled water. The egg water was made by adding 1.5 ml of stock salts solution, and 1 L of distilled water (Westerfield, 2000). The different treatment groups added the appropriate amount of 0.1 M solution of NH_4NO_3 to the egg water. The control treatment group added no ammonium nitrate for a final concentration of 0 mg/L. The low treatment group added 6.2 mL of ammonium nitrate for a final concentration of 50 mg/L. The medium treatment group added 12.5 mL of ammonium nitrate for a final concentration of 100 mg/L. The high treatment group added 25 mL of ammonium nitrate for a final concentration of 200 mg/L (Burgett et al, 2007). Twelve beakers, three replicates of each concentration, were filled with about 100 mL of the appropriate treatment solution, and set aside until the day that the embryos arrived.

The embryos were picked up from Carnegie Institution of Washington in Baltimore, Maryland at 11:30 a.m., after being fertilized at 9:30 a.m. on June 30, 2008. The embryos were transported back to FSU. At 3:30 p.m., 20 healthy embryos were placed in each beaker with the prepared solution. If an embryo had 30% epiboly, had no fungus, and the head and tail were visible, the embryo was considered healthy, on-time and useable. Once in the treatment solutions, these embryos were put inside an incubator with a temperature of 28.5° C. This was done because the temperature was the same as that used in the previous study on wood frog tadpoles (Burgett et al, 2007).

Over the next four days, the students observed the embryos at different stages of embryonic development. Around 9:30 a.m. each day, each student acquired a beaker from the incubator, a pipette, a stereoscope and two petri dishes. The pipette was used to gather the embryos in addition to some of the solution into one of the petri dishes. The petri dish was placed under the stereoscope and the individual embryos were observed. A checklist of criteria was used to determine whether the embryos were developing on-time, delayed, or dead. This checklist of criteria was made using parts of the book *Stages of Embryonic Development of the Zebrafish* by Kimmel et al. The embryos were considered on-time if they had more than three of the criteria, delayed or abnormal if they did not have at least three of the criteria, and dead if the embryo looked like a black cloud. After checking the embryos, they were placed back into the incubator, and the dead ones were removed. At the end of the study, the zebrafish were poisoned with tap water, which contains chlorine, and then discarded.

ANOVA tests were used to obtain the statistic analysis of the collected data. The first ANOVA test compared the combined count of the delayed and dead embryos in the control, low, medium and high treatment groups at 96 hours. Additional ANOVA tests were conducted

comparing the effects of each specific ammonium nitrate concentration at 96 hours. These tests were appropriate for this study because they compared the variability between two or more data sets. These tests determined whether or not there was significant difference between the number of delayed and dead embryos in varying concentrations of ammonium nitrate.

RESULTS

Figure 1 below shows the effects of ammonium nitrate on zebrafish embryonic development over a 96-hour period. Three key observations are higher concentrations of ammonium nitrate show increased amounts of delayed embryos; over time, the impact of ammonium nitrate becomes more evident; and there is a noticeable difference in the data between 24 - 48 hours and 72 - 96 hours.

