The Effects of Atrazine on Developing Zebrafish Embryos

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Abstract:

The aim of this experiment is to showcase the effects that Atrazine causes in the first three days of development in zebrafish embryos. The observations of the zebrafish embryos were made periodically on the varying concentrations of Atrazine, which were 0 mg/L (control), 2.5 mg/L, 5.0 mg/L, and 7.5 mg/L, respectively. The times the observations were made and recorded were 24, 48, and 72 hours post-fertilization, a time frame that a healthy zebrafish embryo should being hatching over. Atrazine is by far the most widely used herbicide in the United States, but the research on the pros of the substance greatly outweighs that of the research on the cons. That just what this experiment sets out to do: bring light to the darker side of Atrazine that so often goes unnoticed. The control group developed just as expected; nearly all of the embryos survived to the final day of observation, and were all on track to hatch within a matter of hours. This was a stark contrast to the highest concentration of 7.5 mg/L, in which none of the embryos survived the first day. Although the concentration of 5.0 mg/L is thought to be just survivable enough to cause mutations while keeping the embryos intact, the 5.0 mg/L proved to be hardly suitable for development as the survival rate of the embryos after 72 hours was a mere 6.7%. The concentration of 2.5 mg/L went on to yield the most astounding results, although the survival rates were still not optimal. Nonetheless, the most prominent deformations dealt with the hearts of the zebrafish, as all of the 2.5 mg/L possessed an astoundingly slow and feeble heartbeat as compared to the healthy control embryos. In a few select embryos, the only trace that the zebrafish were still living was an incredibly minute heartbeat that barely seemed strong enough to carry blood throughout the extremely deformed embryo; these embryos were no more than a mass of cells, with no regular formation of a spine or other features. The zebrafish are known to be a more than satisfactory model for human development, and the results prove that overexposure to quite diluted concentrations of the substance would still lead to either extreme cases of mutations or even death. Although these concentrations are more dramatic than the concentration deemed safe for drinking water -3 µg/L-, individuals in the agricultural industry deal with heightened concentrations much greater than this on a daily basis, and the results prove that Atrazine can have devastating effects on such individuals.

Introduction:

The agriculture industry has grown to depend on herbicides in its everyday use. The majority of farmers have adopted the practice of using chemicals to either enhance crop yields or to ward off other unintentional and undesirable plants, and a sizeable portion of this majority have leaned toward the chemical, atrazine (Mergel 2011). The surface benefits appear to be clear: studies have reportedly stated that atrazine boasts anywhere from a 1% to 6.5% increase in crop yields, while at the same time dramatically reducing topsoil erosion by eliminating broadleaf weeds (Mergel 2011). But is Atrazine hiding potentially harmful long-term effects?

After all, Atrazine has only been around for half a century, which means that up until just recently, research on the long-term effects of America's favorite herbicide has not been possible. The USDA decided to take a step in the right direction however, and released a chain of studies that revealed atrazine to be found in 94% of drinking water, the largest percentage out of any other commercially used herbicides (Pesticide Action Network). Although the guideline claims safety in no more than three parts per billion, in farming-based areas - specifically the Midwest-, this limit is often exceeded, while many claim that even three parts per billion has the potential to do damage in the first place (Pesticide Action Network). Without question, an alarming fact. Especially alarming when coupled with other studies that correlate atrazine to having serious effects on the development of the reproductive system, along with being a possible carcinogen (Wirbisky, Sepulveda, Weber, Lin, Jannasch, Freeman 2016).

Human testing is out of question for obvious reasons, and that is exactly where zebrafish can assist in furthering the reaches of knowledge on the chemical. The development of zebrafish are profoundly similar to that of humans, and with their rapid development and ease of raising large quantities, they offer a very advantageous option of studying diseases and health defects that are common in humans without ever endangering a single human life (Sanitoriello, Zon 2012).