Effects of Ammonium Nitrate on Zebrafish Embryos over a 96 hour period

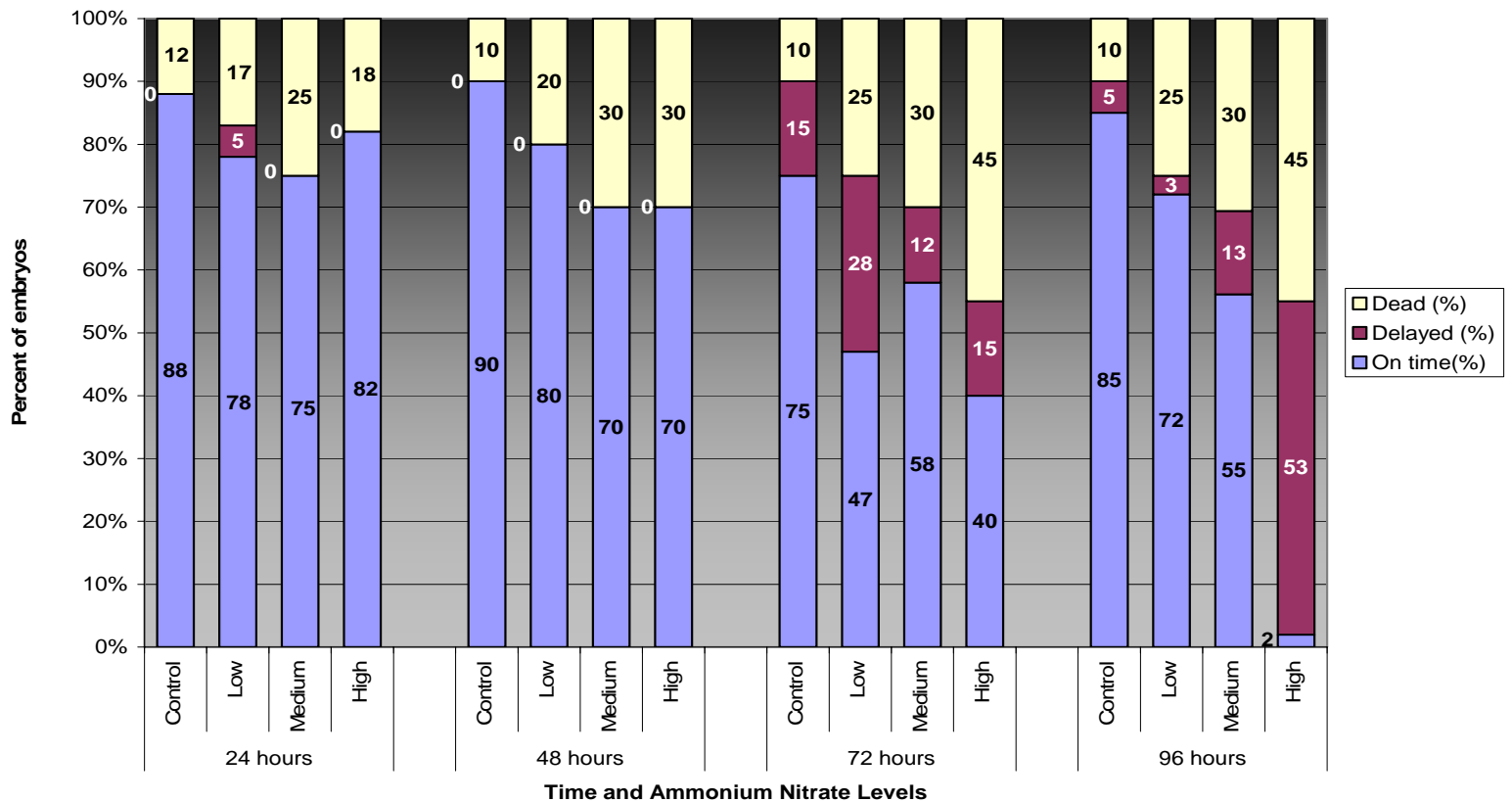


Fig. 1. – The effects of ammonium nitrate on zebrafish embryos over 96 hours.

Delayed behavior was seen most frequently in the high treatment group. After close observation of the data, the difference in death percentage between the high treatment groups and the others were noticed. At 96 hours, the high concentration had the highest percentage of dead embryos and the highest percentage of delayed embryos. These embryos were slow to develop physically. Some of the embryos failed to show avoidance behaviors; the larvae in the high treatment group barely moved and made no effort to avoid the pipettes when threatened by touch. At 96 hours, only 2% of the high treatment group's larvae were on-time and one embryo was still in its chorion.