Zebrafish have come along at a critical time, as the need for wide-scale research on atrazine is in order. There was justified reasoning for banning the use of atrazine in Germany and Italy as far back as 1991, and for the safety of all United States citizens that have no choice but to come into contact with atrazine, it is clear that more research still needs to be done to bring attention to the herbicide's capacity for damage (Pesticide Action Network). Regardless, past research done on the detrimental health effects of Atrazine are proof enough to confidently hypothesize that zebrafish embryos exposed to Atrazine will suffer from significant alterations to their development, and prove the negative health effects that Atrazine has the ability to cause.

Materials:

(credited to UW-Milwaukee SEPA)

- (3) well plates
- Micropipette with disposable tips
- Fine tipped disposable pipette
- 1 mL disposable pipette
- Atrazine solutions of 2.5, 5.0, and 7.5 mg/L
- Instant Ocean solution
- Newly fertilized zebrafish embryos
- Solution of methylene blue
- Viewing microscope
- Dissecting Microscope
- Glass depression slides for Dissecting Microscope
- Incubator at suitable temperature for development (≈ 30°C)
- Latex gloves to handle concentrated Atrazine
- Waste beaker

Procedure:

1. Obtain necessary materials.
2. Clearly label thoroughly cleaned observation plate.
    a. Note the three wells for control group, and the three wells for each varying concentration of Atrazine.
3. Place approximately 10 embryos in each well, making sure that all the excess waste, including dead embryos, is removed with an appropriate-sized pipette and placed in a separate waste container.
4. Once all waste is removed, record the exact number of fertilized embryos remaining under “Number of starting embryos”.
5. Once the embryos are counted, the solutions are to be added. Remove all excess liquid in each respective well, and using a precise pipette, add one mL of the appropriate solution to each well.
    a. Add a sufficient amount of methylene blue to each well until the solutions appear as a faint blue tint; this step is essential in preventing harmful bacteria growth within the well plate.
    b. After step 6 is finished, the well plate may be covered and placed in the incubator for 24 hours.
6. After the allotted amount of time, the well plate may be removed from the incubator and uncovered to make initial observations. Observations should include the amount of embryos dead, alive, or hatched, as well as any noteworthy qualitative observations noticed with the dissecting microscope such as heart rate, spinal development, etc.
7. Once all observations have been properly recorded, all dead embryos may be removed and placed in a separate waste container.
8. The old solutions are now to be replaced with fresh but identical solutions.
9. Additional qualitative observations may be made once the solutions are replaced.
10. Once observations have been properly recorded, the embryos may be returned to their respective wells, and the methylene blue may be added. Once the antibacterial solution is administered, the well plate may be covered and placed back in the incubator for an additional 24 hours.
11. Repeat steps 7-10 for both 48 and 72 hours post fertilization.
12. After all necessary observations for 72 hours post fertilization have been recorded, all embryos, solutions, and any other waste products may be properly disposed of. Thoroughly clean all reusable materials for potential future use with distilled or deionized water, and place in a proper place to dry.

Qualitative Observations:

Control - The embryos developed as expected all throughout the allotted points of observation. Although not all of the embryos hatched after 72 hours post-fertilization, nearly all of them appeared well on their way to hatching, and the heartbeats remained steadfast throughout all three days. See Figures 1 and 2 for healthy control embryos.

2.5 mg/L:

Initial observations - Being adjacent to the control group wells, the difference in water clarity was plain to see between the two groups.

24 hours post-fertilization - Roughly half of the embryos in their own respective wells had died from the exposure, and it was clear under closer examination that the surviving embryos would hold astonishing results if they remained alive long enough for the duration of the experiment. Attributes included a much slower heartbeat than those of the control group, as well as what appeared to be a slightly irregular placement of the eyes.

48 hours post fertilization - The extra 24 hours the surviving embryos had to develop had only exaggerated the aforementioned abnormalities. The hearts of the embryos appeared to be struggling to efficiently pump blood to the rest of the body, and appeared strained in general. As far as bodily development goes, several appeared to be developing in a similar fashion to those of the control group, whereas other embryos have no apparent development past the eyes and heart. These embryos in particular appeared very unlikely to make it another 24 hours, but are by far the most intriguing deformations thus far. See Figures 4 and 6.

72 hours post fertilization - A final observation of the 2.5 mg/L proved to not disappoint. The results and deformations were extraordinary, being that the previously mentioned deformed embryos had only worsened. The embryos were nothing more than blobs of zebrafish, trapped in embryos that they were clearly never going to hatch from. All that was to be made out were a set of eyes and a faintly beating heart. There was absolutely no spinal development in the severely deformed embryos, and they appeared on the brink of death, yet somehow still alive enough to barely maintain a pulse. The Atrazine had clearly taken its toll on these embryos, and viewing these deformed embryos was startling to say the least. See Figures 7 and 8.

5.0 mg/L:

Initial observations - The solution was much cloudier than the 2.5 mg/L, and appeared to be a harsh environment to sustain a healthy development.

24 hours post fertilization - In contrast to the 2.5 mg/L and even the control group, the Atrazine appeared to have put a different sort of strain on the embryos, as the heartbeats of the few remaining were abnormally quick. It seemed that more time was needed to observe this odd display.

48 hours post fertilization - After an additional 24 hours, only two embryos remained. However, these embryos fell into the pattern of the 2.5 mg/L concentration, and have very slow, methodic heartbeats that barely seemed sustainable for life.

72 hours post fertilization - The final 24 hours did not yield much change for the two remaining embryos. Oddly enough, the higher concentration of Atrazine did not yield the mutations seen in the lower concentration, but perhaps this could have been different if more embryos than 30 altogether would have been present, allowing for a wider view and possibly more live embryos.

7.5 mg/L - This concentration proved to be unlivable for the embryos, and none survived for further observation past the initial exposure.

Data Table Analysis:

The data tables are quantitative proof of the ill effects the Atrazine. The control group behaved as it should, with high survival rates and hatches near the end of the lab. However the data tables for the zebrafish embryos exposed to Atrazine demonstrate the example of the killing capabilities the substance has. Mortality rates were relatively high for even the lowest concentration, and worsen exponentially as the concentration is strengthened. The Atrazine prevented any hatching whatsoever in all the groups it was present in, and on top of this caused the aforementioned deformities listed in the qualitative results. Therefore the data tables are quantitative evidence collected through experimentation of the effects that Atrazine has on the development

Chart Analysis:

Chart 1 - This chart gives a stunningly clear visual representation of the rates of survival of the zebrafish embryos in solutions of different Atrazine concentrations. The control group, which is only in the experiment for comparison, shows the levels of survival at which the embryos should be at. Meanwhile at the concentrations of the 2.5 mg/L, which were closest to the Instant Ocean in levels of Atrazine, the number of surviving embryos was nearly halved, which shows significant support to the hypothesis of Atrazine having effects on the development of zebrafish embryos.

Chart 2 - This chart gives insight to the hatching patterns of the zebrafish embryos over the course of the 72 hour experiment. As shown in the data table, the zebrafish embryos were not subject to hatching early on in the experiment. The control is the norm to compare to, and that particular group only began to hatch at around the third day of observation. As far as the groups in the solutions of Atrazine are concerned, no hatching was ever close to occurring in the affected embryos over the course of the experiment. The opposite may occur in other toxicants as far as inducing premature hatching, but in the case of the embryos exposed to Atrazine, the embryos were completely unable to hatch in the harsh conditions the Atrazine created.

Photographic Data/Observations:

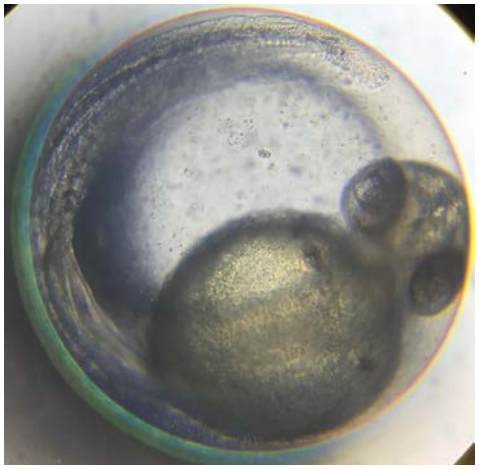

Figure 1

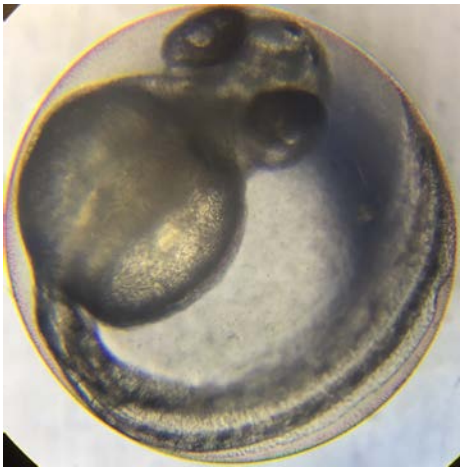

Figure 2

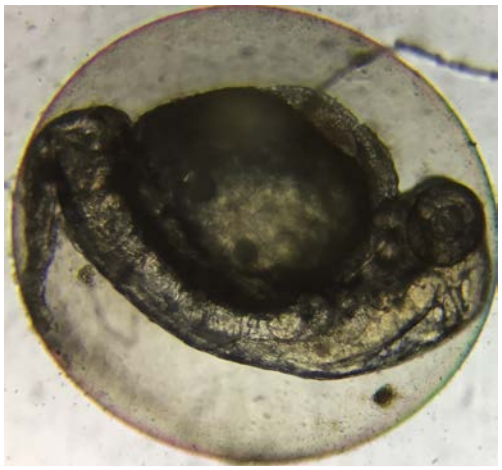

Figure 3

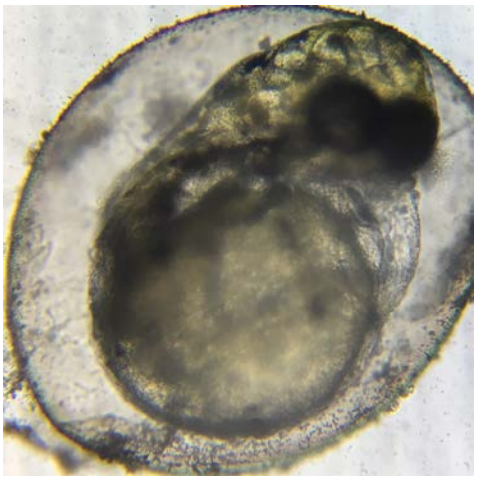

Figure 4

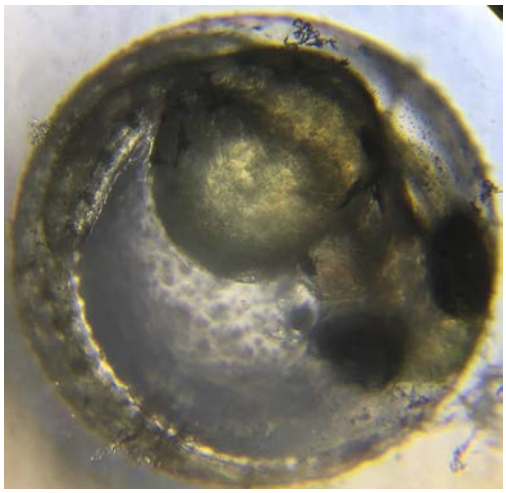

Figure 5

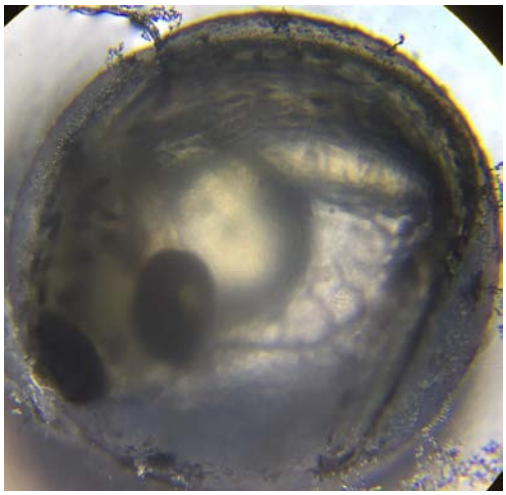

Figure 6

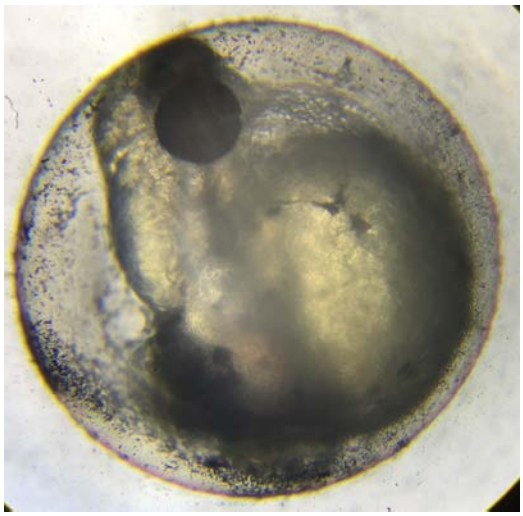

Figure 7

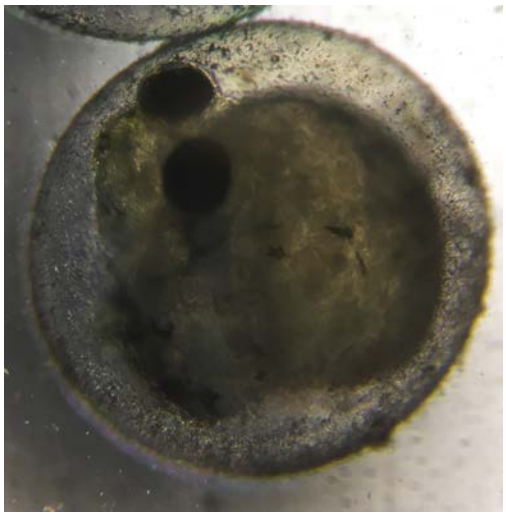

Figure 8

Image Notes/Explanations:

Figures 1 and 2- These are examples of healthy, control group embryos late into development, or 72 hours post-fertilization. Notice the spinal development is normal, and while video is not possible, the heartbeats were completely regular and strong, showing signs of a healthy embryo.

Figures 3 and 5 - These show both show embryos that were exposed to 5.0 mg/L of Atrazine. On top of being the only two embryos to survive the entire 72 hours, which speaks volumes on its own, these embryos were clearly affected by their exposure. The spinal development is slightly irregular with the embryo in Figure 3, whereas 5 appears to have undergone relatively normal development. The main concern with these embryos were the heartbeats, which as previously stated in the qualitative observations, was much too low to support any long-term growth or further development. The embryos in these figures appear healthy, but were actually on the edge of death even after 48 hours post-fertilization.

Figure 4 - This is a view of one of the more deformed embryos that the 2.5 mg/L solution had to offer. This image was captured 48 hours post-fertilization, and although a follow-up view 72 hours post-fertilization would have been optimal to see a more complete development, this embryo was one of the many to be taken by the Atrazine. Instead of a side view like most of the other photos offer, this image lends itself the advantage of a differing view, although it is still plain to observe that the all-around development has been altered by the embryo's exposure to the Atrazine. The heart seems to be placed in an abnormal spot on the body, and like its counterparts in the 2.5 mg/L, the heartbeat is noticeably weak and slow.

Figure 6 - This provides another view of an embryo 72 hours post fertilization in the 2.5 mg/L solution. This embryo in particular can be seen to have a rough shape of a normal embryo, but the heart is clearly out of normal position and, like the others, is incredibly slow in comparison to those of the control group. The most notable tendency of this zebrafish was its habitual fluttering of its underdeveloped fins. The poor embryo appeared restless, as it could not grasp that it was not under normal developmental conditions, and was attempting to swim about with absolutely no success. The fluttering was mesmerizingly regular, with the embryo attempting it once every 4-5 seconds.