As time went on, the percentage of delayed and dead embryos increased in the medium and high concentrations. Within 24 hours the medium and high concentrations had the highest percentage of deaths. At 48 hours, 10% of the control treatment group embryos were dead compared to the high treatment groups 30% of dead embryos. All of the concentrations experienced an increase of dead embryos until 72 hours, with an exception to the control treatment group embryos, but no embryos died between 72 and 96 hours.

Figure 1 shows that at 72 hours delayed development increased, as compared to the 24 and 48 hour periods. With an exception to the 5% of delayed embryos at 24 hours, no delayed embryos appeared until 72 hours of development. The percentage of delayed embryos decreased for the control and low treatment group at 96 hours, but the medium and high treatment group saw an increase in delayed embryos. An example of the delay increase is the jump from 15% delayed embryos to 53% delayed embryos in the high treatment group between 72 and 96 hours. At 96 hours, the high treatment group showed a large decrease in the percent of on-time embryos.

Irregularities in the data were noted. The control treatment group had a decrease in the percent of dead embryos from the 24-hour period to the 48-hour period. This occurred because of a fungus contamination in one of the control beakers. That data was unusable after 24 hours. The data for this beaker was still included in the 24 hour data. During the 72-hour period, a control beaker was broken, causing the elimination of more data from the 72 and the 96 hour data.

The ANOVA test compared the number of the delayed and dead embryos in the four different treatment groups at 96 hours. Based on the wording of the group's hypothesis, the delayed and dead embryos were combined in the ANOVA so the group could either reject or accept the null hypothesis. At an alpha level of 0.01, the initial ANOVA test has an F-ratio greater than the F-critical, and a p-value of 0.001257 showing a significant difference in the data. Since this comparison was between all four treatment groups, the area of significance could not be located. Further ANOVA tests compared the different treatment groups to each other. One of the additional ANOVA tests shows that there was a significant difference between the medium and high concentrations; the F-ratio was greater than the F-critical and the p-value was 0.002143. Figure 1 and the ANOVA tests show that there is a significant difference in the number of delayed and dead embryos in the different treatment groups at 96 hours.

CONCLUSIONS AND DISCUSSION

The purpose of this study was to determine the effects of ammonium nitrate on the embryonic development of zebrafish. The group's hypothesis was that higher concentrations of ammonium nitrate would lead to higher percentages of delayed and dead embryos. The null hypothesis was that higher concentrations of ammonium nitrate would not lead to higher percentages of delayed and dead embryos. Based on Figure 1, the highest concentration of ammonium nitrate did have the greatest number of delayed and dead embryos. After performing

the ANOVA test, the group accepted the hypothesis and rejected the null hypothesis. At an alpha level of 0.01, the F-ratio was greater than the F-critical, allowing the group to reject the null hypothesis. The p-value was less than 0.01, indicating a significant difference between the number of delayed and dead embryos in all concentrations of ammonium nitrate in this study.

Additional ANOVA tests were used to discover the most harmful level of ammonium nitrate to zebrafish development. These tests found a significant difference between the effects of the high concentrations of ammonium nitrate and the effects of all other levels of ammonium nitrate. Figure 1 illustrates how harmful high concentrations of ammonium nitrate can be for the development of zebrafish.

Over time, the condition of the zebrafish worsened. The effects of high concentrations of ammonium nitrate also increased. This suggests that if the embryos were in the solution longer, more embryos would have died. The number of delayed and dead embryos was low at 24 and 48 hours and was high at 72 and 96 hours. According to the checklist, by 72 hours the embryos should have hatched. Once outside of the chorion, larvae may be more susceptible to the ammonium nitrate. The chorion acts as a protective membrane. Once the chorion is gone, the movement of the larvae allows more nitrates in, causing the increase in delayed and dead embryos. The surviving embryos in the high group did not show avoidance behavior during the 72 and 96 hour periods. For example, the embryos did not move away from the pipette or interact with other embryos.