Figures 7 and 8 - The final two figures provide the most extreme deformities observed in the entire experiment. The stunt in development is nothing short of incredible. These images were taken only minutes after those of Figures 1 and 2, and the differences between the four figures are fascinating. There is no observable spinal development whatsoever in both of these embryos, and are immensely behind in development as compared to the normal embryos in the

control group. Figure 8 especially appeared to be nothing more than a mass of zebrafish with eyes and a heartbeat. Even the embryonic sac appeared to have been altered, and looks rather unhealthy in its own right. In short, there is no way to differentiate and pick out any individual body part, as it is all mashed into one intriguing mass.

Data Analysis:

An unpaired t-test was used to test the statistical significance of the final results of this experiment. A t-test takes two groups that are independent of each other and uses the mean values of these groups in order to calculate whether or not the difference between the groups is significant and not because of chance. A P-value less than .05 reveals statistical significance. The t-test can only handle comparing two separate groups at a time, therefore the control has to be tested individually against each of the varying solution groups. If each of these individual tests come out with a P-value less than .05, it can be concluded that the results of this experiment have statistical significance, and have repeatable outcomes that were not caused by chance, but rather by the effects the Atrazine had on the developing zebrafish embryos.

The 2.5 mg/L solution t-test resulted in a P-value of .0034, which is considered to be very statistically significant. This is well under the P-value for being considered statistically significant, and provides mathematical support that the Atrazine will consistently affect the embryonic development of zebrafish, even in the lowest concentration of Atrazine present in the experiment.

When the 5.0 mg/L solution was tested against the control, the P-value was calculated out to be .00037, which is considered to be extremely significant. Using common sense, the statistical significance makes sense, as there were only two embryos to even survive past the first observation date. This high death rate correlates with the result of the t-test, as a 6.7% survival rate is no coincidence in this case.

The 7.5 mg/L solutions t-test obviously has the lowest P-value of .0001, as there were no surviving embryos even after just 24 hours. The only way to achieve a lower P-value in this case would mean having a larger pool of zebrafish to experiment on in the first place, which would only serve to increase the statistical soundness that a concentration of 7.5 mg/L will efficiently kill any zebrafish embryo in the vulnerable stage of early development.

Results:

This experiment was specifically designed to observe the detrimental effects on embryos exposed to Atrazine. Previous experiments have shown to cause problems in zebrafish, and this experiment in particular was an attempt to pinpoint the threshold of concentrations that would yield the most noteworthy deformations and results possible. The experiment was done over the course of 72 hours with observations taking place every 24 hours after initial fertilization, and the embryos in varying concentrations of Atrazine were compared to a control group in a normal environment of Instant Ocean solution.

In this experiment, there were independent and dependent variables, as all sound experiments should have. The independent variable was Atrazine, the toxicant that the embryos were exposed to in differing concentrations. The dependent variables were the survival and hatching rates of the embryos, on top of any other observations that were made to observe the effects the Atrazine had, such as heart rate and other deformities in development. The control in this experiment was the group in the uncontaminated Instant Ocean solution, where the fish were only exposed to the normal conditions that they were meant to be raised in. The control group is kept in order to have a standard for comparison across all aspects of the experiment; the results of the zebrafish being affected by the toxicant would not be significant if there was no norm to compare to.

Discussion:

The proposal that drives the entire experiment and gives it a purpose throughout is the first matter that will be touched on: the hypothesis. The original hypothesis was just this: zebrafish embryos exposed to Atrazine will suffer severe alterations in their development. This was clearly supported across all aspects of data, observations, and statistical analysis. As the concentration of Atrazine increased from the control group to 7.5 mg/L, the mortality rate of the fished increased exponentially, major deformities were observed, and as previously stated, the statistical significance was proven in a t-test of the quantitative results. The 7.5 mg/L was undoubtedly too much for the fragile embryos to handle, which was expected. The highest concentration was meant to be a sort of shot in the dark, a means of testing the limits of how much Atrazine can the embryos truly handle, without dying, and still maintain severe deformities and extraordinary results. The low survival rates of the 5 mg/L were not anticipated, as previous research points to a concentration of 15 mg/L as having the most profound effects on the zebrafish embryos.

Aside from survival rates, the qualitative observations were able to shine on their own merit, as they proved to only solidify the already strongly supported hypothesis. Aside from the two surviving zebrafish during the 24 hours post-fertilization, the heartbeats of the zebrafish embryos were remarkably low. Even those two same embryos were slowed down far past the normal rate of the control group just 24 hours later; the heightened heartbeat was most likely due to the poisoned embryos being under a great amount of distress, and a short while later were slowed to the expected rate by the toxicant. This agrees with previous research, as a slowdown of movements and complications with the heart and circulatory system have been documented. Another significant deformity found was the development of the spine, which was all but nonexistent in two of the embryos exposed to the 2.5 mg/L of Atrazine.

A limitation of this experiment would have been the amount of time the experiment had to be finished in. With more time, that threshold of deformities and prime results could have been delved into much deeper, and the point at which all this occurs could have easily been found had ample time been given. A flaw in the experiment was the amount of live embryos initially used in the well plates. There were solid results in the 2.5 mg/L wells, and the 7.5 was clearly too concentrated for survival, but perhaps with a larger pool of embryos, more

detrimental developmental effects could have been observed in the 5.0 mg/L wells. The untapped potential of this concentration is due to not having enough live embryos to observe as the experiment moved into its later stages. More results from this concentration would have been optimal to observe, as if the 2.5 mg/L produced such fine results, the 5.0 mg/L should, in theory, outdo the deformities already observed.

The results of this experiment coincide nicely with other experiments previously done on the detrimental health effects Atrazine can cause. However, they do not line up with the research done in favor of Atrazine. Although banned in the European Union, the research done in the United States remains persistent enough to allow the continued use of Atrazine. Use of the substance is proof that many believe this herbicide to either be harmless enough to use, or too essential to give up. The results of this experiment greatly beg to differ, however, and as far as the data goes to show, Atrazine clearly has the potential to wreak havoc on any living organism it comes into contact with, and should not be trusted for widespread agricultural use due the the dangers it poses.

References:

Ganzel, B., "Farming 1970's to Today." *Living History Farm*. Ganzel Group Inc.

<http://www.livinghistoryfarm.org/farminginthe70s/pests_05.html>.

Mergel, M., (2011). Atrazine - History and Uses., from

http://www.toxipedia.org/display/toxipedia/Atrazine - History and Uses

Pesticide Action Network | Atrazine . (n.d.). from http://www.panna.org/resources/atrazine

Santoriello, C., & Zon, L. I. (2012). JCI - Hooked! Modeling human disease in zebrafish. from

http://www.jci.org/articles/view/60434

Weber, G. J., Sepúlveda, M. S., Peterson, S. M., Lewis, S. S., & Freeman, J. L. (2013).

Transcriptome Alterations Following Developmental Atrazine Exposure in Zebrafish Are

Associated with Disruption of Neuroendocrine and Reproductive System Function, Cell Cycle, and Carcinogenesis. *Toxicological Science*, 132(2), 458-486. from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3595526/

Wirbisky, S. E., Weber, G. J., Sepulveda, M. S., Lin, T., Jannasch, A. S., & Freeman, J. L. (2016). An embryonic atrazine exposure results in reproductive dysfunction in adult zebrafish and morphological alterations in their offspring. *Nature*. from http://www.nature.com/articles/srep21337