Many limitations were faced throughout the experiment. The group only studied 240 zebrafish embryos. This was a limitation because the group only had a small sample size of the embryos. Another shortcoming to the study was the zebrafish were only observed for 96 hours. The ammonium nitrate could have had more effect on the embryos after 96 hours. Also, the

embryos' small size required the zebrafish to be looked at through a microscope. Some of the criteria from the checklist were difficult to identify under the microscope.

Since the experiment was done by students, student inexperience occurred. One example was when an embryo-filled beaker from the control treatment group was accidentally dropped on the ground. In addition, another student from the control treatment group neglected to remove the embryos contaminated with fungus. Finally, because the checklist this study used was chosen by the teacher, the criteria could have been too stringent.

Suggestions for future studies are that the group should test both the embryonic stage and the adult stage. The zebrafish group should also conduct the experiment more than once. This would allow this study to improve technique and find more data. Another suggestion for future studies would be to have the embryos birthed in the ammonium nitrate solution. Having the embryos birthed in ammonium nitrate might have affected their development. In addition, each group member could have more than 20 embryos to create a larger sample size. If each group had a stronger microscope, the group could get a better visual of the zebrafish organs. A more accurate checklist to compare the zebrafish would also improve the study. A less stringent checklist would allow future studies to identify the embryos as delayed or dead more accurately.

At the end of the experiment, the group still had a few questions not answered by this study. For example, would changes occur if the embryos were harvested in the solution? What would happen if another study was conducted on the impact of ammonium nitrate on the adult stage of zebrafish? Would this study have better observations if the embryos were observed more often during the day? Would the results have changed if the group used more embryos? Most importantly, will humans show a similar impact to nitrogen as the zebrafish did?

Even though this study has many questions left unanswered, this study has supported evidence that zebrafish are negatively impacted by ammonium nitrate. This study showed that concentrations of ammonium nitrate of 200 mg/L were the most harmful to zebrafish embryos. At the rate ammonium nitrate is increasing, vertebrates such as humans may begin showing sublethal or even lethal effects. If levels of ammonium nitrate reach 200 mg/L, humans may eventually suffer the consequences of ammonium nitrate's impact. However, humans have the ability to change these impacts on the nitrogen cycle.

Currently, very little is being done about the abundance of nitrogen. To prevent humans from suffering, humans should try to place less nitrogen in the environment. For example, humans should try to find an alternative to synthetic fertilizers. This study was conducted on zebrafish because both humans and zebrafish share a similar vertebral developmental process. By lowering ammonium nitrate's concentrations, humans may lower the chances of sharing a similar affect to ammonium nitrate as the embryos in this study.

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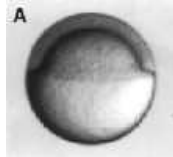
APPENDIX

Criteria for Zebrafish Embryos

Based on *Stages of Embryonic Development of the Zebrafish*, by Kimmel et. al.

At 6 hours:

- 30% epiboly
If yes, then place embryo in experimental group
If no, then discard embryo



At 24 hours:

- Tail extended away from body
- Notochord visible
- Optic vesicle (eye) visible
- At least 20 somites
- Heartbeat visible (side of yolk)
If yes to 3 or more, then embryo is on-time
If meet less than 3, then embryo is delayed / abnormal



At 48 hours:

- Tail straightened
- Movement
- Pigmentation
- Dorsal and/or ventral stripes visible
- Otic vesicle (ear) visible
If yes to 3 or more, then embryo is on-time
If meet less than 3, then embryo is delayed / abnormal



At 72 hours:

- Hatched
- Protruding mouth
- Pectoral fins
- Yolk smaller than head
- Avoidance behavior present (larva moves away from pipette)
 - If yes to 3 or more, then embryo is on-time
 - If meet less than 3, then embryo is delayed / abnormal



At 96 hours:

- Dorsal fin
- Gill slits
- Swim bladder
- Yellow bodies
- Avoidance behavior