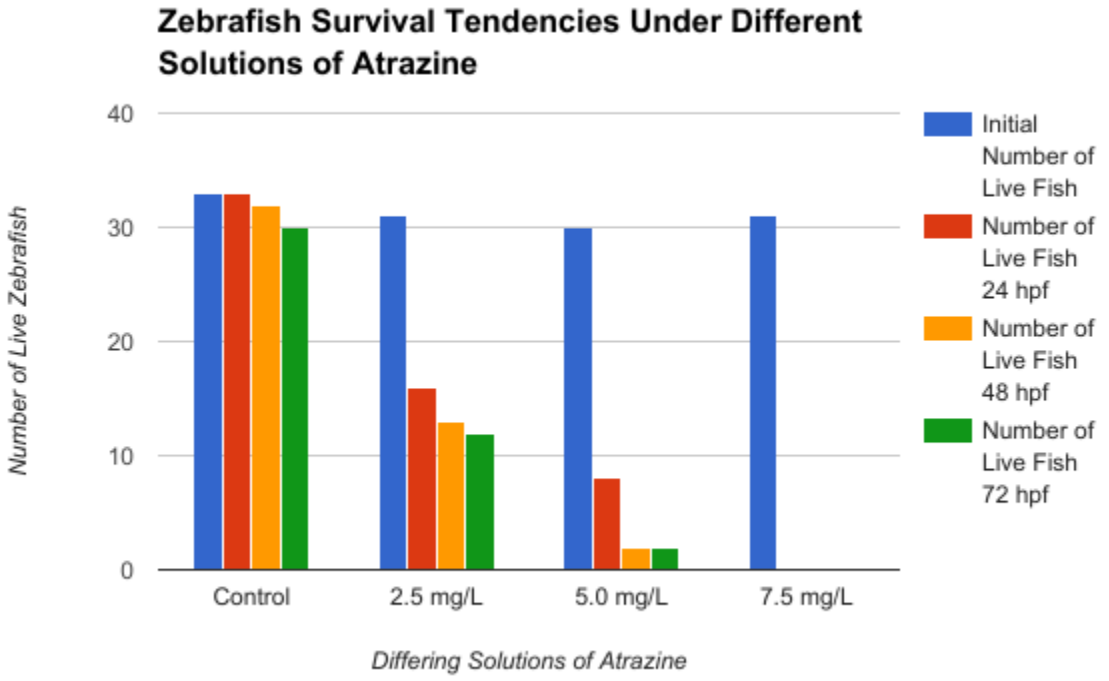

Chart 1

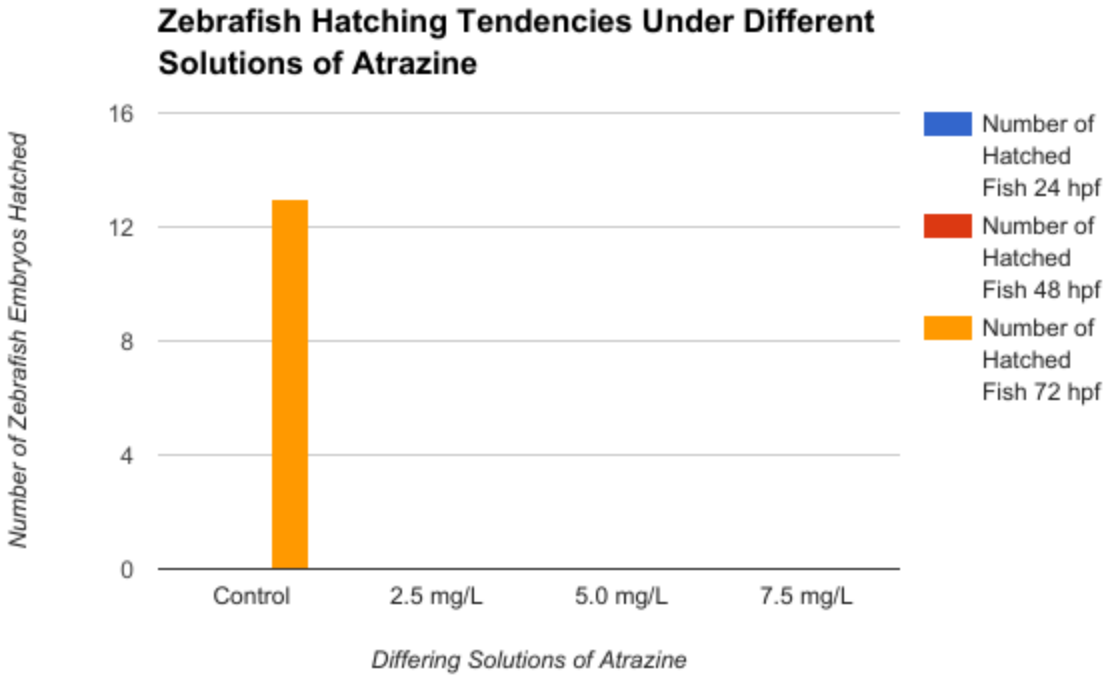

Chart 2

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